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**NMR AND BIOCHEMICAL ANALYSIS OF
PROTEIN-PROTEIN INTERACTIONS**

Insights on dopamine synthesis regulation and heterochromatin formation

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Abstract

Molecular recognition between proteins plays a fundamental role in almost all biological processes. Therefore, the structural characterization of protein-protein interactions represents a fundamental tool to shed light on the molecular mechanisms of biological events. Moreover, it can elucidate the structural basis of the function for proteins without a detectable catalytic activity.

The regulation of dopamine synthesis is an example of the importance of protein-protein interactions in the modulation of a biological function.

L-Dopamine, one of the catecholaminergic neurotransmitters, has many functions in the brain, including regulation of behavior and cognition, motor activity, and sleep.

The enzyme responsible for the biosynthesis of its precursor L-DOPA, named Tyrosine Hydroxylase, is therefore tightly regulated by different mechanisms. One of the most important is the interaction with protein modulators. 14-3-3 proteins bind to the N-terminal regulatory domain of Tyrosine Hydroxylase, leading to the stabilization of the active conformer. Several published data suggest that also α -synuclein, a natively unfolded protein linked to the pathogenesis of Parkinson Disease, could play a role in dopamine synthesis, through the inhibition of Tyrosine Hydroxylase. Interestingly, experimental evidence exists also for the interaction between 14-3-3 protein and α -synuclein: the binding has been supposed to have functional significance in the network regulation of dopamine synthesis.

The first part of this research is focused on the characterization of the interactions among these three key protein modulators of dopamine biosynthesis using a combined biochemical and spectroscopic approach.

The work can be divided into different levels. Molecular biology, biochemical and spectroscopic methods were used to obtain and characterise each protein sample separately. Subsequently, we analysed the interaction between each pair of protein partners using both biochemical and biophysical tools, in particular NMR spectroscopy.

Electrophoretic and chromatographic results revealed that α -synuclein and 14-3-3 proteins fail to form a stable complex in solution, while NMR experiments suggest a transient interaction involving only the first N-terminal residues of monomeric α -synuclein. However, a strong indication of interaction comes from aggregation experiments followed by fluorescence polarization, where 14-3-3 proteins were demonstrated to slow down the α -synuclein aggregation rate. Taken together, these results suggest that 14-3-3 proteins may bind to the α -synuclein oligomeric species, thus opening a possible chaperon function of 14-3-3 proteins to prevent α -synuclein aggregation and fibrillation.

On the other hand, both enzymatic and NMR experiments indicated that α -synuclein does not inhibit Tyrosine Hydroxylase and there is no direct interaction between the two proteins in the experimental conditions tested.

Finally, we verified, using biochemical tools, the formation of a complex with high affinity between 14-3-3 proteins and Tyrosine Hydroxylase phosphorylated in Serine 19, and carried out preliminary crystallization screenings of the complex.

Moreover, this research includes the NMR structural investigation of a second example of protein-protein interaction, involved in a different biological function. We analysed the molecular recognition mechanism between Heterochromatin protein 1 (Hp1 β) and a twenty-one residues peptide that mimics the histone 3 N-terminal tail of the nucleosome. The Hp1 β /histone 3 interaction leads to the packaging of the DNA in tertiary and quaternary structures, altogether named heterochromatin. As heterochromatin prevents access of the transcriptional machinery to the DNA, its formation represents a regulatory mechanism to inhibit gene expression in specific regions of the genome.

As necessary premise to study this interaction by NMR, we assigned, using heteronuclear 3D experiments, the ^1H , ^{15}N and ^{13}C backbone chemical shifts of Hp1 β protein. At present, we completed the assignment of the resonances from the domain that mediates the interaction with histone 3, called chromo-domain. The assignment of the signals from the other two parts of the molecule, named hinge region and chromo-shadow domain, is in progress. We then characterised the tumbling of the different parts of Hp1 β protein by cross-correlation experiments. Residual dipolar couplings were used to compare the structure of the chromo-domain domain in the full-length protein with that published for the domain produced as deleted mutant.

$[\text{H},^{15}\text{N}]$ HSQC experiments were obtained titrating Hp1 β with the histone 3 derived peptide. The results of the chemical shift perturbation analysis allowed us to map the residues of Hp1 β protein that are responsible for the interaction with the histone 3 N-terminal tail.

Riassunto

Il riconoscimento molecolare tra proteine riveste un ruolo fondamentale nella gran parte dei processi biologici. Pertanto, la caratterizzazione strutturale delle interazioni proteina-proteina rappresenta una chiave di lettura fondamentale per far luce sui meccanismi molecolari alla base dei processi biologici. Inoltre tale caratterizzazione può chiarire le basi strutturali della funzione di quelle proteine che mancano di attività catalitica rilevabile. La regolazione della sintesi di dopamina è un esempio dell'importanza delle interazioni proteina-proteina nella modulazione delle funzioni biologiche. La dopamina, uno dei neurotrasmettitori catecolaminergici, regola molte funzioni del sistema nervoso, tra cui quelle legate al comportamento ed ai processi cognitivi, all'attività motoria e al sonno. L'enzima responsabile della sintesi del suo precursore L-DOPA, è la Tirosina Idrossilasi. Data l'importanza della sua attività catalitica, l'enzima è finemente regolato da molteplici meccanismi. Uno dei più importanti è basato sull'interazione con modulatori proteici.

Le proteine 14-3-3 legano il dominio regolatore ammino-terminale della Tirosina Idrossilasi, stabilizzandone la conformazione cataliticamente attiva. Diversi dati pubblicati in letteratura sostengono che anche l' α -sinucleina, una proteina "natively unfolded" correlata all'insorgenza del morbo di Parkinson, potrebbe avere una funzione nella sintesi della dopamina, attraverso l'inibizione della Tirosina Idrossilasi. Di particolare interesse sono le evidenze sperimentali che dimostrano l'interazione tra α -sinucleina e proteine 14-3-3, che potrebbe giocare un ruolo importante nella complessa rete di regolazione della sintesi della dopamina.

La prima parte di questa ricerca è focalizzata sulla caratterizzazione delle interazioni fra i tre modulatori proteici della sintesi della dopamina, attraverso l'utilizzo combinato di tecniche biochimiche e spettroscopiche.

Il lavoro si articola in più livelli. Metodi di biologia molecolare, biochimica e spettroscopia sono stati impiegati per produrre e caratterizzare i campioni di proteine per i successivi studi. Abbiamo poi analizzato l'interazione di ciascuna coppia di proteine, utilizzando sia tecniche di biochimica e di biofisica, in particolare spettroscopia di risonanza magnetica nucleare (NMR). Dai risultati elettroforetici e cromatografici è emerso che α -sinucleina e proteine 14-3-3 non formano un complesso stabile in soluzione, mentre esperimenti NMR suggeriscono che vi sia una interazione debole che coinvolge solo i primi residui della regione ammino-terminale della α -sinucleina. Tuttavia, esperimenti di aggregazione seguendo il segnale di polarizzazione di fluorescenza, portano ad una forte indicazione dell'interazione tra le due proteine, dimostrando che le proteine 14-3-3 sono in grado di rallentare la velocità di aggregazione dell' α -sinucleina. Complessivamente i risultati suggeriscono che le proteine 14-3-3 potrebbero legarsi alle specie oligomeriche della α -sinucleina, aprendo quindi la strada ad una possibile funzione di "chaperon" nell'inibizione del processo di aggregazione e fibrillazione dell' α -sinucleina.

Invece, sia gli studi di attività enzimatica che NMR, indicano che l' α -sinucleina non è un inibitore della Tirosina Idrossilasi, e che non c'è interazione diretta tra le due proteine nelle condizioni sperimentali utilizzate.

Infine, mediante tecniche biochimiche, abbiamo verificato che le proteine 14-3-3 e Tirosina Idrossilasi fosforilata sulla serina 19, formano un complesso ad alta affinità. Abbiamo quindi purificato il complesso su larga scala, ed effettuato lo screening preliminare di cristallizzazione.

La seconda parte del lavoro di ricerca si occupa dell' indagine strutturale, mediante NMR, di un secondo esempio di interazione proteina-proteina che è alla base di una differente funzione biologica. Abbiamo analizzato il meccanismo di riconoscimento molecolare tra "Heterochromatin protein 1" (Hp1 β) ed un peptide di ventuno residui che mima la regione ammino-terminale della proteina istonica, appartenente al complesso proteico nucleosoma. L'interazione tra Hp1 β e l'istone III porta all'impacchettamento del DNA in strutture terziarie e quaternarie, definite complessivamente eterocromatina. Dal momento che l'eterocromatina impedisce l'accesso dell'apparato di trascrizione al DNA, la sua formazione rappresenta un meccanismo di regolazione per inibire l'espressione genica in specifiche regioni del genoma.

Il requisito fondamentale per studiare questa interazione mediante NMR, è l'assegnazione, utilizzando esperimenti tridimensionali eteronucleari, delle risonanze di ^1H , ^{15}N e ^{13}C della catena polipeptidica della proteina Hp1 β . Ad oggi abbiamo completato l'assegnazione dei segnali relativi al dominio che media l'interazione con la proteina istonica III, definito "chromo-domain". L'assegnazione dei segnali relativi alle altre due regioni della molecola, definiti "hinge-region" e "chromo-shadow domain", è tuttora in corso. Abbiamo poi caratterizzato le proprietà di "tumbling" delle diverse regioni della proteina Hp1 β , mediante particolari esperimenti di rilassamento di magnetizzazione, definiti di "cross-correlation". I valori di "residual dipolar coupling" sono stati utilizzati per confrontare la struttura del "chromo-domain" compreso nella proteina intera, con la struttura pubblicata dello stesso dominio prodotto come mutante di delezione.

Tramite esperimenti di titolazione per risonanza magnetica nucleare abbiamo infine individuato i residui amminoacidici della proteina Hp1 β che sono coinvolti nel riconoscimento molecolare del peptide. I risultati dell'analisi delle differenze nei valori di "chemical shift", ha consentito di mappare le regioni della proteina Hp1 β che sono coinvolte nell'interazione con la parte ammino-terminale dell'istone III.

Aims of the project

The aim of the experimental work described in this thesis is the study of the molecular recognition mechanism between proteins using a combined biochemical and spectroscopic approach. Specifically, NMR methods were used to characterize protein-protein binding at high resolution. The choice of NMR spectroscopy as the tool for the structural characterisation is justified by the possibility to work under physiological conditions and to analyse both stable-high affinity complex and weak-transient interactions. Moreover, it is a potent method to combine both dynamic and structural information, thus providing a complete characterization of the molecular basis of protein recognition and interaction.

The project considers two different examples of protein-protein interaction as molecular basis of biological functions.

The first part of the research is focused on the characterisation of the interaction between three key protein modulators of dopamine biosynthesis: α -synuclein, 14-3-3 proteins and Tyrosine Hydroxylase. This study is part of a wider research project in the group of Padova, aimed to unravel the molecular mechanism that leads to a failure of the regulation of dopamine metabolism that has been proposed to play a role in the pathogenesis of Parkinson Disease.

The first aim of this experimental part is to verify the interaction between each of the possible protein pairs. The second aim is to investigate, at an atomic level, each hetero-protein complex, to unravel the structural factors that govern the interaction. Molecular biology, biochemical and spectroscopic methods are employed to obtain and characterize both α -synuclein and Tyrosine Hydroxylase samples for the subsequent interaction studies. For the experiments involving 14-3-3 proteins, we worked with the ζ and η isoforms, kindly provided by the crystallographic group of Prof H. L. Monaco at the Verona University. The choice of ζ and η proteins is based on the published data that document the interaction of these isoforms with both α -synuclein and Tyrosine Hydroxylase.

Subsequently, we want to use both biochemical and biophysical tools to analyze the interactions. NMR provides the tool to map the interface of protein complex.

The second part of the research is focused on the analysis of the molecular recognition between Heterochromatin protein 1 (Hp1 β) and a twenty-one residues peptide that mimics the histone 3 tail of the nucleosome. This is a fundamental interaction for the regulation of the heterochromatin synthesis that represents one of the ways used by eukaryotic cells to silence gene expression. The experiments were carried out during a six months visiting period in the structural biology department of Prof C. Griesinger, under the supervision of Prof M. Zweckstetter at the Max Planck Institute of Göttingen, Germany. Hp1 β samples were prepared by the Max Planck group of Chromatin biochemistry.

As necessary premise to study this interaction by NMR, we want to assign, using heteronuclear 3D experiments, the ^1H , ^{15}N and ^{13}C backbone chemical shifts of Hp1 β protein. NMR relaxation and residual dipolar coupling experiments are the tools chosen to characterize the dynamic properties and the structure of the different regions of the

proteins. Further NMR experiments allow us to map the binding site on the Hp1 β structure.

Chapter I.

Introduction

Introduction 1.1

**Protein-protein interactions in the
regulation of the dopamine
synthesis**

Overview

L-Dopamine (DA), one of the catecholaminergic neurotransmitters, has many functions in the brain, including important roles in behavior and cognition, motor activity, and sleep.

Pathologically, a loss of DA-containing neurons projecting from the *substantia nigra* to the dorsal striatum leads to Parkinson Disease (PD). Oxidative stress seems to have a key role in neurodegeneration: specifically the self-oxidation of DA in the cytoplasm, out of the synaptic vesicles, generates reactive oxygen species and quinones toxic for dopaminergic neurons (Barnham et al., 2004).

Therefore, although dopamine plays an essential function in the nervous system, it can also be potentially toxic if there is a failure in the control of its cytoplasmic level. This is indeed tightly regulated by different mechanisms. One of the most important comes from the modulation of the biosynthesis of its precursor 3,4-dihydroxy-L-phenylalanine (L-DOPA) by the reaction catalyzed by Tyrosine Hydroxylase (TH). TH is a non-heme iron enzyme that catalyzes the conversion of L-tyrosine to L-DOPA using molecular oxygen as oxidant and tetrahydrobiopterin (BH₄) as source of electrons.

In the following introduction the most important details on the enzyme structure and on the catalytic mechanisms of L-tyrosine hydroxylation will be reviewed. In particular the short term regulatory mechanisms of enzymatic activity will be reported to emphasize how catecholaminergic neurons can modulate so subtly Tyrosine Hydroxylase activity, in response to intracellular signals, like the increment of Ca²⁺ concentration.

Several reports indicate that 14-3-3 proteins and α -synuclein represent key protein regulators of dopamine synthesis, with different effects on enzyme activity modulation. Moreover experimental evidence exists also for the interaction between TH and 14-3-3 proteins, suggesting that one of the physiological significance of this interaction is just a further regulatory point of Tyrosine Hydroxylase activity.

The biochemical and structural characterization of the mechanisms of molecular recognition among these three protein partners represents the aim of my PhD project. Therefore particular attention will be given to the published works that describe these interactions. Biochemical and biophysical data are of particular interest, as they already confirm the possibility to study the complexes in solution using the recombinant forms.

An overview of the biological functions, regulation and structure details of 14-3-3 proteins and α -synuclein will conclude the chapter.

1.1.1 Tyrosine Hydroxylase: structural details

The first and rate-limiting step in the biosynthesis of the neurotransmitters dopamine, norepinephrine and epinephrine is catalyzed by the enzyme Tyrosine Hydroxylase (TH; tyrosine 3-monooxygenase; EC 1.14.16.2).

TH belongs to the family of tetrahydrobiopterin dependent aromatic amino acid hydroxylases which also includes Phenylalanine Hydroxylase (PH) and Tryptophan Hydroxylase (TpH), that catalyze the rate limiting step of phenylalanine catabolism and serotonin synthesis, respectively. Tetrahydrobiopterin dependent aromatic amino acid hydroxylases are non-heme iron dependent enzymes, requiring one iron atom in the ferrous form per subunit and stoichiometric amount of tetrahydrobiopterin for activity (Fitzpatrick, 1999).

The enzymes from eukaryotic sources have a common three-domains structure.

The N-terminal domain, responsive for regulatory properties, has 100-170 residues and a pairwise homology of about 25%. This is followed by a 270 residue catalytic domain exhibiting 80% sequence homology and a 65% pairwise sequence identity. The C-terminal 40 residues form a tetramerization domain with a 60% pairwise homology. (Goodwill et al., 1998). Deletion experiments on rat protein demonstrated that the highly conserved catalytic domain can promote the reaction independently from the other domains (Hufton et al., 1985).

Crystal structures have been determined for the iron-bound forms of rat TH (Goodwill et al., 1997 and Goodwill et al., 1998) and human PH (Erlandsen et al., 1997 and Fusetti et al., 1998). Both enzymes show an iron coordination of two histidines, a glutamate, and two or three water molecules.

Due to the high homology sequence with the rat enzyme (84% identity), the overall fold of human Tyrosine Hydroxylase catalytic and tetramerization domains can be derived from the description of the two crystal structures solved at 2.3 Å for the rat protein (Goodwill et al., 1997 and 1998). The PDB accession codes are 1toh and 2toh. A rat TH model made from 1toh PDB, is shown in figure 1.1.1, together with an insight of the iron coordinating region. Below there is the sequence scheme for human enzyme. The catalytic domain is organized as basket-like arrangement of helices (49%) and loops (42%). The tetramerization domain consists of two strands and a 40 Å long α -helix characterized by an hydrophobic heptad repeat that forms a coiled coil at the core of the tetramer. The active site is founded in a 17 Å deep cleft at the centre of the catalytic domain basket. The cleft is lined mostly by four α -helices. Two loops are arranged to form the opening of the active site. The iron atom, 10 Å from the enzyme surface, is in an active-site cleft. The iron is coordinated by two histidines (His360 and His365), one glutamic acid (Glu405) and two water molecules in a square pyramidal coordination geometry (Goodwill et al., 1998). The same ligands system is found in the human enzyme (Schüenemann et al., 1999). A third water molecule is the 6th ligand in a different coordination geometry, as shown with Mössbauer and X-ray absorption measurements with the protein in solution (Schüenemann et al., 1999).

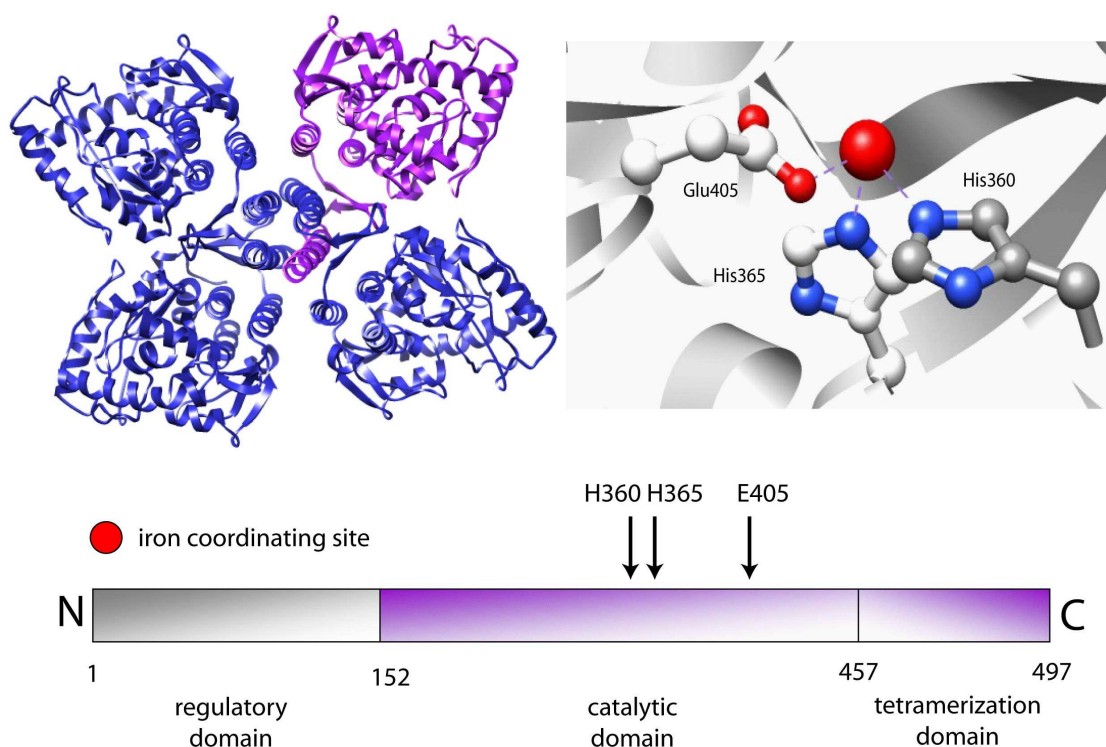


Figure 1.1.1 Structural model of catalytic and tetramerization domains of rat Tyrosine Hydroxylase (PDB accession code 1toh). The three iron coordinating residues are shown in ball-and-stick format. Below the sequence scheme of the human enzyme.

The crystal structure of rat TH in the presence of iron and the cofactor analogue 7,8-dihydrobiopterin (PDB accession code 2toh) shows the mode of pterin binding.

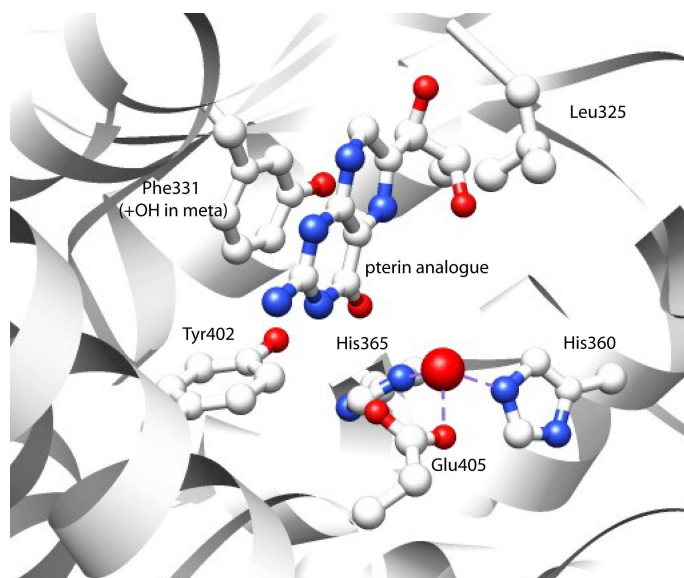


Figure 1.1.2 Active site cleft of Tyrosine Hydroxylase (PDB accession code 2toh). The pterin analogue and some of its ligands (Phe 331, Tyr402, Glu 405 and Leu 325) are shown in ball-and-stick format, as the iron coordinating residues.

The pterin binds on one face of the large active-site cleft, forming an aromatic π -stacking interaction with Phe331. This phenylalanine residue of TH is found to be hydroxylated in the meta position, most likely through an autocatalytic process, and to consequently form a

hydrogen bond to the main-chain carbonyl of Gln341 which anchors Phe331 in the active site. The bound pterin forms hydrogen bonds with Tyr402, Glu405, Leu325 and Leu326, and with an iron-coordinating water. The iron is 5.6 Å from the pterin 4a carbon which is hydroxylated in the enzymatic reaction.

No structural changes are observed between the pterin bound and the apo enzyme. (Goodwill et al., 1998).

While the catalytic core of the rat enzymes is 52% identical, as regards to the other tetrahydrobiopterin dependent aromatic amino acid hydroxylases, the N-terminal domains show very low levels of identity (14%), consistent with the different regulatory mechanisms of the three enzymes. Therefore, for this regulatory part, is not convenient to derive structural information from the crystal structure of Phenylalanine Hydroxylase (PDB accession number 1PHZ), that is the only one containing the N-terminal regulatory domain.

Circular dichroism analysis on human TH N-terminal domain revealed that it is mostly unstructured with few β -sheet and α -helix contribution, according to that found for the similar rat TH N-terminal domain (84% identity) (Obsilova et al., 2008). However, since deconvolution values are usually affected by a large error, a more precise analysis is required in order to characterize the secondary structure of the protein.

1.1.2 Tyrosine Hydroxylase: catalytic cycle and regulation mechanism

Tyrosine hydroxylase (TH; tyrosine 3-monooxygenase; EC 1.14.16.2) catalyzes the conversion of L-tyrosine to L-DOPA, which is the initial and rate-limiting step in the biosynthesis of the catecholamine dopamine, norepinephrine and epinephrine (Nagatsu et al., 1964). These molecules play several important roles in cell signalling and in neuronal transmission, for the regulation of vegetative, motor and psychic functions.

Therefore, the control of Tyrosine Hydroxylase activity represents a key point in the modulation of these processes.

TH activity is modulated by two mechanism. Medium- to long-term regulation of gene expression: enzyme stability, transcriptional regulation, RNA stability, alternative RNA splicing and translational regulation; short-term regulation of enzyme activity: feedback inhibition, allosteric regulation and phosphorylation (Kumer and Vrana 1996).

Splicing variants from the single human gene give rise to four TH isoforms differing in their amino acid sequence at their N-terminal regulatory domain (Grima et al. 1987; Le Bourdelles et al. 1988; Nagatsu 1995; Kappock and Caradonna 1996; Kumer and Vrana 1996) The shortest TH 1 variant is homologous to the same subunit from most other species. The TH 2,3 and 4 variants have inserts that lead to the expression of proteins containing 4, 27 and 31 (4 + 27) amino acid residues immediately N-terminal to Ser31 of the TH1 isoform. The four isoforms differ in potential phosphorylation sites, have slightly different catalytic activities and distribution. As an example TH1 and TH2 forms are the most prominent in brain, adrenal medulla and human neuroblastoma cells (Haycock 1991, 1993, 2002), while TH3 and TH4 mRNA are mainly in pheochromocytoma adrenal gland. Pancreatic islets express almost exclusively TH-2 (Kumer & Vrana, 1996).

Particular mutations in TH gene are the cause of autosomal recessive Segawa syndrome, also known as DOPA-responsive dystonia. Some cases present with parkinsonian symptoms in infancy and are referred to as autosomal recessive infantile parkinsonism (Ludecke et al., 1995-1996; Swaans et al., 2000).

The short term regulation mechanisms have direct effect on the enzymatic activity. It is therefore necessary to give details on the catalytic cycle before describe the regulation mechanisms.

TH belongs to the family of tetrahydrobiopterin dependent aromatic amino acid hydroxylases that are non-heme iron dependent enzymes, requiring one iron atom in the ferrous form per subunit and stoichiometric amount of tetrahydrobiopterin for activity (Fitzpatrick, 1999).

The redox reaction catalyzed by TH (figure 1.1.3) is the hydroxylation of the side chain of L-tyrosine, utilizing tetrahydrobiopterin (BH₄) as the source of electrons and molecular oxygen as oxidant.

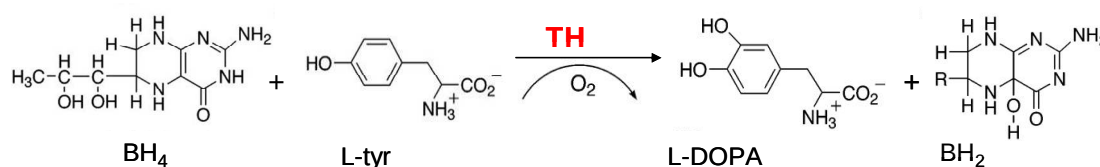


Figure 1.1.3_ The reaction catalyzed by Tyrosine Hydroxylase

The reaction products are the hydroxylated amino acid (L-DOPA) and a 4a-hydroxylated pterin (BH₂), then recycled through de-hydration by 4a-carbinolamine dehydratase and reduction by dihydropteridine reductase. Molecular oxygen is the source of the oxygen atom incorporated into the pterin product as well as the oxygen atom incorporated into the side chain of aromatic amino acid (Fitzpatrick, 1999).

Subsequent steps in the catecholamine biosynthetic pathway are catalyzed by aromatic amino acid decarboxylase (AADC), which converts L-DOPA to L-dopamine, dopamine-β-hydroxylase (DBH), that catalyse the hydroxylation of L-dopamine to norepinephrine, and phenylethanolamine-N-methyl transferase (PNMT), that converts norepinephrine to epinephrine (Kumer & Vrana, 1996).

A common catalytic mechanism was proposed to explain the general activity of aromatic amino acid hydroxylation, in order to support the kinetic data of all pterin-dependent enzymes (Fitzpatrick, 2003).

Figure 1.1.4 shows a scheme of the catalytic mechanism. The reaction can be divided into two partial reactions: first the formation of an hydroxylating intermediate and then the oxygen transfer to the amino acid substrate.

Computation studies support an initial binding of oxygen to Fe(II) to form a species described as Fe(III)O₂⁻. It then reacts with pterin to give Fe(II) μ-peroxypterin. Heterolytic cleavage of the OO bond of such an intermediate would generate the hydroxyl-pterin and a ferryl-oxo species.

This Fe(IV)O product is the electrophilic hydroxylating intermediate required for an electrophilic aromatic substitution reaction. (Bassan et al., 2003)

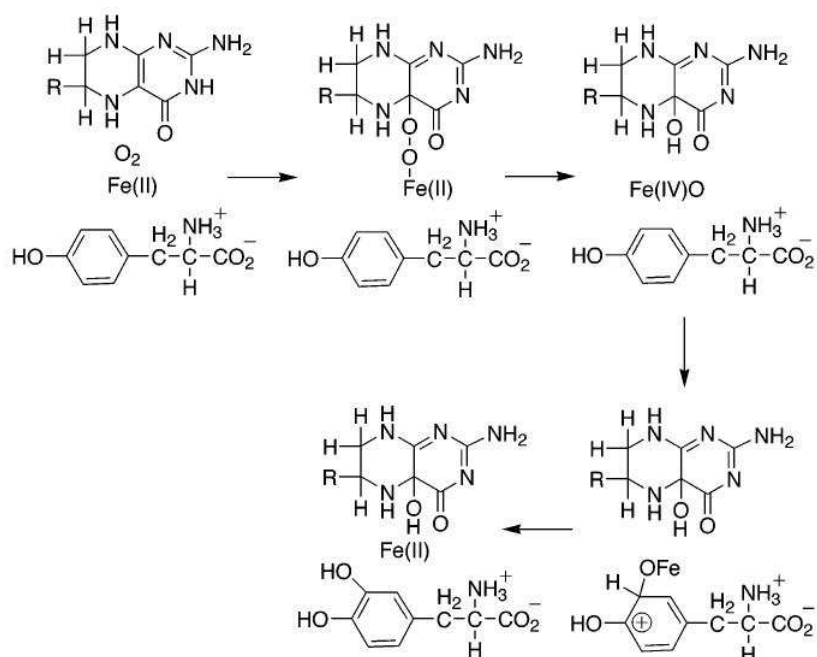


Figure 1.1.4_ Proposed catalytic mechanism of L-tyrosine hydroxylation.
Adapted from Fitzpatrick et al., 2003.

The requirement for TH activity is the protein coordination to iron in the Fe²⁺ state. TH coordinating Fe³⁺ has no catalytic activity and can bind catechols: TH is therefore regulated by a feedback inhibition in which catechols binding stabilizes the enzyme in the inactive Fe³⁺ form. Two mechanisms of feedback inhibition by the catechols have been proposed. First, catechols can compete with BH₄ for binding at the catalytic site of the enzymes thus inhibiting cofactor interaction. This is a reversible inhibition and acts as a sensor of the local concentrations of the catechols. Second, catechols can bind almost irreversibly with the ferric iron at the TH catalytic site. The consequence is the decrease of the enzyme activity and its stabilization (Kumer and Vrana 1996).

Several catechols can inhibit TH but the physiologically relevant, and with the highest affinity are dopamine, norepinephrine and epinephrine (Ramsey and Fitzpatrick 2000).

Phosphorylation of TH induce structural changes that lead a conformation that is still inhibited but with decreased affinity for catechols (Ramsey and Fitzpatrick 1998). Dissociation of catechols allows the Fe³⁺ to be reduced by BH₄, thereby returning the enzyme to the fully active state. One possible role of tetrahydrobiopterin in vivo, in addition to its role in normal enzyme turnover, may be to return the ferric enzyme to the active ferrous state (Goodwill et al., 1998).

TH is also inhibited by the substrate L-tyrosine (Quinsey et al. 1998) and by excess BH₄ (Alterio et al. 1998). Allosteric effectors, such as heparin, polyanions and phospholipids, have all been shown to increase TH activity by decreasing the K_M for BH₄ (Kumer and Vrana 1996).

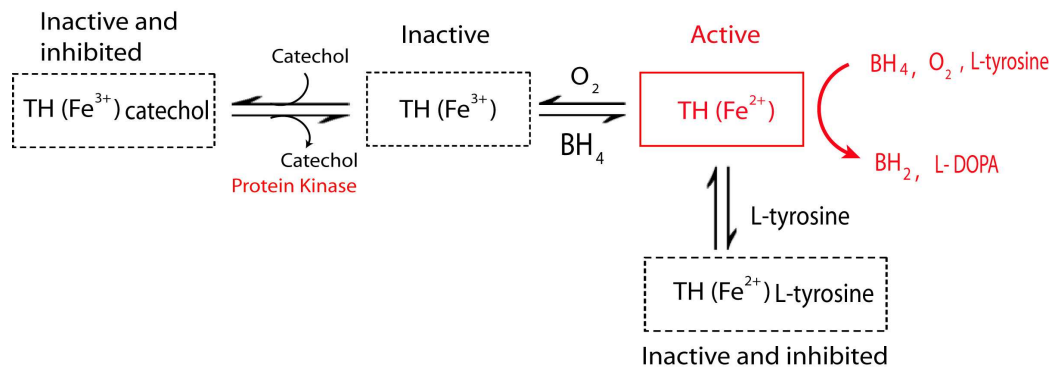


Figure 1.1.5_ Short term regulation mechanisms of Tyrosine Hydroxylase.
Adapted from Dunkley et al., 2004.

Besides the product feedback and substrate inhibition mechanisms, phosphorylation represents another way to activate the enzyme: by release of the feedback catechol inhibitors, TH activation by phosphorylation is the primary mechanism responsible for the maintenance of catecholamine levels in tissues after catecholamine secretion (Dunkley et al., 2004).

The TH N-terminal domain can be phosphorylated, both in vivo and in vitro experiments, at four sites: serine residues (Ser) 8, 19, 31 and 40 in the rat enzyme (Haycock 1990). The human isoforms have a threonine at position 8 (Grima et al. 1987).

In vitro studies demonstrate that TH can be phosphorylated by a wide range of protein kinases, including protein kinase C (PKC) (Albert et al. 1984), calcium- and calmodulin stimulated protein kinase (CaMPK) II (Vulliet et al. 1984), protein kinase G (PKG) (Roskoski et al. 1987), MAPK activated protein kinases (MAPKAPKs) 1 and 2 (Sutherland et al. 1993), p38-regulated/activated kinase (PRAK) and mitogen- and stress-activated protein kinase (MSK) 1 (Toska et al. 2002). The effects of Ser40 phosphorylation on kinetic parameters are the best characterized: the increment on TH activity is due to a decrease of the K_M for the cofactor tetrahydrobiopterin (BH₄) and an increment of the K_i for inhibitory catecholamines (Haavik et al. 1990, 1997; Almas et al. 1992).

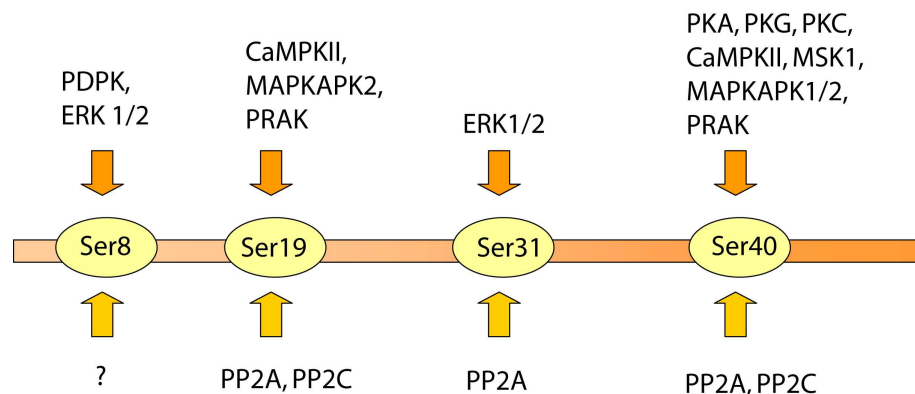


Figure 1.1.6_ kinases and phosphatase able to modify TH enzyme is shown.
Adapted from Dunkley et al., 2004.

Phosphatase, such as PP2A and PP2C, reverse this modification making this regulatory mechanism reversible. In figure 1.1.6 a scheme of the kinases and phosphatase that can modify TH enzyme is shown.

Through the post-translational modifications operated by different kinases and phosphatase, TH activity is tightly modulated in response to the neurotransmission signalling network.

1.1.3 Stimulus-coupled interaction of Tyrosine Hydroxylase with 14-3-3 proteins

Due to its relevance on neuronal and endocrine functions, Tyrosine Hydroxylase activity is controlled by several mechanisms, as describe in 1.2. The phosphorylation-dependent regulation of enzyme activity is believed to be of functional significance with respect to stimulus-response coupling.

One of the strongest experimental evidence supporting this hypothesis comes from cell culture experiments, where phosphorylation of Ser19 by CaM kinase II results in an increment of the Tyrosine Hydroxylase activity in response to a variety of stimuli that increase intracellular Ca^{2+} (Itagaki et al., 1999). These experiments also demonstrate, in agreement with previous reports (Yamauchi et al, 1981), that phosphorylation of Ser19 requires an additional protein factor for the activation of TH. This was identified as the 14-3-3 proteins (Ichimura et al., 1987). 14-3-3 denotes a family of highly conserved proteins expressed in seven distinct isoforms in human cells (β , γ , η , ϵ , ζ , σ , and τ). Through the binding to more than 200 different mostly phosphorylated proteins 14-3-3 play a key role in regulatory network of several cellular process, as intracellular signalling, protein turnover, cell cycle and apoptosis (Van Heusden, 2005). They mostly recognise their binding partner through a phosphorylated consensus motif containing either a phosphorylated serine (pS) or a phosphorylated threonine (pT) residue. 14-3-3 proteins act as adapter that modulates the activity, conformation, stability, interaction, or intracellular localization of target proteins. A detailed description of their structure and physiological roles will be found in chapter 1.8. The tetrahydrobiopterin dependent aromatic amino acid hydroxylases represent an example of enzymes regulated by the binding to 14-3-3 proteins. Both Tryptophane Hydroxylase (TPH) and Tyrosine Hydroxylase (TH) active conformations are stabilised by the binding of these adapter proteins at N-terminal regulatory domain (Ichimura et al., 1988), but the structural details of this interaction are still unknown.

In the work of Itagaki et al. of 1999, CaM Kinase II phosphorylates both Ser19 and Ser40 of TH. To discriminate their relative importance on 14-3-3 protein recognition, the mutant on S19A was tested for the ability to form the complex with 14-3-3 protein: the results show that only phosphorylation of TH at Ser19, and not at Ser40, is necessary to trigger its binding when cells are depolarized to induce Ca^{2+} influx. The protein complex was purified from the cell cultures with specific antibodies. Furthermore, the binding was confirmed using the recombinant purified versions of the two proteins, with TH phosphorylated by CaM Kinase II. Enzymatic tests revealed a 2,6 fold enhancement of TH activity, while surface plasmon resonance measurements provided an equilibrium dissociation constant of 3 nM.

Competition studies with phospho-peptides based on the TH sequence, including pSer-19 or pSer-40, confirmed that only phosphorylation on Ser19 can lead to the high affinity binding with 14-3-3 proteins.

The mechanism proposed by Itagaki to explain the Ca^{2+} -dependent activation of TH starts from the elevation of intracellular Ca^{2+} concentration, in response to a specific stimulus. Then the Ca^{2+} cellular sensor calmodulin (CAM) activates CaM kinase II which subsequently phosphorylates TH at Ser-19. Finally 14-3-3 proteins recognise and bind to TH phosphorylated on Ser19 (pSer19TH) to promote its activity, possibly by preventing de-phosphorylation and the conversion to the conformer with high affinity for catechols inhibitors (figure 1.1.7). These findings suggest that both phosphorylation of Tyrosine Hydroxylase and its binding to 14-3-3 proteins, have a role on the increment of catecholamine synthesis that occurs in response to stimuli that increase the intracellular Ca^{2+} concentration.

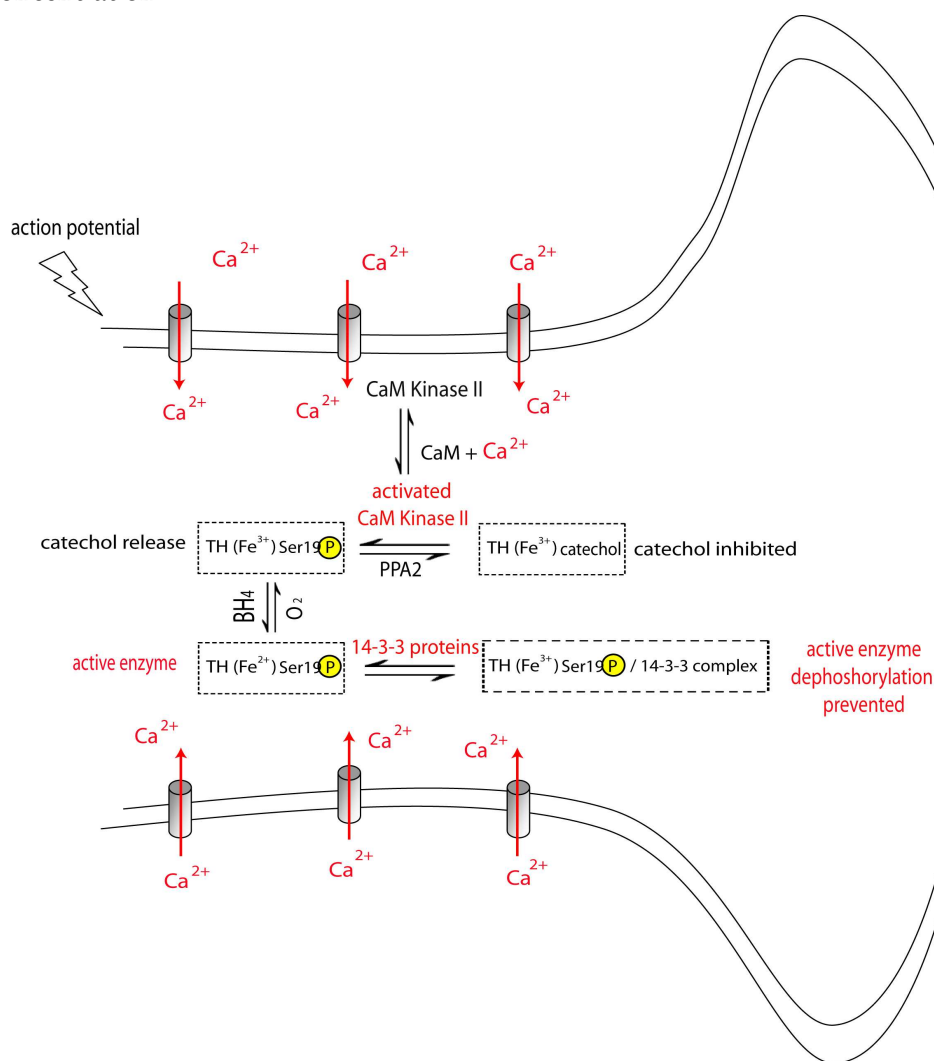


Figure 1.1.7_ Schematic illustration of the mechanism proposed by Itagaki et al., 1999 of Ca^{2+} mediated stimulus coupled interaction of TH with 14-3-3 proteins.

1.1.4 Experimental evidence of recombinant Tyrosine Hydroxylase / 14-3-3 proteins interaction

While phosphorylation of Tyrosine Hydroxylase on Ser-31 on at Ser40 results in a direct increase in its enzymatic activity (Sutherland et al., 1993, Ramsey et al., 1998 and Wu et al., 1992), the activation of the enzyme through Ser19 phosphorylation requires the binding of 14-3-3 protein. Here, we reported the most important published evidence that the formation of an high affinity complex can occur between the recombinant forms of the two proteins.

The results of Itagaki reveal the formation of a strong complex (K_d of 3 nM) between recombinant pSer19,40 TH and 14-3-3 proteins, with a 2.6 fold enhancement of the enzymatic activity. The binding was confirmed using several 14-3-3 isoforms (η , β , θ). Competition studies with phospho-peptides demonstrate that phosphorylation of TH on Ser19, and not on Ser40 is the fundamental requirement for 14-3-3 binding.

Similar data were obtained by Kleppe et al. in 2001, who investigated how phosphorylation of different sites on human TH can affect 14-3-3 ζ proteins binding. While phosphorylation of TH on Ser31 and Ser40 does not result in an high affinity 14-3-3 binding, phosphorylation of both Ser19 and Ser40, leads to the formation of a strong complex with 14-3-3 ζ isoforms.

In agreement with the results obtained by Itagaky, competition studies with phospho-peptides that mimic the ten residues surrounding Ser19 and Ser40, show that while pSer40 peptide is a weak inhibitor of pSer19,40 TH/14-3-3 interaction, pSer19 strongly inhibits the binding between the two proteins.

Experimental evidence of the formation of an high affinity complex also derives from the spectroscopic analysis performed by Obsilova et al., 2008. The work investigated whether 14-3-3 η protein binding affects the conformation of the regulatory domain of human TH (THR) phosphorylated at both Ser19 and Ser40. Fluorescence and circular dichroism studies revealed that phosphorylation on Ser19 and Ser40 by itself does not induce significant conformational changes on the TH regulatory domain. On the other hand, time-resolved tryptophan fluorescence measurements demonstrate that the interaction between 14-3-3 protein and phosphorylated TH1R decreases the solvent exposure of tryptophan residues at positions 14 and 34 and induces structural change in the proximity of residue 73. Moreover, experiments of proteolysis indicated that 14-3-3 protein binding can protect the first 33 residues of the TH regulatory domain

1.1.5 Possible role of α -synuclein on Tyrosine Hydroxylase activity

At present, 14-3-3 proteins represent the only known proteins that modulate Tyrosine Hydroxylase, and that are able to enhance its activity by a direct protein-protein interaction. The previously mentioned data described the formation of an high affinity complex, dependent on Ser19 phosphorylation, between the two recombinant proteins.

Published data suggest that also α -synuclein has a role on Tyrosine Hydroxylase activity modulation (Perez et al., 2002). α -Synuclein is a small protein usually localized to pre-synaptic terminals in mammalian brain (Jakes et al., 1994). It is unfolded in its native state, but it can acquire a mostly helical structure in association with negatively charged membrane systems (Bisaglia et al., 2005), and a β -sheet structure in protofibrils and fibrils (Serpell et al., 2000).

Although a huge amount of work was done to elucidate the role of this protein in the pathogenesis of Parkinson disease (Dev et al., 2003), its physiological functions are still undefined.

α -Synuclein has been described to specifically interact with numerous proteins involved in signal transduction, ubiquitin-proteasome system, vesicular recycling, synaptic functionality, regulation of oxidative stress, and mitochondrial function (Dev et al., 2003, Zhou et al., 2004). However there is not a clear correlation between all these interactions and a defined function. A detailed description of the structure and possible physiological and pathological role of α -synuclein, will be provided on chapter 1.9.

The work of Perez et al., 2002, suggests that α -synuclein, like 14-3-3 proteins, may also bind to TH but, in contrast, inhibit its activity. The binding seems not TH-phosphorylation dependent. α -Synuclein and 14-3-3 proteins would therefore play antithetic roles in dopamine synthesis, through the inhibition or activation of Tyrosine Hydroxylase, in response to different signalling pathways.

The first clue of α -synuclein and Tyrosine Hydroxylase interaction comes from “pull down” experiments where the complex of the two proteins was selectively isolated from brain homogenates using antibodies (Perez et al., 2002) or affinity column (Zhou et al., 2004).

To explore the consequences of this interaction, the effect of recombinant α -synuclein on TH activity was investigated in a cell-free system, showing a dose-dependent inhibition (Perez et al., 2002). Moreover over-expression of α -synuclein in neuronal cell lines significantly reduced TH activity, with a corresponding reduction in dopamine synthesis (Perez et al., 2002). However recent reports demonstrate that α -synuclein can interact and modulate the activity of several kinases and phosphatase (Zhou et al., 2004.) Some of them are able to regulate Tyrosine Hydroxylase, as Protein Kinase C (Ostrerova et al., 1999) and phosphatase PPA2 (Peng et al., 2005). Moreover α -synuclein can also bind to calmodulin (Lee et al., 2002; Martinez et al., 2003), the Ca^{2+} sensor that activate Cam Kinase II in response to intracellular Ca^{2+} increment.

Therefore it will important to verify whether the TH inhibition by α -synuclein is due to a direct binding, or is mediated by a indirect effect on the network system that regulate TH phosphorylation.

1.1.6 Interaction between α -synuclein and 14-3-3 proteins

Among the numerous putative binding partners of α -synuclein, the interaction with Tyrosine Hydroxylase seems to be of functional significance, with respect to a possible role in the regulation of dopamine synthesis. As described in the previous chapter, it was suggested that 14-3-3 proteins and α -synuclein are antithetic modulators of TH activity. Moreover several line of evidence, from proteomic (Zhou et al., 2004), immunological (Ostrerova et al., 1999) and biophysical (Sato et al., 2006) studies, reported that the two proteins can interact both in vivo and in vitro.

The first insight derived from a work of Ostrerova et al. in 1999, where they demonstrated that the first 60 residues of α -synuclein and the fragment spanning the residues 40 to 100 of 14-3-3 proteins are more than 40% homologous. Moreover, the complex of the two proteins was isolated both from brain homogenate and neuronal cell culture using specific antibodies.

The interaction was furthermore confirmed by Zhou et al. in 2004. They identified in the bovine brain homogenate the ensemble of proteins trapped by α -synuclein immobilized on a chromatographic matrix. By quantitative proteomics, 14-3-3 θ , τ , ζ and ϵ isoforms, and Tyrosine Hydroxylase were detected as α -synuclein binding partners.

The surface plasmon resonance analysis (Sato et al., 2006) provided a K_d value of 1.1 μ M for the complex formed in solution between recombinant α -synuclein and 14-3-3 η isoform. Besides a possible regulatory role on dopamine synthesis, α -synuclein and 14-3-3 proteins interaction was suggested to be of functional significance in cell death mechanisms. Xu et al. in 2002, demonstrated that dopamine-dependent neurotoxicity in human dopaminergic neurons is mediated by a 54–83-kD soluble protein complex that contains both α -synuclein and 14-3-3 proteins.

1.1.7 Hypothesis of overall regulatory mechanism

To emphasise the importance of the protein interaction network on the regulation of dopamine synthesis, a scheme that summarises the relations among Tyrosine Hydroxylase, α -synuclein and 14-3-3 proteins is shown.

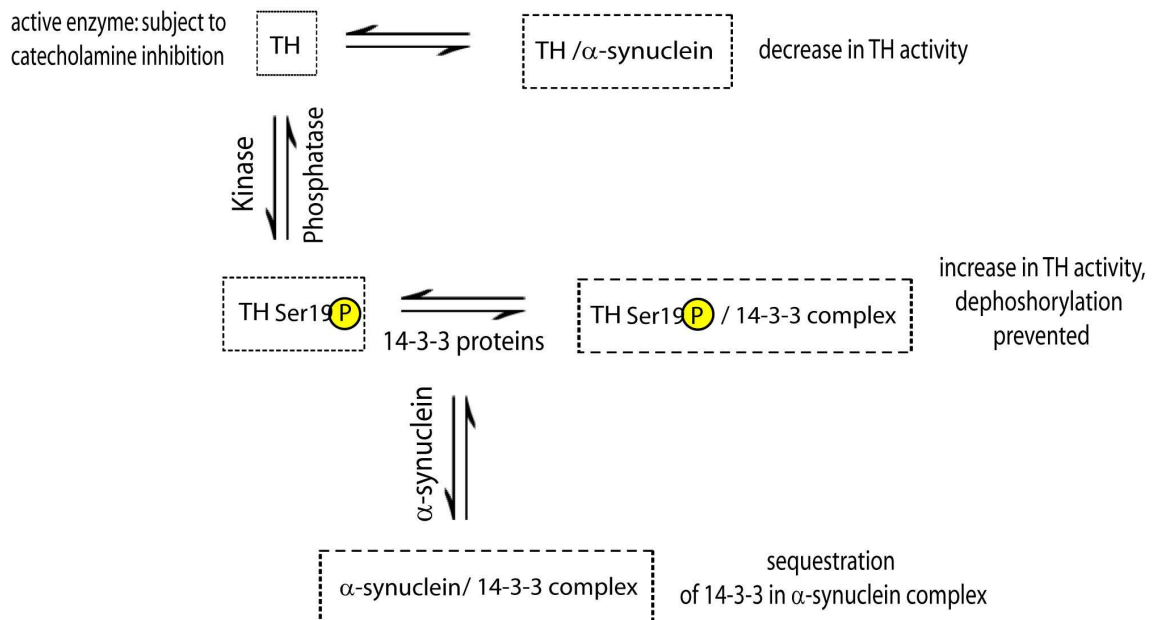


Figure 1.1.8_ Schematic representation of the general regulatory mechanisms of dopamine synthesis mediated by the interactions among Tyrosine Hydroxylase, α -synuclein and 14-3-3 proteins.

1.1.8 14-3-3 proteins: structure, function and regulation

Physiological functions

14-3-3 proteins are a family of highly conserved acidic proteins, expressed in all eukaryotic cells (Gardino et al., 2006). The name “14-3-3” derived from the combination of their elution position on DEAE-cellulose chromatography and their migration pattern on starch-gel electrophoresis, during an early effort to classify all bovine brain proteins in the late 1960s (Moore et al., 1967). The terminology remained till now: however, although they comprise approximately 1% of brain soluble protein (Berg et al., 2003), they are expressed in almost all tissues. The subcellular localization is mainly cytoplasmatic but they were detected also at the plasma membrane and inside the intracellular organelles (Van Heusden et al., 2005). The human genome contains seven distinct 14-3-3 genes denoted β , γ , ϵ , η , σ , τ and ζ . α and δ are the phosphorylated isoform of β and ζ proteins, respectively (Fu et al., 2000).

14-3-3 proteins commonly form dimers, with an acidic isoelectric point of 4-5. Each subunit has a molecular mass of approximately 30000.

While some isoforms prefer to adopt strictly homodimeric forms, such as human 14-3-3 σ and γ , others preferentially form heterodimers, as human 14-3-3 ϵ (Chaudhri et al., 2003; Jones et al., 1995).

The biological functions ascribed to 14-3-3 proteins depend on their ability to bind and regulate a wide variety of proteins. At the moment approximately 200 different cellular proteins, involved in almost every cellular process, such as cell cycle progression, the DNA damage response, apoptosis, protein trafficking, signal transduction, cytoskeletal rearrangements, metabolism, transcriptional regulation of gene expression, development and stress response, and cell adhesion, are described as binding partners for 14-3-3 proteins (Aitken, 2006). By the binding and activity modulation of such a wide variety of proteins, 14-3-3 have a fundamental role in the coordination of key points in the biological functions network.

Typically the basis of 14-3-3 proteins molecular recognition is a phosphorylated motif on their binding partners. The optimal binding motifs, recognised by all 14-3-3 isoforms, were described as RSXpSXP or RXF/YXpSXP sequences, where pS denotes phospho-serine (Gardino et al., 2006). Other binding motifs contained a phospho-threonine (Fu et al., 2000). As result, 14-3-3 proteins were the first protein family to be regarded as phospho-serine/threonine binding molecules. Moreover, other 14-3-3 binding partners do not require Ser or Thr phosphorylation, like the platelet glycoprotein Ib-IX-V (Du et al., 1996). The molecular basis of 14-3-3 proteins action was suggested to be mediated by different mechanisms, depending on the binding partner. The most important mechanisms include conformational changes, physically occlusion of structural features, scaffolding, or change on cellular localization (Yang et al., 2006) of the target protein. Some examples are here reported.

In the contest of neurotransmission regulation, several enzymes are activated by the binding to 14-3-3 proteins. Tryptophan and Tyrosine Hydroxylases interact with 14-3-3 proteins, resulting in increased activity (Ichimura et al., 1995; Banik et al., 1997; Ichimura et

al., 1987). In the example of Serotonin-N-acetyltransferase, it was shown that binding to a 14-3-3 protein results in a conformational change that leads to full activation (Obsil et al., 2001). 14-3-3 proteins can also mediate protein-protein interactions between proteins of signal transduction pathway involved in cell growth, differentiation and oncogenic transformation, thus acting as an adapter molecule. The best example is the regulation of Raf1, a serine/threonine kinase: 14-3-3 proteins can both stimulate the rapid transition between the inactive and active form of this kinase (Yip-Schneider et al., 2000) and mediate the association with its activity modulators, such as Bcr, and protein kinase C (Berg et al., 2003; Braselmann et al., 1995). This function can be justified by the ability of 14-3-3 hetero-dimers to bind simultaneously two different targets.

14-3-3 proteins can also regulate the subcellular localization of their binding partners. For example they can bind to phosphorylated sequences of nuclear proteins, resulting in the shielding of the nuclear localization signal required for nucleus import (Sekimoto et al., 2004). In the context of cell surviving-death equilibrium, 14-3-3 proteins have a critical role by sequestering the pro-apoptotic factor Bad in the cytoplasm, thus impairing its role on cell death with a signalling pathway that would start from mitochondria (Fu et al., 2000).

Recently, a new function was attribute to 14-3-3 proteins (Yano et al., 2006). It was demonstrated that 14-3-3 ζ can function as a stress-induced molecular chaperone, able to dissolve and help in the refolding of thermal aggregated proteins, both in cell-cultures and in vitro experiments. This function was specifically tested for few substrates, like apocytochrome C and citrate synthase, but a possible role in the aggregation of neurodegenerative related proteins, like α -synuclein of Parkinson Disease, or prion protein in Creutzfeldt-Jacob disease, was not investigated.

Pathological implications

As the 14-3-3 proteins are associated with almost all cellular processes, they are involved in many human diseases, mostly comprising tumour and neurological disorders. A clear correlation was found between the σ -isoform and human cancers (Yang et al., 2003).

The deletion of the gene encoding the 14-3-3 ϵ isoform leads to the Miller-Dieker syndrome, a severe neuronal migration defect associated with profound mental retardation and epilepsy (Toyo-oka et al., 2003).

Moreover 14-3-3 proteins were detected in the cerebrospinal fluid (CSF) in several neurological disorders. β , γ , ϵ and η isoforms were identified in cerebrospinal fluids from patients with Creutzfeldt-Jakob's disease (Berg et al., 2003) The presence of these isoforms in cerebrospinal fluids is being used as a diagnostic test for the prion diseases (Hsich et al., 1996). Moreover, 14-3-3 proteins seem to aggregate in some disease-specific inclusions within the brain. Isoforms ϵ , γ , ζ and θ are abundantly present in the Lewy bodies of Parkinson's disease.

14-3-3 proteins: structure, regulation and determinants of binding specificity between different isoforms

The X-ray crystal structures of all human 14-3-3 proteins bound to peptides have been solved: the crystallographic coordinates can be found in the Protein Data Bank (PDB codes: ζ : 1QJB, β : 2C23, σ : 1YWT, η : 2C74, γ : 2B05, τ : 2BTP, ϵ : 2BR9). Moreover, even the complex structure between serotonin *N*-acetyltransferase and isoform ζ has been deposited (PDB code: 1ib1).

The structural comparisons among the isoforms revealed a similar overall architecture, with few divergent regions responsive for the different ligand binding specificity.

14-3-3 protein structure is a cup-shaped dimeric, resembling a flattened horseshoe with a central channel 35 x 35 x 20 Å. Each monomeric subunit consists of a bundle of nine α -helices, organized in an anti-parallel fashion. Residues within the first four α -helices form the dimer interface, as well as the floor of the channel. Homo-dimer interface is maintained by three salt bridges and hydrophobic contacts. The residues involved are conserved in all human 14-3-3 isoforms (Gardino e al., 2006)

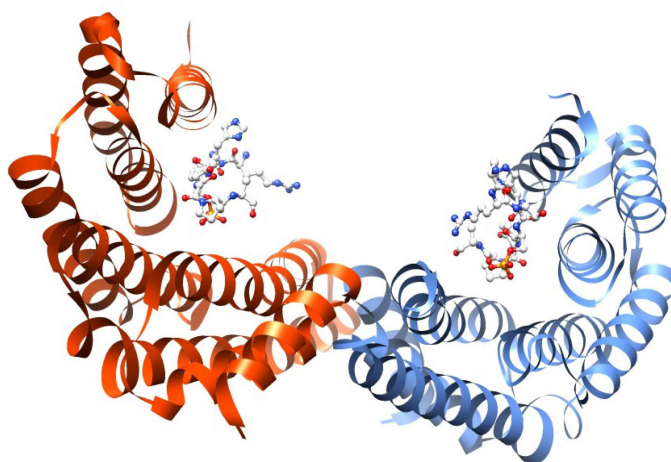


Figure 1.1.9_ Crystal structure of human 14-3-3 ζ dimer in complex with phospho-peptide RSHpSYPA (ball-and-stick; PDB code 1QJB).

A key feature of the central channel is the presence of an amphipathic groove in each monomer, formed by a cluster of charged and polar residues on one side, and a patch of hydrophobic residues on the other. It was identified as the site of interaction with phosphorylated and non-phosphorylated ligands. The interior of the channel is highly conserved in all 14-3-3 proteins, suggesting a common mode of target recognition (Fu et al., 2000).

Mutational analysis and co-crystallization studies demonstrated that a basic cluster in the groove, including K49, R56 and R127, is responsible for the recognition of phospho-Ser or phospho-Thr in the binding partners (Aitken, 2006).

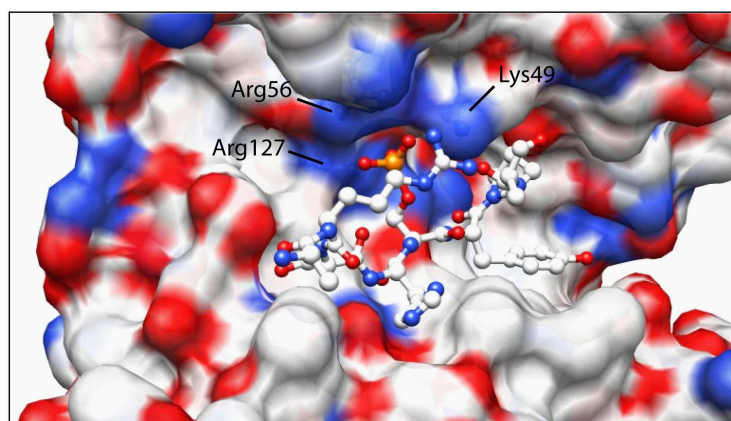


Figure 1.1.10_ Close up view of the amphipathic groove in the surface model of human 14-3-3 ζ structure in complex with the phospho-peptide RSHpSYPA (ball-and-stick; PDB code 1QJB). Red detect oxygen atoms, while blue detects nitrogen atoms. The basic cluster formed by Lys49, Arg56 and Arg127, makes contacts with the phosphate group (red and orange) of the peptide.

The alignment of 14-3-3 isoforms sequences (figure 1.1.11) show highly conserved regions, containing residues that are important for peptide binding and residues involved in dimerization, and less conserved regions. Residues displaying more sequence variability correspond to loops exposed on the outer surface of the protein. It was shown that these residues make additional secondary contacts with ligands, providing isoform substrate specificity (Gardino et al., 2006; Obsil et al., 2001). Mutational studies suggest that the loop that connects the two α -helices at the C-terminal part is strongly involved in ligand discrimination independent of phosphorylation (Wilker et al., 2005). The carboxyl tail, composed of a stretch of acidic amino acids, is the most divergent region in the sequence. This region is not visible in the crystal structures. However, a variety of biophysical experiments and modelling studies suggest that it may fold back into the empty peptide-binding pocket to protect against non-specific interactions with inappropriate ligands (Truong et al., 2002; Obsilova et al., 2004).

The complex structure of 14-3-3 ζ bound to serotonin *N*-acetyltransferase (Obsil et al., 2001) reveals that two target molecules bind a 14-3-3 dimer, occupying both central grooves. The conformation of 14-3-3 does not change upon ligand binding, implying a structural rigidity of the 14-3-3 molecule. Interestingly, different regions of serotonin *N*-acetyltransferase were found to interact outside of the phospho-peptide-binding groove, thus implying that the sequence variability among the different 14-3-3 isoforms justify the affinity for specific protein partners.

An important difference between the seven isoforms comes from a recent work on dimerization equilibrium: the results demonstrated that ϵ , γ and τ isoforms are almost dimeric, while β and η isoforms are in equilibrium with the monomeric form with a dimer/ratio of 70:30 (Yang et al., 2006). The oligomerization state represents one of the regulatory mechanism of 14-3-3 function, as some isoforms were found to be inactive in the monomeric form (Tzivion et al., 1998).

Another regulatory mechanism of 14-3-3 function is protein phosphorylation, which controls both its dimerization and ligand binding. Phosphorylation on Ser58 at the 14-3-3 ζ

dimer interface compromises dimer formation, although it does not affect peptide binding (Powell et al, 2003).

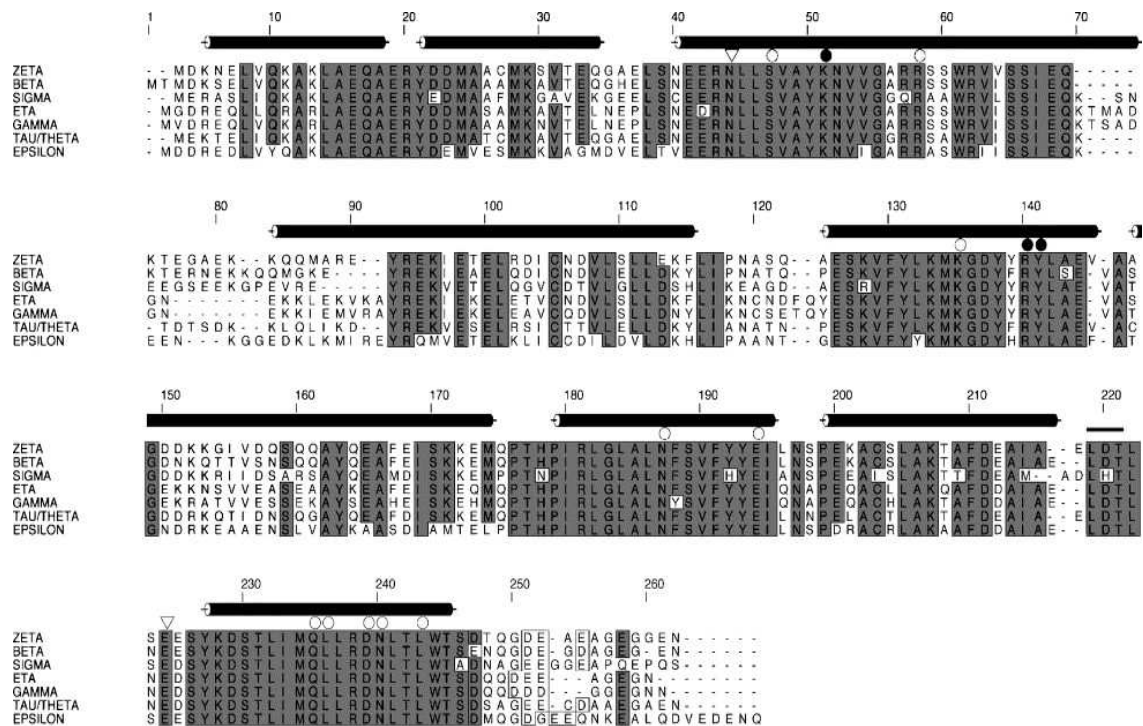


Figure 1.1.11_ Structure based sequence alignment of the seven human 14-3-3 isoforms. α -helix are shown above the alignment. Grey boxes detect the residues that are conserved across the isoforms, while empty boxes detect the highly divergent acidic residues of the C terminus. Residues involved in critical interactions with the ligand are indicated by circles above the alignment. Adapted from Gardino et al., 2006.

1.1.9 Structural insight on physiological function and pathological relevance of α -synuclein

Between the end of the 1980s and the early 1990s (Clayton et al., 1998) a novel protein family, relevant to several neural processes, was discovered and classified as synuclein. Among the three genes α , β , and γ , α -synuclein resulted of raising interest as it was found in the insoluble deposits typical of Alzheimer's disease and Parkinson's disease (PD). By now, a plethora of experimental evidence supports a role of this protein in the pathology of PD (Dev et al., 2003), the second most common neurodegenerative disorder, which affects 1–2% of the population above age 65 and 4–5% above age 85.

The term “synucleinopathies,” was introduced to indicate the ensemble of human neurodegenerative disorders where α -synuclein play a major role in the pathogenesis. They include also dementia with Lewy bodies and multiple-system atrophy (Spillantini et al., 1998).

α -Synuclein is a small (14.5 kDa, 140 amino acids), highly acidic protein abundantly expressed in the brain, where it is concentrated in presynaptic nerve terminals (Maroteaux et al., 1988; Jakes et al., 1994).

The analysis of the sequence (figure 1.1.12) shows that the N-terminal region (approximately residues 1-95) is almost entirely composed of variants of an imperfect 11 amino acid repeat (XKTKEGVXXXX, where underlined characters refer to the consensus motif), which strongly resembles that found in the amphipathic helices of the exchangeable apolipoproteins (Segrest et al., 1992; Davidson et al., 1998). The fragment corresponding to the sequence 61–95, originally named NAC (non-amyloid- β component), was observed in amyloid plaques associated to Alzheimer's disease (Ueda et al., 1993). The NAC is the most hydrophobic portion of the protein, and several studies ascribed this region as responsible for α -synuclein aggregation by β -sheet formation (Bodles et al., 2001; Giasson et al., 2001). The carboxy terminus of α -synuclein, which is rich in acidic residues, has been shown to regulate fibril formation (Murray et al., 2003).

Three point mutations A30P (Polymeropoulos et al., 1997) A53T (Kruger et al., 1998) and E46K (Zarranz et al., 2004), have been reported in the literature as linked to autosomal dominant early-onset PD. Moreover, familiar forms of PD have been found with triplication (Singleton et al., 2003) and duplications (Chartier-Harlin et al., 2004) of the entire synuclein locus.

The published biophysical works emphasize the high versatility of α -synuclein structure, as it can adopt different conformations depending on the experimental conditions. α -Synuclein is unfolded in its native state, but it can acquire a mostly helical structure in association with negatively charged membrane systems (Bisaglia et al. 2005; Ulmer et al., 2005), and a β -sheet structure in protofibrils and fibrils (Serpell et al., 2000). Therefore, to achieve a deep understanding of α -synuclein structural properties, it is important to consider the role of the different variables of the chemical environments that govern the equilibria among the accessible conformations. In figure 1.1.13 the scheme of the equilibria between the accessible conformations of the protein is presented.

The functions ascribed to α -synuclein include physiological regulation of certain enzymes, transporters, neurotransmitter vesicles recycle, as well as roles in neuronal survival. Specifically, α -synuclein is thought to regulate dopamine neurotransmission via effects on vesicular dopamine storage and modulation of the Tyrosine Hydroxylase activity (Dev et al., 2003). In the following sections the structural insights and the physiological or pathological implications of the different α -synuclein conformations will be reviewed. The last paragraph will describe the large variety of proteins that was shown to interact with α -synuclein and that can provide insights on its cellular role.

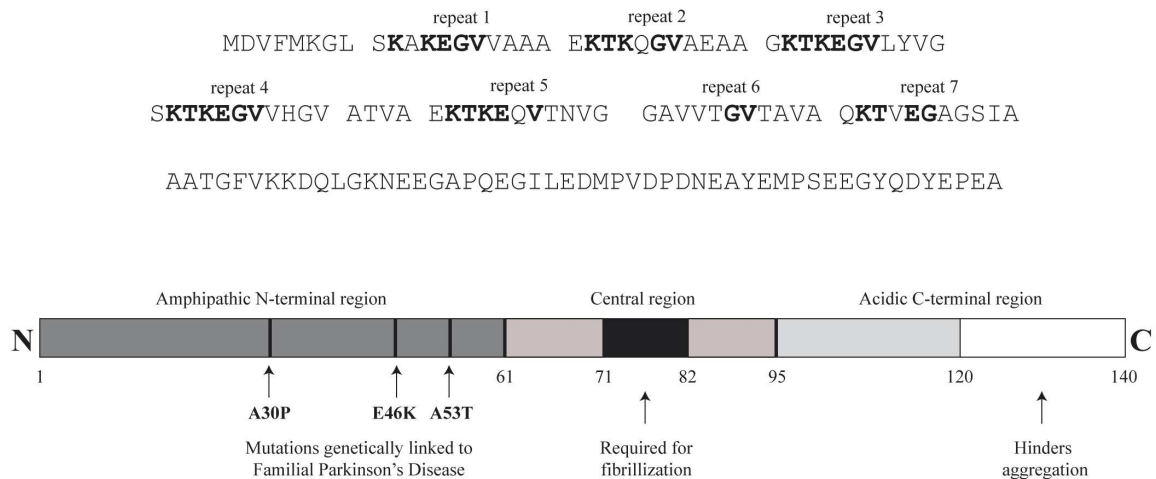


Figure 1.1.12 α -Synuclein sequence. The imperfect 11-mer repeats are emphasized, and the more conserved residues are represented in boldface. In the scheme below, the three different regions that compose the protein are indicated. Adapted from Bisaglia et al., 2009.

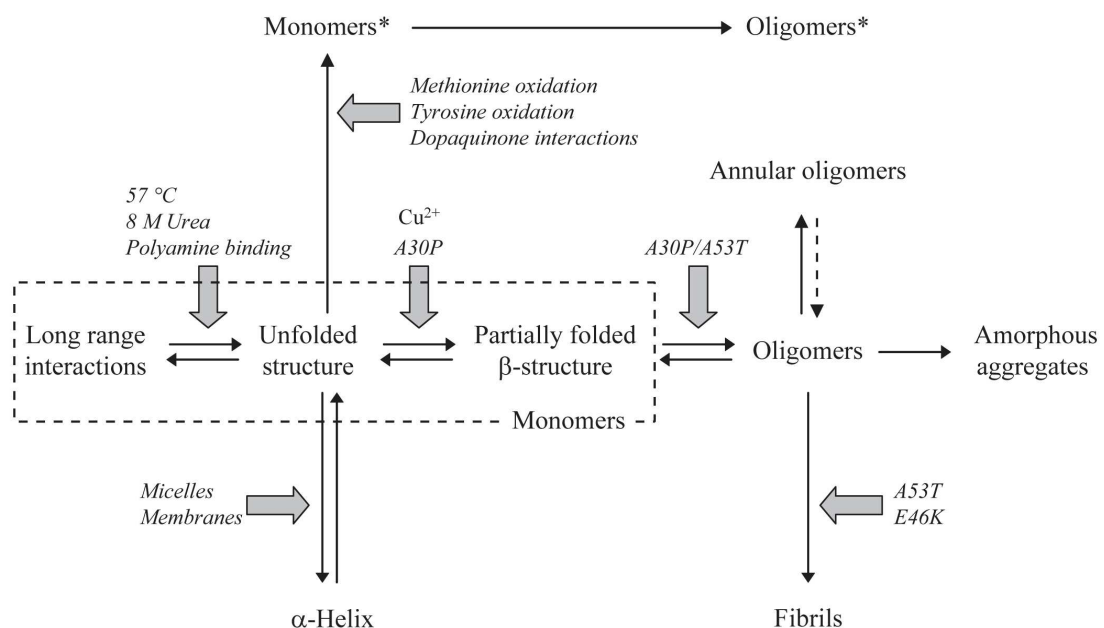


Figure 1.1.13 Equilibria between the different accessible α -synuclein conformations. Adapted from Bisaglia et al., 2009.

Monomeric α -synuclein

Biophysical studies revealed that α -synuclein lacks a well defined secondary structure in solution (Eliezer et al., 2001; Chandra et al., 2003). Therefore, based on its natively unstructured conformation, α -synuclein was classified as an intrinsically unstructured protein (Uversky, 2002; Tompa, 2005). To obtain a complete description of the structure of a natively unfolded protein, the characterization of an ensemble of structures rather than the determination of a unique conformation is required (Weinreb et al., 1995). At present, the combination of an array of biophysical techniques, as NMR, EPR, circular dichroism, fluorescence, IR and atomic force microscopy, provided important insights on the ensemble of dynamically interchanging structures of α -synuclein.

NMR methodology made available most of all the structural details. Site-directed spin labelling and Paramagnetic relaxation enhancement methodology allowed to detect long-range interactions. The work of Dedmon et al. in 2005, identifies long-range contacts between the C-terminal tail and the central region of the protein. Specifically, contacts between residues 120–140 and residues 30–100 were detected. These results were confirmed by Bertoncini et al. in 2005, that explored, with the same methodology, the effect of both temperature and polyamine binding on these long-range interactions. The results obtained at 15 and 37°C verified the contacts described by the previous work. Moreover the C- terminus of α -synuclein was found to adopt an almost fully extended conformation at 47°C that was also induced by polyamine binding. This observation suggests that a release of long-range interactions in the α -synuclein monomer to a fully unfolded state renders the hydrophobic patches of the NAC region accessible, triggering oligomerization and aggregation (Bertoncini et al. 2005).

The analysis of C_{α} and C_{β} secondary shifts at -10°C provided additional information on α -synuclein structure. Residues from 30 to 140 showed a mild propensity toward the α -conformation. The results were confirmed by Kim et al. in 2007, that revealed that, at -15°C, the region between residues 39 and 98, shows both secondary shifts and $^3J(H^N, H^{\alpha})$ coupling values typical of β -conformations. These conclusion ascribed to the region around 40-100, a key role in protein aggregation. Furthermore, the fragment from residues 1 to 38, did not show any propensity for either α -helix or β -sheet.

In contrast to these findings, the analysis of the C_{α} secondary chemical shifts by Bussel et al. in 2001 indicated that, on average, helical conformations populate the N-terminal region (6-37) about 10% of the time.

The application of other biophysical techniques, such as time-resolved fluorescence energy transfer, Raman, and FTIR, led to convergent results: an highly dynamic distribution of secondary structures for α -synuclein, interchanging on the microsecond time scale (Lee et al. 2004 and 2005; Maiti et al., 2004). Single-molecule atomic force microscopy provided new insights on the characterization of the conformational heterogeneity of this protein. Three distinct classes of structures in equilibrium were identified: random coil (38.2%), β -like structures (7.3%), and conformations stabilized by short- and long-distance weak interactions (54.5%) (Sandal et al., 2008).

Membrane bound α -synuclein

A key feature in the sequence of α -synuclein is the presence of seven imperfect 11-amino acid repeats in the first 93 residues. These are characterised by an highly conserved hexamer motif (KTKEGV), which is also a typical feature of the amphipathic α -helices of apolipoprotein family (Davidson et al., 1998). In agreement with this observed sequence similarity, α -synuclein has been reported to bind to acidic synthetic membranes and to undergo a conformational transition to a helical state (McLaurin et al., 2000; Chandra et al., 2003).

The protein-lipid interaction was demonstrated to be very sensitive to several factors, as the chemical properties of the membrane, the ionic strength of the solution, the curvature of the phospholipid surface, and the mass ratio of α -synuclein to lipid (Davidson et al., 1998; McLaurin et al., 2000). While this interaction was extensively studied *in vitro*, the binding of α -synuclein with membranes *in vivo* is not characterised. An important clue comes from the work of Kim et al. in 2006, where they demonstrated that α -synuclein binds with high affinity to detergent-resistant membranes isolated from HeLa cells or synaptic vesicles, without the help of other proteins.

The NMR structure of SDS micelle-bound α -synuclein was determined by Ulmer et al. in 2005, and it can be found on the Protein Data Bank (accession code: 1XQ8). The structure (figure 1.1.14) consists of two curved α -helices between residues Val3-Val37 and Lys45-Thr92, connected by a short linker in an anti-parallel arrangement, followed by a short extended region (Gly93-Lys97) and a predominantly unstructured mobile tail (Asp98–Ala140).

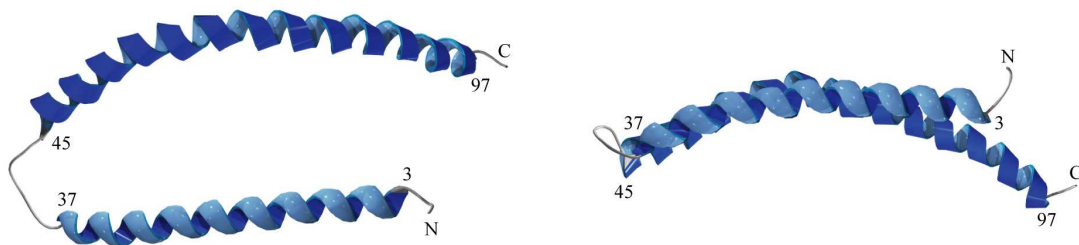


Figure 1.1.14. Structure of micelle-bound α -synuclein (PDB code 1XQ8). The dynamically disordered C-terminal tail has been omitted.

EPR measurements obtained using different singly spin-labelled α -synuclein mutants bound to small unilamellar vesicles provide additional information (Jao et al., 2004; Bortolus et al., 2008; Drescher et al., 2008). However, there are still contradictor results on the presence or absence of an helical break around residues 42-43. The experimental discrepancies can be justified by an inter-conversion between the broken and extended helical (Drescher et al., 2008).

In agreement with its nerve terminal localization and the ability to interact with membranes, it was suggested that α -synuclein can play an important function at the synapse. α -Synuclein knockout mice suggest that the protein plays an important role in the recycle of synaptic vesicles (Abeliovich et al., 2000; Yavich et al., 2004; Liu et al., 2004).

α -synuclein oligomerization and fibril formation

An early indication of the involvement of α -synuclein in the pathogenesis of Parkinson's disease derived from the discovery that it is one of the major components of the pathological hallmarks of the disease, called Lewy bodies (LBs). LBs are intra-neuronal proteinaceous inclusions where α -synuclein was found accumulated in fibrils (Spillantini et al., 1998).

In vitro, recombinant α -synuclein forms amyloid fibrils that have morphology similar to those isolated from LBs (Serpell et al., 2000). Due to the key role of α -synuclein aggregation in the PD pathogenesis, a great effort has been made to understand the structural properties of these fibrils and the mechanism of the fibrillation process. The results obtained from several techniques, as X-ray diffraction, spin label EPR, and solid-state NMR, provide a structural description of α -synuclein fibrils.

Organized, distinct domains were identified, with a tightly packed core region, from residue 35 to 98, characterized by stacking interactions and strong immobilization. The core contains turns or loops along with extended portions of β -strands. Specifically, several regions, characterised by β -structures (regions 37–43, 53–59, 62–66, 68–77, and 90–95) were identified. An increased mobility and almost no stacking interactions were also detected for residues before 35 and after 99, beyond the N- and C-terminal boundaries of the core region (Serpell et al., 2000; Chen et al., 2007; Vilar et al., 2008).

To evaluate the kinetics of fibrillation, fluorescence thioflavin T assays were amply used. This tool provided evidence for sigmoidal behaviour of fibril formation, suggesting a nucleation-dependent lag phase in the aggregation mechanism (Wood et al., 1999; Conway et al., 2000). Soluble oligomeric species are present during the lag phase: by means of AFM, discrete non-fibrillar α -synuclein oligomers have been observed: “spheres”, “chains” and “rings”. It is still unclear whether the transient oligomers are on the pathway to fibrils or are off-pathway dead ends (Fink, 2006).

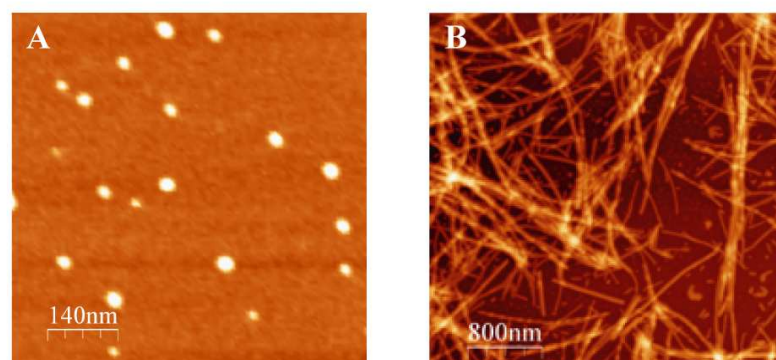


Figure 1.1.15_ AFM images of α -synuclein oligomers. Adapted from Bisaglia et al., 2009.

It has been proposed that annular pre-fibrillar oligomers, rather than fibrils themselves, may be the pathogenic species (Conway et al., 2001). This because it was shown that dopaminergic neurons that contain LBs appear to be healthier than neighboring neurons (Tompkins et al., 1997). Moreover, other studies indicate that α -synuclein annular oligomers can modify the permeability of vesicles by pores formation of pores (Volles et al., 2002, Lashuel et al., 2002).

α -Synuclein binding partners

α -Synuclein is intrinsically unstructured in its native state (Uversky, 2002; Weinreb et al., 1996), however, it has been described to specifically interact with numerous proteins, which possibly alter its native state conformation conformations (Uversky et al., 2001).

The lack of a well-defined secondary or tertiary structure seems to play a key role in the molecular recognition of their partners. The major advantages of structural disorder are the potential binding strength with low specificity, increased speed of interaction, effective regulation by degradation, and ability to bind partners with different structural properties. Furthermore the inherent flexibility of natively unstructured proteins may allows their local and global structure to be modified in response to different molecular targets (Dyson et al., 2002). The possibility of one protein to interact with multiple cellular partners provides the key to regulate complex protein network. The functional importance of structural disorder is related to signal transduction, cell-cycle regulation, gene expression, and chaperone activity (Tompas et al., 2005).

A quantitative proteomic analysis (Zhou et al., 2004) identified more than 250 proteins as soluble α -synuclein binding partners in dopaminergic culture cells. These proteins display different structure and function properties, thus it is hard to derive a common recognition motif. The binding partners of α -synuclein are involved in several key biological processes, as signal transduction, the ubiquitin-proteasome system, vesicular recycling, synaptic functionality, regulation of oxidative stress, and mitochondrial function (Dev et al 2003, Zhou et al., 2004). The great versatility on α -synuclein protein recognition can be justify with a possible regulatory role in the connection points of biological network. A scheme of the major classes of α -synuclein interactors is shown in figure 1.1.16.

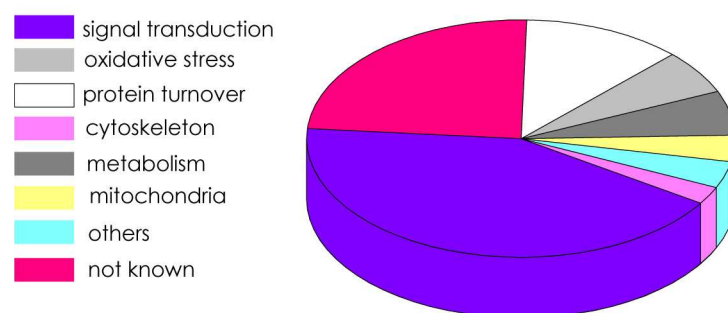


Figure 1.1.16_ Major classes of α -synuclein associated proteins from quantitative proteomic analysis. Adapted from Zhu et al., 2004.

The results derived from the proteomic approach confirmed most of the α -synuclein binding partners identified in previous works (Zhou et al., 2004).

Moreover, the screening by phage display methods, carried out by Woods et al., 2007, allowed the identification of the binding partners that selectively recognise α -synuclein folded on phospholipid vesicles: 20 proteins have been identified with roles in diverse cellular processes related to membrane trafficking, ion channel modulation, redox metabolism, and gene regulation.

Among the wide array of binding partners identified, some proteins are of particular interest in the context of dopamine metabolism. Several data reported that α -synuclein is able to bind to and modulate the activity of dopamine transporter DAT (Torres et al., 2001; Lee et al., 2001) and phospholipase D2 (Jenco et al., 1998; Payton et al., 2004). While the first is involved in the dopamine import from the synaptic cleft, the second has a role on the vesicles recycle and neurotransmitters storage. Moreover, as described previously, α -synuclein was found to interact with both Tyrosine Hydroxylase (Perez et al., 2002) and 14-3-3 proteins (Ostrerova et al., 1999; Sato et al., 2006), suggesting a direct role on dopamine synthesis.

α -Synuclein seems to be also involved the neurotransmitter metabolism, as it is able to inhibit aromatic amino acid decarboxylase activity in dopaminergic cells (Tehrani et al., 2006) and to interact with the signal transduction regulators of the Tyrosine Hydroxylase phosphorylation state: specifically, among the binding partners, we also find Calmodulin (Lee et al., 2002; Martinez et al., 2003), Protein Kinase C (Ostrerova et al., 1999) and Phosphatase PPA2 (Peng et al., 2005).

This interaction network supports a strong role of α -synuclein in the regulation of dopaminergic function. However, most of the interaction are not characterised by biophysical methods, and a great effort will be required to analyse these protein complexes.

An other interesting finding is the ability of several molecular chaperones to interact with α -synuclein in conditions that lead to its fibrillation. Both Hsp70 (Dedmon et al., 2005) and Dj-1 (Zhou et al., 2006) were shown to inhibit α -synuclein aggregation, by binding to the pre-fibrillar species. However it was found that these chaperones are not able to bind to monomeric α -synuclein, implying that specific pre-fibrillar conformations contain structural determinants for chaperone molecular recognition.

Introduction 1.2

**Protein-protein interactions in the
regulation of the heterochromatin
formation**

1.2.1 Heterochromatin function and regulation

Core Histone Proteins and Chromatin function

In higher eukaryotes, genetic information, encoded by DNA, is organized in a highly conserved structural polymer, with the assistance of histone and non histone proteins. The polymer, termed chromatin, is the physiologically relevant form of eukaryotic genomes and it supports and regulate critical function of the genome.

Chromatin represents a scaffold for many genetic events and shows different degrees of condensation, including a relatively open form, termed euchromatin, and a highly condensed form, named heterochromatin (Bottomley, 2004).

The dynamic changes between these two forms is of functional relevance during many genetic processes. Structural reorganizations can determine the accessibility of the protein machinery required for DNA replication, gene expression, DNA repair or recombination events (Eissenberg et al., 2000). The reorganization of chromatin architecture is therefore an eukaryotic molecular mechanism that regulates the functions of the genome (Cheung et al., 2003).

The fundamental repeating unit of chromatin is the nucleosome, which consists of 146 base pairs of DNA wrapped around an octamer of core histone proteins (H) containing one H3-H4 tetramer and two H2A-H2B dimers. Crystal structures were published for both the histone octamer, and of the nucleosome particle (Arents et al., 1991; Luger et al., 1997). The structure representation of the nucleosome complex is shown in figure 1.2.1.

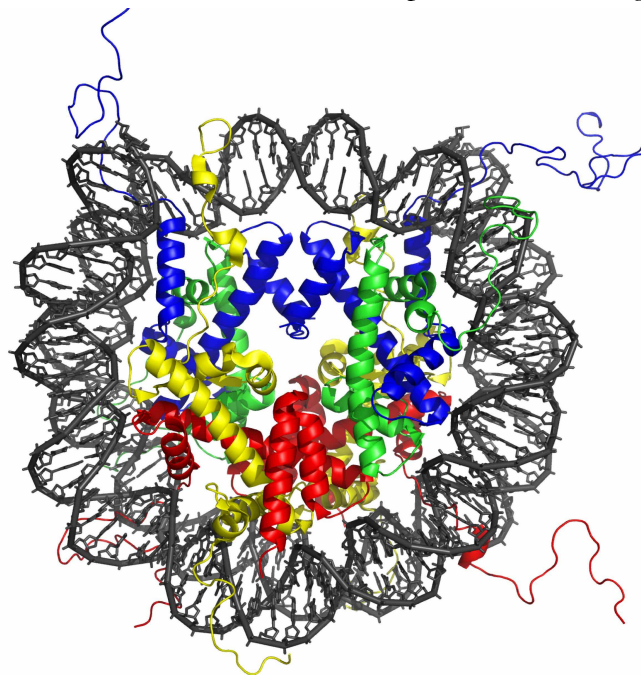


Figure 1.2.1_ Model structure of the nucleosome core particle, based on the crystallographic data deposited by Luger et al., 1997. The four core histone molecules, H2A, H2B, H3, and H4 have been illustrated with different colours: yellow, red, blue and green, respectively.

The four core histone molecules, H2A, H2B, H3, and H4, are among the most evolutionarily conserved proteins (Van Holde, 1996). DNA binds to the positively charged histone surfaces through H-bonds and electrostatic interactions. In the chromatin fibre,

successive nucleosomes are separated by 10–80 base pairs of linker DNA. Histones are composed of flexible amino-terminal tails protruding outward from the nucleosome, and globular carboxy-terminal domains making up the nucleosome scaffold. The amino-termini of histones H3 and H4 protrude from the nucleosome core and contain dense clusters of modifiable residues. Post-translational modifications such as acetylation, methylation and ubiquitination of lysine residues, phosphorylation of serine and threonine residues, and methylation of arginine residues, create new chemical environment that makes chromatin a “signaling platform” to link signal transduction and gene expression. The modifications on N-terminal histone tails leads to change of the chromatin architecture, through the modulation of the contacts between histones and DNA. Because these modifications are reversible, they can act as chromatin-based “on/off” switches that regulate a multitude of DNA-related processes. In figure 1.2.2, an overview of the possible post-translational modifications on N-terminal histones tail is presented.

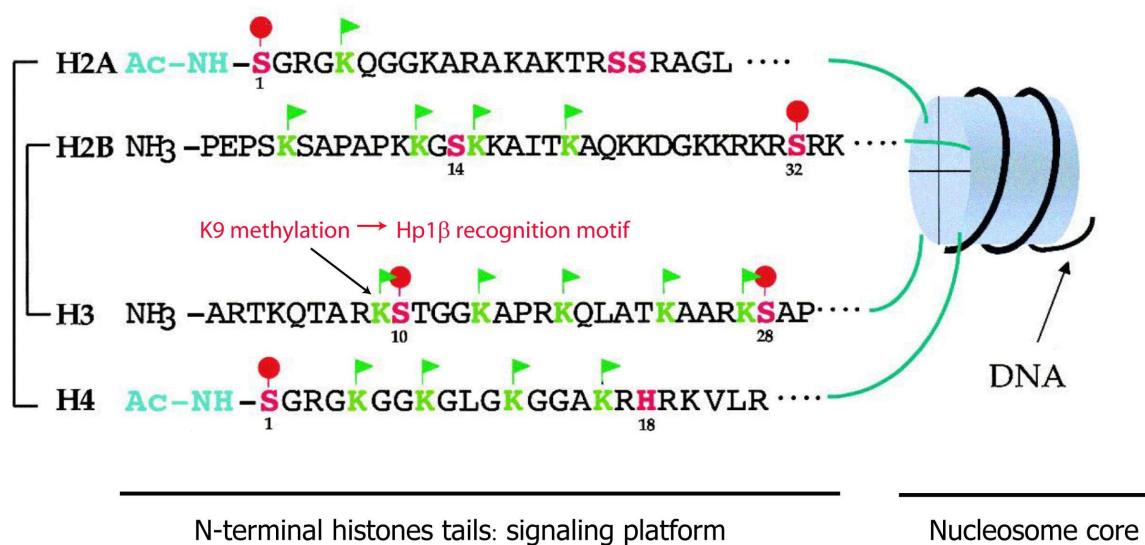


Figure 1.2.2_ Schematic representation of a nucleosome core particle and of the amino acids sequence of the N-terminal tails of the four human histones. Flags represent known post-translational modification sites: phosphorylation, acetylation and methylation. Methylation in K9 is emphasized by the black arrow. Adapted from Cheung et al., 2000.

Histones methylation: regulation of heterochromatin synthesis by interaction with Hp1β protein

Histones modification represents one of the fundamental mechanism to regulate the genome functions, especially in the contest of the transmission of genetic information throughout subsequent generations. It is well recognized that heritable changes in phenotype or gene expression can occur without mutations in the nucleotide sequence. These changes, studied in the epigenetic field, may remain through cell divisions for the remainder of the cell's life and may also last for multiple generations. The remodeling of chromatin structure, without modification of the DNA sequence, represents one of the most important epigenetic mechanisms. In the eukaryotic nuclei, the level of packaging of the chromatin in higher order structures determines the accessibility of the transcription machinery and finally the gene expression (Cheung et al., 2000). Histones modification primarily act either by promoting DNA packaging into repressive heterochromatin, in

which DNA is largely inaccessible, or by relaxing chromatin into the less compact euchromatin. The formation of heterochromatin is indeed a regulatory mechanism to inhibit gene expression in specific regions of the chromosome. Specifically, the packaging involves a particular set of post-translational modifications, in particular methylation. Histones lysines can be mono-, di- or tri-methylated, while arginines can be mono- or di-methylated. Methylation of histone 3 at lysine 9 (H3K9) is one of the most significant examples of the effect of a covalent modification on the chromatin remodelling. Di- or tri-methylation of H3K9 creates a binding site for the chromo domain of Heterochromatin protein 1 (HP1 β). Through the binding to chromatin, HP1 β stabilizes the packaging of the DNA in a structure not readily accessible to transcription, with a final silencing effect on gene expression (Jacobs et al., 2001).

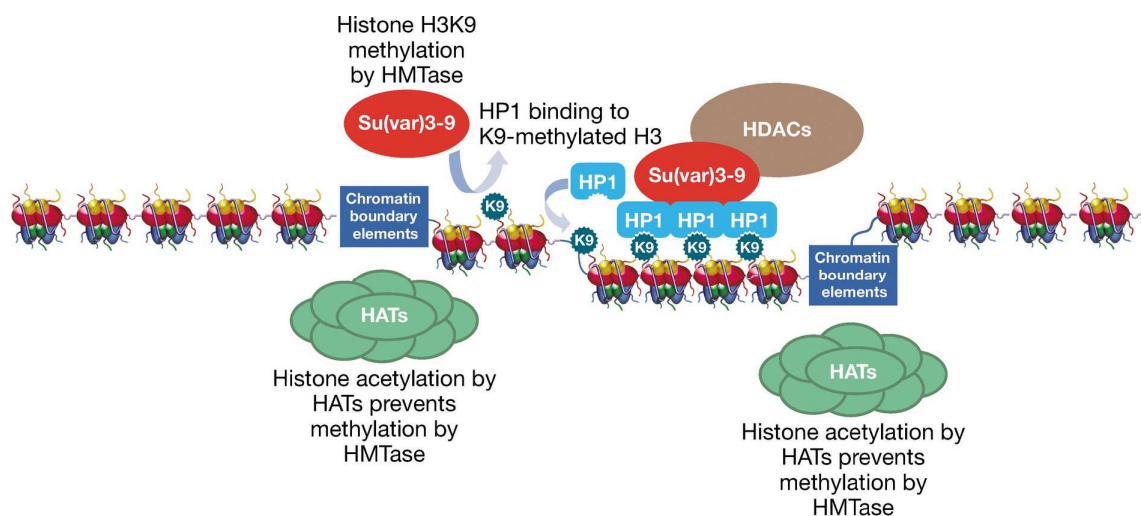


Figure 1.2.3_ Schematic representation of heterochromatin assembly.
Adapted from Shilatifard., 2006.

An interesting model describing the steps of heterochromatin assembly can be found on the review of Shilatifard of 2006. The model proposes that, when histone deacetylase complexes (HDACs) deacetylate histones, methyltransferases (Su(var)3–9 HMTase) can methylate histone 3 on lysine 9. The HP1 β protein can recognize and bind to the lysine 9-methylated histone 3 by its N-terminal chromo domain, and continues the assembly of heterochromatin. The spreading of heterochromatin occurs through self-association of HP1 β molecules, and the use of the HP1 β C-terminal domain, named chromo-shadow, to recruit additional histone 3 lysine 9 methyltransferase machinery. This process can be stopped by “boundary elements,” which are considered to be potential sites for the recruitment of histone acetyltransferases that would prevent the methylation of K9 of histone 3.

1.2.2 Hp1 β : structural details and interactions

HP1 β protein is characterised by an N-terminal chromo domain (CD) connected to a slightly larger, C-terminal domain termed the chromo shadow domain (CSD). The hinge region linking the two motifs is unstructured in solution, enabling the chromo and chromo shadow domains to move independently in the intact protein (Brasher et al., 2000). NMR structures were deposited for the deleted mutants 10-80 and 102-171, corresponding to the two domains alone (PDB code: 1ap0, Ball et al., 1997; 1dz1, Brasher et al., 2000).

Although they are structurally related, with a core of three β -sheets packed against an α -helix, there are some important functional differences: while the chromo domain is monomeric and is responsible for the recognition of the methylated histone 3 N-terminal tail, the chromo shadow domain forms a tightly associated symmetric dimer and binds to non-histone proteins (Eissenberg et al., 2000).

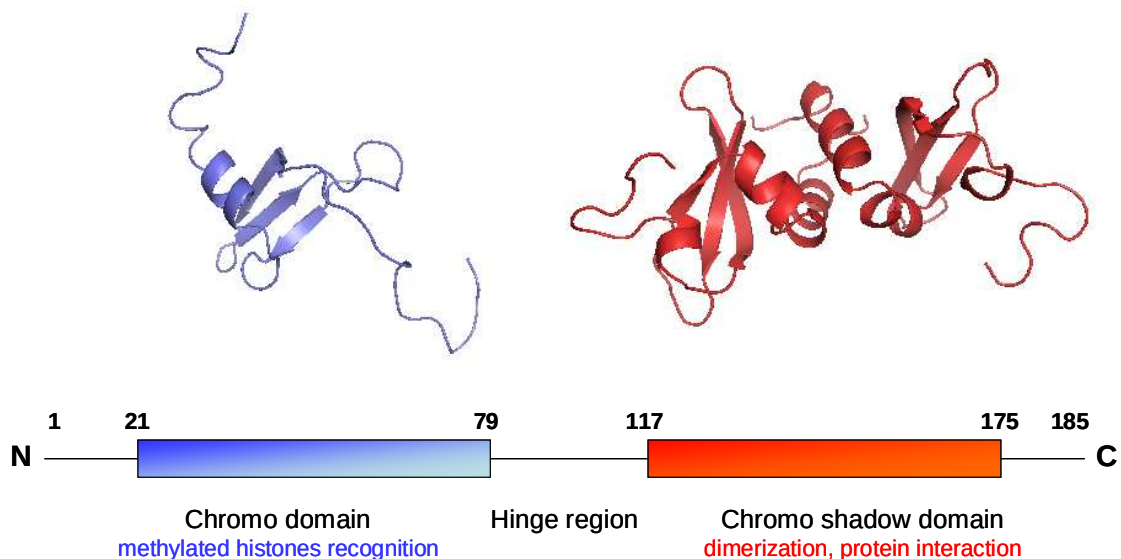


Figure 1.2.4_ Human Hp1 β structure details. In blue the NMR structure of chromo domain 10-80 (Ball et al., 1997). In red the NMR structure of shadow chromo domain dimer 102-171 (Brasher et al., 2000). The common motif consists of three stranded beta sheet packed against an alpha helix..

The NMR studies of the chromo-domain of HP1 β bound to histone H3 methylated on Lys 9 (Jacobs et al, 2001; Nielsen et al, 2002), reveals that the chromo domain is characterised by an hydrophobic pocket that can bind mono-, di- or tri-methyl-Lys 9 with increasing relative affinity (dissociation constant (K_D) $\sim 2 \mu\text{M}$ for tri-methyl-Lys). The binding pocket for the N-methyl groups is provided by three conserved aromatic side chains, Tyr 21, Trp 42 and Phe 45, which reside in two regions that become ordered upon the binding of the peptide. Residues Val 23, Leu 40, Trp 42, Leu 58 and Cys 60 appear to be responsive for the recognition of the polypeptide (Nielsen et al., 2002).

Chapter II.

Results

Protein-protein interaction in the regulation of the dopamine synthesis

2.1 Recombinant protein: cloning, expression and purification

2.1.1 α -Synuclein expression and purification from bacteria periplasm

Since recombinant α -synuclein is an unfolded protein, several methods, including sample denaturation treatment, can be used to purify it from *Escherichia coli* with high efficiency. For this reason, we decided to produce the protein without any affinity tag, to avoid a further step of tag removal that would elongate the procedure and decrease the purification yield.

Moreover, a great improvement in the purification protocol comes from the periplasm fractioning strategy. As demonstrated by Ren et al., 2007, α -synuclein naturally contains a signal, localised in the N-terminal region, that addresses the protein into the periplasm compartment of the bacteria. Therefore, optimizing the protocol published by Huang et al., 2005, 30 mg of highly purified protein can be obtained from 1 litre of culture with few steps: the key starting point is the release of α -synuclein contained in the periplasm by osmotic shock. Details of the procedure can be found in 5.2.11.

Protein expression, using pET28a vector (where α -synuclein was previously cloned by dott. Tessari), was induced with 0.1 mM IPTG when the culture cells reached an O.D. of 0.3. After 5 hours of growth at 37 °C, cells were harvested and treated with the osmotic shock buffer, that is, a high osmolarity solution (40% sucrose) that prepares the conditions for the following osmotic shock. Very few proteins are released in this step, as shown in figure 2.1, lane 4: probably, this is due to the breakage of some cells during the resuspension of the cell pellet. Osmotic shock release of the periplasmic proteins is achieved resuspending the cell pellet in an $MgCl_2$ solution. The supernatant obtained is a partially purified sample, enriched of recombinant α -synuclein. The following 10 min boiling step in 20 mM Tris-HCl buffer, pH 8.0, allows the removal, by precipitation, of most of all the protein contaminants, as shown in lane 7. α -Synuclein and few other smaller proteins resist the treatment and remain in solution (lane 8).

The fundamental step that allows the improvement of the efficiency of the already published protocol (Huang et al., 2005), is the removal of nucleic acid contaminants from the sample. With two steps of ammonium sulphate fractioning, at 35% and 55% of saturation, recombinant α -synuclein can be separated from both DNA and RNA fragments. These contaminants remain in the 55% saturated ammonium sulphate solution, while the protein is selectively precipitated and, after collection by centrifugation, is resuspended in water. Overnight dialysis against water at 4 °C removes the excess salts. The sample, after the addition of concentrated Tris-HCl (pH 8.0) up to 20 mM, is ready for the anionic exchange chromatography.

As shown in figure 2.1_B the anionic exchange chromatography further improves the preparation purity. The chromatographic fractions that contained α -synuclein were taken

and subsequently dialysed against water at 4 °C, overnight. The last dialysis step is performed against MQ water, and then the protein was lyophilized and stored at 4 °C.

The same protocol was used to purify ^{15}N -labelled α -synuclein, expressed in *Escherichia coli* grown in ^{15}N -enriched minimal medium. The yield and quality of the final purified sample are comparable to those described above.

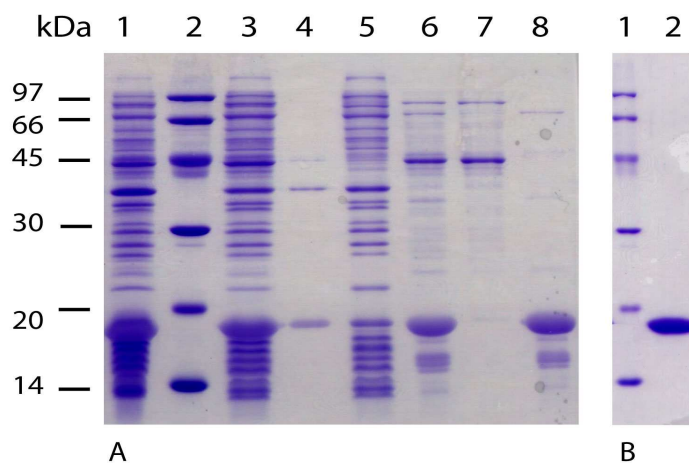


Figure 2.1_ 13% SDS-PAGE of α -synuclein purification from the periplasmic fraction. **A_** Lane 1: whole cell extract after 5h of induction; lane 2: MM markers; lane 3: pellet from cells washed with Osmotic Shock Buffer; lane 4: supernatant from cells washed with Osmotic Shock Buffer; lane 5: pellet from cells treated with an MgCl_2 solution; lane 6: supernatant from cells treated with an MgCl_2 solution; lane 7: pellet from the periplasmic fraction after boiling; lane 8: supernatant from the periplasmic fraction after boiling. **B_** Lane 1: MM markers; lane 2: purified α -synuclein by anionic exchange chromatography.

2.1.2 Characterization of purified α -synuclein

Before starting the protein-protein interaction experiments, the quality of the purified α -synuclein sample was analyzed by several methods, as shown in figure 2.2.

The Far-UV CD spectrum (figure 2.3_A) confirms the unstructured nature of α -synuclein; this property determines a non classical behaviour in Size Exclusion Chromatography (figure 2.3_C): the protein, due to the lack of a well-defined secondary and tertiary structure, elutes like a globular molecule of about 60 kDa as determined by comparison with the calibration curve (not shown). Moreover, the chromatographic profile confirms that the protein preparation is highly homogenous.

The HPLC reverse phase analysis revealed that contaminants are present only in trace (figure 2.3_D). The purified peak was then subjected to Mass Spectrometry analysis (figure 2.3_B): the analysis gives the expected MM for human α -synuclein: 14460 daltons. There are also, as minor components, the +64 daltons oxidised form (on the four Met residues), and a degradation product of 11685 daltons.

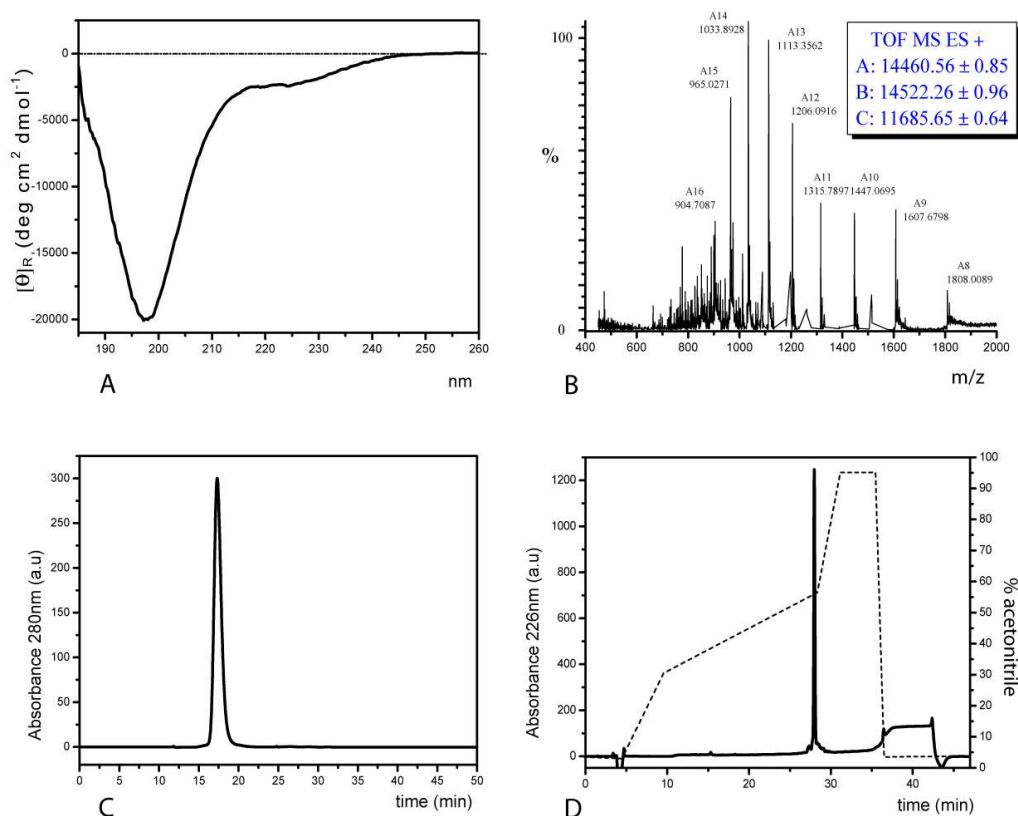


Figure 2.2_ Characterization of purified recombinant α -synuclein. **A.** FAR-UV circular dichroism spectrum. **B.** Mass Spectrometry analysis. **C.** Size-Exclusion Chromatography profile on a Superdex 75 HR 10/30 column. **D.** HPLC Reverse phase Chromatography profile on a C4 column.

2.1.3 Tyrosine Hydroxylase purification

To purify full length Tyrosine Hydroxylase (TH), many protocols can be used, depending on the features of the recombinant protein produced. The purification of the wild type form (expressed in pET3a), with respect to the histidine tagged form (expressed in pET28b), is more challenging and gives a lower yield and purity level. Nevertheless, for the NMR titration experiments, we decided to work with the wild type one, to avoid possible non-specific interactions due to the presence of the affinity tail. For the activity tests, both forms can be used, as the histidine tag does not interfere with the enzymatic reaction.

Moreover, to overcome the problems of secondary cleavages, the enzyme coding sequence was also cloned in the pPROEX vector. This system allows the cleavage of the affinity tag with TEV (Tobacco Etch Virus), an highly specific protease that has no secondary recognition motifs in the TH polypeptide chain.

Below, the purification protocols and results for the wild type and histidine tagged recombinant TH are described. Both include an affinity chromatography step: the protocol for the wild type protein takes advantage of the natural affinity of the TH N-terminal domain for the heparin matrix; the protocol for the histidine tagged form uses IMAC chromatography. Both wild type and histidine tagged proteins resulted catalytically active:

the K_m of 26.6 μ M, determined with the fluorimetric assay described in 5.2.19, is in agreement with our previous results and with published data (Nagashima et al., 1999). The preliminary expression and solubility tests for the protein cloned in pPROEX vectors are also shown.

Purification of TH wild type (pET3)

Starting from the protocol published by Sura et al., 2004, the best result was obtained by fractionating the crude bacterial extract with streptomycin to remove the nucleic acids, followed by $(\text{NH}_4)_2\text{SO}_4$ precipitation and heparin chromatography. The final preparation, although it exhibits some impurities, contains the TH enzyme as the major component (figure 2.3_A, lane 7). The heparin chromatographic fractions that contained TH were dialysed against 20 mM Hepes buffer (pH 7.0), 10% glycerol, 0.1 M KCl, at 4 °C, overnight. The samples were then concentrated with Amicon device, subsequently frozen in liquid nitrogen and stored at - 80 °C.

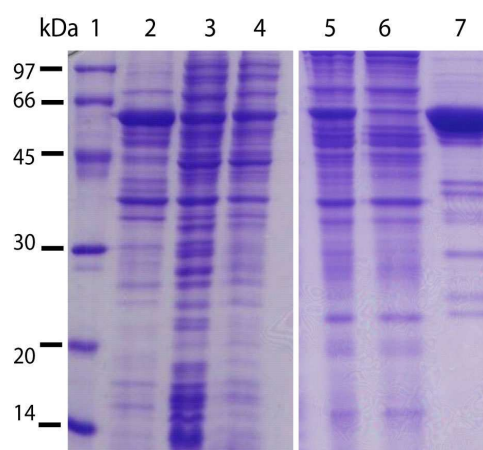


Figure 2.3_ Wild type TH purification. A. 12% SDS-PAGE . Lane 1: MM markers; lane 2: insoluble fraction; lane 3: soluble fraction; lane 4: sample after treatment with 0.9% streptomycin; lane 5: sample after precipitation with 40% ammonium sulphate; lane 6: Heparin flow through; lane 7: purified TH by heparin chromatography.

The low yield obtained, 2 mg per litre of culture, mainly depends by the low protein solubility, which results in most of the recombinant protein being lost in the inclusion bodies.

Purification of TH with an N-terminal histidine tag (pET28)

The purification of histidine tagged TH gives an higher yield with respect to the wild type form. Although, as shown in the SDS-PAGE of figure 2.4, more than 50% of the protein produced is lost in the inclusion bodies, the IMAC chromatography is very efficient: the chromatographic fraction containing the recombinant protein (lane 8) present few contaminants. The next steps are comparable to that described in the previous section. Per litre of culture, 5.5 mg of purified Histidine tagged TH were obtained.

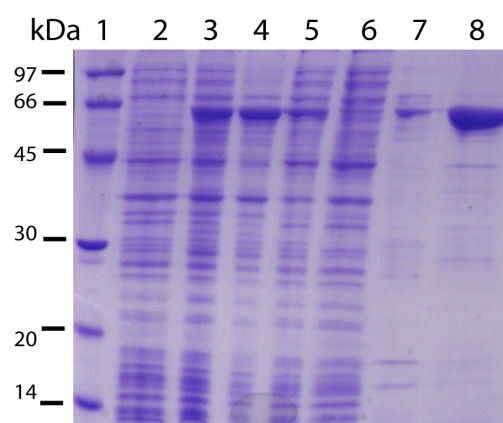


Figure 2.4_ Histidine-tagged TH purification. **A.** 12% SDS-PAGE. Lane 1: MM markers; lane 2: whole cell extract before induction; lane 3: whole cell extract after 3 hours of induction at 28 °C; lane 4: insoluble fraction; lane 5: soluble fraction; lane 6: IMAC flow through; lane 7: purified histidine tagged TH by IMAC chromatography.

TH with an N-terminal Histidine tag (pPROEX): cloning, expression and solubility test

The coding region of full length TH (1-497) was amplified by PCR using synthetic oligonucleotides containing the restriction sites *NarI/KpnI*, suitable to clone into pPROEX vectors. After digestion with the restriction enzymes, the PCR product was inserted into the *NarI/KpnI* linearized pPROEX vector. PCR colony screening and DNA sequencing provide the final product pPROEX_TH 1-497.

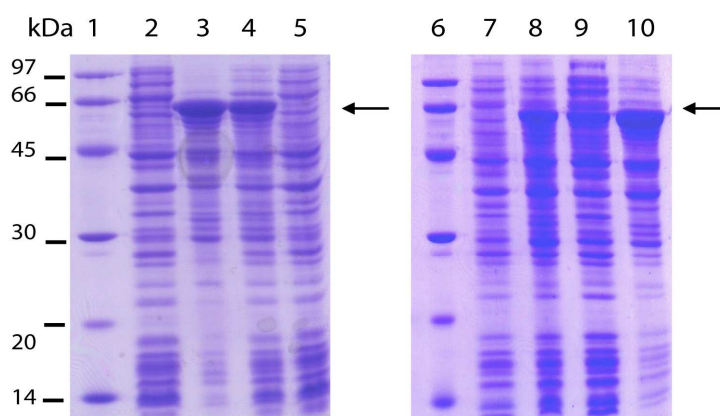


Figure 2.5_ 12% SDS-PAGE of histidine tagged TH (pPROEX) expression and solubility test. Lane 1: MM markers; lane 2: whole cell extract before induction; lane 3: whole cell extract after 3 hours of induction at 28 °C; lane 4: insoluble fraction; lane 5: soluble fraction; lane 6: MM markers; lane 7: whole cell extract before induction; lane 8: whole cell extract after overnight induction at 24 °C; lane 9: soluble fraction; lane 10: insoluble fraction.

Preliminary expression tests were carried out with small scale cultures: the bacteria were grown at 37 °C and then induced with 0.2 mM of IPTG at two different temperatures for different times. The first test, performed at 28 °C for 3 hours, resulted in an efficient

production (lane 3 of the SDS-PAGE of figure 2.5), but the recombinant protein is completely insoluble (lane 4). The reason why the histidine tagged TH, cloned in the pPROEX system, is less soluble than the same clone in pET28 system, probably resides in the high content of hydrophobic residues of the TEV protease recognition motif. The second test, performed overnight at 24 °C, resulted in a smaller total production (lane 8). However, half of the protein is soluble (lane 9) and therefore can be purified by IMAC (work in progress).

2.1.4 Cloning and solubility test of Tyrosine Hydroxylase deleted mutants

Deleted mutants of Tyrosine Hydroxylase were cloned to evaluate if one of them had the proper solubility features to prepare a highly concentrated sample for NMR interaction experiments. Moreover, they could help to map the possible interaction region on the enzyme polypeptide chain. Based on the known structure of the rat protein, we decided to clone sequence fragments corresponding to the domains alone, or in different pair combinations: the N-terminal regulatory domain from 1 to 152; the N-terminal regulatory domain plus the catalytic domain, from amino acid 1 to 457; the catalytic domain, from amino acid 151 to 457, and the catalytic domain plus the tetramerization domain, from amino acid 151 to 497. The first three mutants were cloned into pET3 and pET28 expression vectors, to express the proteins both with and without the N-terminal histidine tag. The latter mutant was cloned in the pPROEX vector as an N-terminal histidine tag form: only in this case, the affinity tag can be removed specifically with TEV protease, as this enzyme does not have secondary recognition motifs in the Tyrosine Hydroxylase sequence, like Thrombin (the protease used with pET28 expression) does. The coding regions of these mutants were amplified by PCR using synthetic oligonucleotides containing the NdeI/BamHI restriction sites, suitable for cloning into pET vectors, or NarI/KpnI, to clone into pPROEX vectors. After digestion with the restriction enzymes, the PCR products were inserted into the NdeI/BamHI linearized pET3 and pET28 expression vectors, or into the NarI/KpnI linearized pPROEX vector.

Figure 2.6 shows the cloning strategy for the deleted mutants and the PCR products separated on 1.5% agarose gel.

PCR colony screening and DNA sequencing provided the final products: pET3_TH1-152; pET3_TH1-457; pET3_TH151-457; pET28_TH1-152; pET28_TH151-457; pPROEX_TH151-497.

The next step consisted in the screening of expression conditions with small-scale cultures. Specifically, two temperatures (28 °C and 37 °C), three IPTG concentrations (0 mM, 0.25 mM and 0.5 mM) and different times (1, 2, 3, 4 hours up to overnight) were tested.

The results are shown in figure 2.7_A and B. The data obtained with pET28 vectors expression are not shown, as they are comparable to those obtained with pET3a vector system.

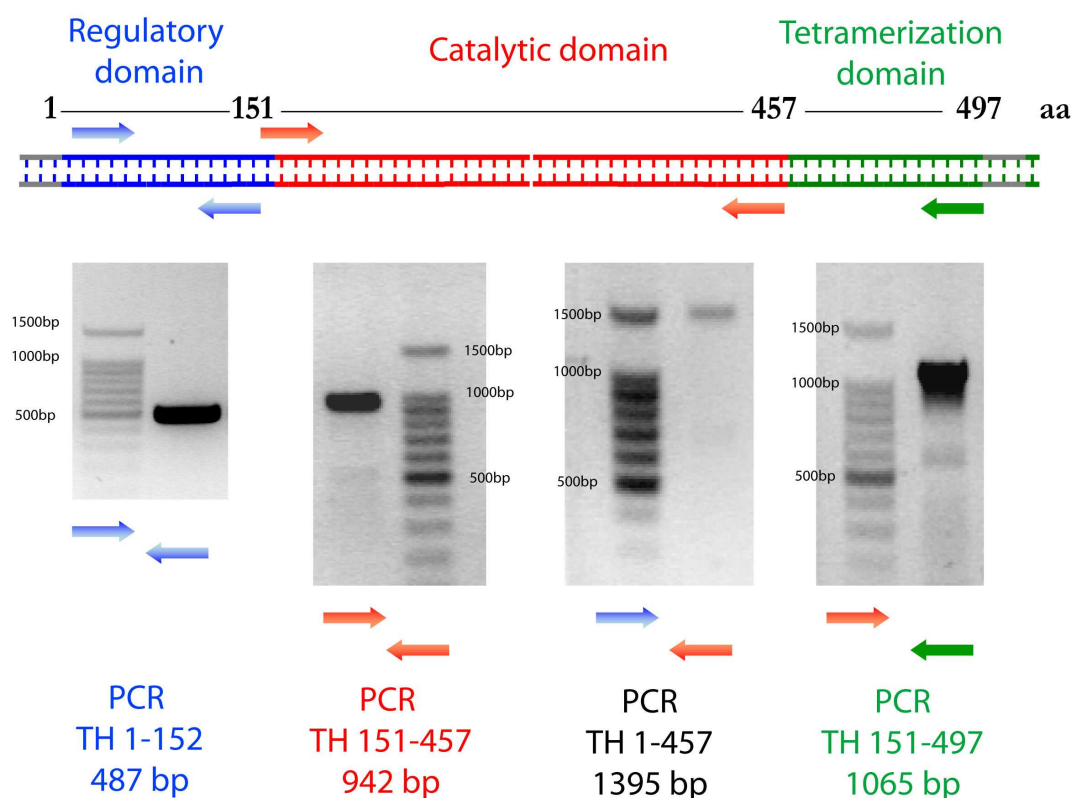


Figure 2.6_ Cloning strategy for human Tyrosine Hydroxylase deleted mutants. The PCR products are shown in the 1.5% agarose gels. From left to right: TH 1-152 insert, TH 151-457 insert, and TH 1-457 insert, all obtained with primers containing BamHI/NdeI restriction sites; TH 151-497 insert, obtained with primers containing NarI/KpnI restriction sites.

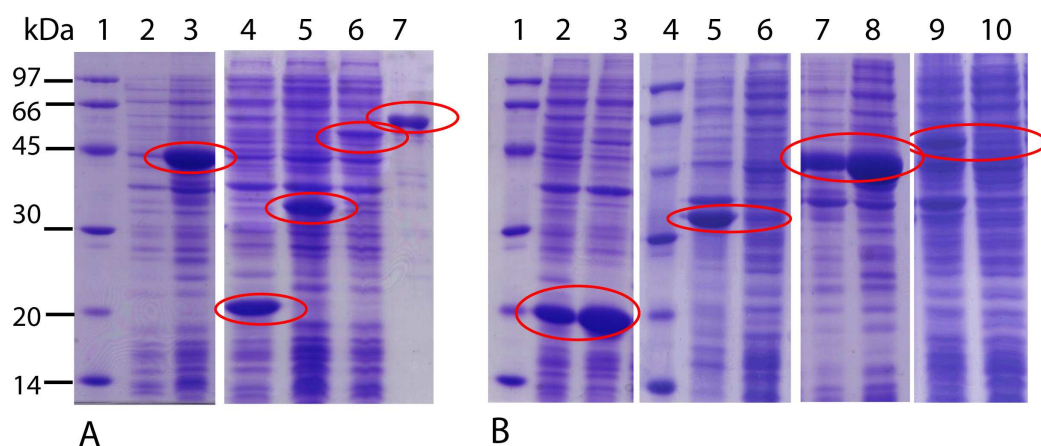


Figure 2.7 A. SDS-PAGE of TH deleted mutant expression. Lane 1: MM markers; lane 2: whole cell extract before induction; whole cell extract after TH 6his_151-497 (lane 3); TH 1-152 (lane 4); TH 151-457 (lane 5); TH 1-457 (lane 6); lane 7: purified wild type TH. **B.** SDS-PAGE of TH deleted mutant solubility test. Lane 1: MM markers; lanes 2 and 3: soluble and insoluble fractions of cells expressing TH 1-152; lanes 4 and 5: insoluble and soluble fractions of cells expressing TH 151-457; insoluble and soluble fraction of cells expressing TH 151-497 (lanes 6 and 7) or TH 1-457 (lanes 8 and 9).

The TH 1-152 mutant is expressed at the highest level at 37 °C, overnight and without IPTG. Most of the protein is insoluble in all the growth and induction conditions tested. The TH 151-457 mutant is expressed in good amounts at 37 °C, with 3 hours of induction, both with 0.25 and 0.5 mM IPTG. Also in this case, most of the protein is found in the inclusion bodies, in all the conditions tested. The TH 1-457 mutant gave the worst results, both in terms of total amount produced and solubility. The result obtained at 37 °C, with 4 hours of induction with 0.25 mM IPTG, is shown. The best protein production yield and solubility were obtained for the TH 151-497 mutant, expressed at 28 °C for 3 hours, with 0.25 mM IPTG.

The only deleted mutant with good solubility is the one that contains the tetramerization domain. Probably, this part of the protein is essential to stabilize the enzyme structure, preventing non specific interaction that lead to protein aggregation. Another explanation for the poor solubility of most deleted mutants, is the cloning strategy: It is possible, for example, that we should have chosen a different sequence length to clone the catalytic core alone, or in combination with the N-terminal part, to stabilize it as a monomer. This because adding, or removing few residues can sometimes determine the fate of the folding of the deleted mutant.

Based on these preliminary results, we decided to focus on the purification of TH 1-152 and TH 151-497.

The first one was chosen because, among the three most insoluble mutants, is that with the best chance to be purified: it is found, in a small percentage, in the soluble portion and, due to its low MW, it has more possibilities to be successfully refolded from the inclusion bodies.

TH 151-497 purification

Since TH 151-497 mutant was cloned in the pPROEX system, in an N-terminal histidine tagged form, IMAC Chromatography was used to purify it with high efficiency, followed by affinity tag removal with TEV proteolysis.

As shown in the SDS-PAGE of figure 2.8, most of the recombinant protein binds efficiently to the chromatographic matrix. However, other contaminants are present in the elution fractions (lanes 6, 7 and 8). Therefore, a further chromatographic step was included to improve the sample quality. The chromatographic fractions were combined together, concentrated with an AMICON ultrafiltration device, and subjected to a size exclusion chromatography step to remove both the contaminants and the imidazole salts. Lane 9 of the SDS-PAGE shows that, after size exclusion chromatography, most of the contaminants were removed from the sample. At this point, the purification yield is 12 mg per litre of culture.

To cleave the affinity tag, TEV proteolysis was carried out at 30 °C for 3 hours: since TEV had been produced as a recombinant histidine tag enzyme, the removal of both the protease and the cleaved histidine tag was achieved purifying the sample with a further IMAC chromatographic step. The flow through, containing TH 151-497 without affinity tag, was then concentrated and finally subjected again to size exclusion chromatography to

give the sample shown in lane 10 of the SDS-PAGE. The last chromatographic step was used to simply change the buffer system: alternatively, dialysis can be used.

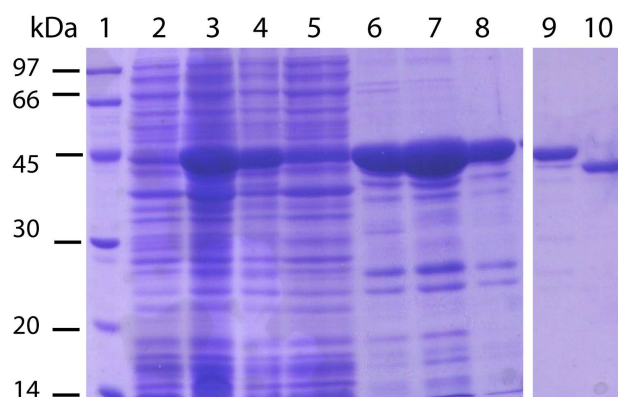


Figure 2.8_ TH 151-497 expression and purification. **A.** 12% SDS-PAGE. Lane 1: MM markers; lane 2: whole cell extract before induction; lane 3: whole cell extract after 4 hours of induction; lane 4: soluble fraction; lane 5: IMAC flow through; lanes 6 to 8: fractions from TH 151-497 IMAC elution; lane 9: purified TH 151-497 by size exclusion chromatography; lane 10: TH 151-497 after tag removal by TEV proteolysis.

TH 151-497 characterization

The purified TH 151-497 sample was analyzed with different methods: the fluorimetric assay results showed that the recombinant protein is active (data not shown) but the K_M has not been measured yet. The sample is very homogeneous, as the protein elutes in a well-defined, unique peak in a Superdex 200 Chromatography column (figure 2.9_B). The comparison with the calibration curve (not shown) allowed us to confirm the tetrameric state of the mutant, as it elutes like a globular protein of about 180 kDa. HPLC reverse phase chromatography results (figure 2.9_A) confirmed the homogeneity of the sample.

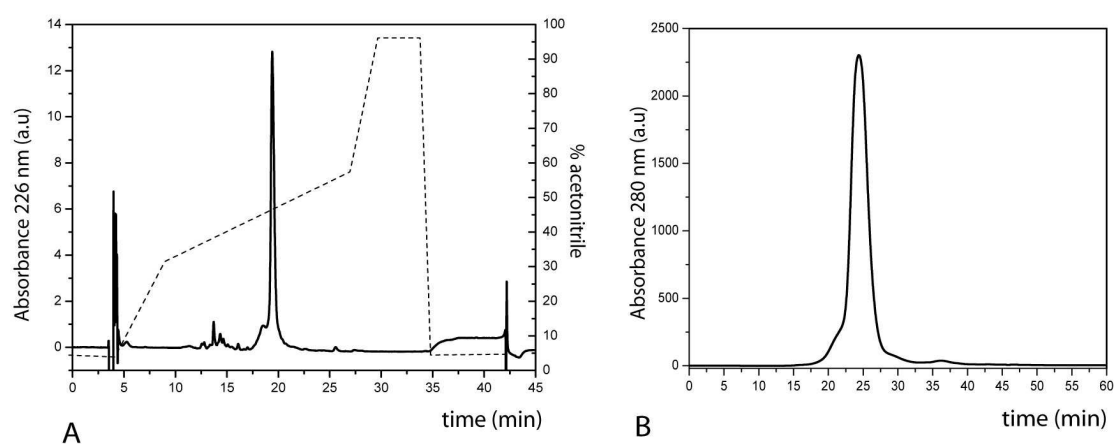


Figure 2.9_ Characterization of purified TH 151-497. **A_** HPLC Reverse phase Chromatography profile on a C4 column. **B_** Size exclusion chromatographic profile with Superdex H/R 200.

The FAR UV circular dichroism spectrum of figure 2.10 demonstrates that the protein retains the characteristic helical secondary structure that is present both in the catalytic and in the tetramerization domain.

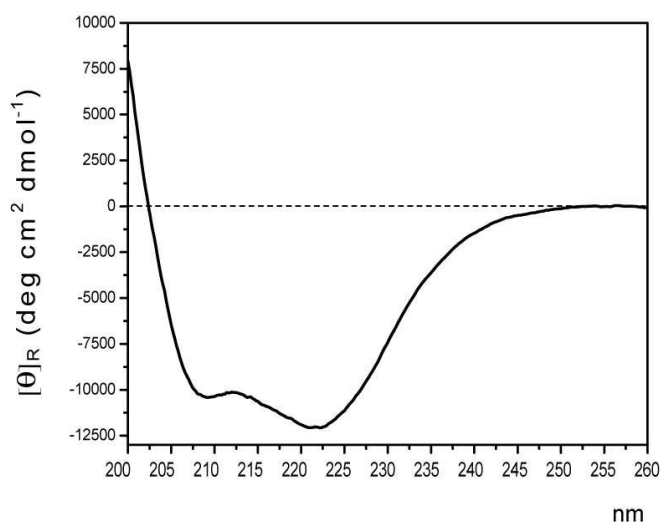


Figure 2.10_ FAR UV circular dichroism spectrum of TH 151-497.

TH 1-152 purification from inclusion bodies

Extensive work was carried out to purify TH 1-152 from the soluble fraction of the bacterial extract: heparin and cationic exchange for the protein without tag, and IMAC for the histidine tagged protein, were tested without success. Most of the soluble recombinant protein did not bind to any of these matrices. The reason for the complete lack of affinity is the strong binding of TH 1-152 to nucleic acids present in the bacterial extract, probably due to its high isoelectric point (estimated pI, based on the sequence: 9.6). Therefore, many protocols were tested to selectively remove the contaminants from the soluble protein sample. Selective nucleic acids precipitation with $(\text{NH}_4)_2\text{SO}_4$, streptomycin, or polyethylene-imine results in the simultaneous precipitation of TH 1-152. Digestion with DNase and RNase, followed by dialysis, was inefficient.

Therefore, we decided to change strategy switching to purification from inclusion bodies. Also in this case, there was the problem of nucleic acids contamination till we chose to purify the inclusion bodies with solutions containing sodium deoxycholate. This detergent allowed the efficient separation of TH 1-152 from nucleic acids. Sodium deoxycholate was always used to purify that protein from inclusion bodies.

The final optimised protocol for TH 1-152 purification starts with washing steps of the inclusion bodies, collected by centrifugation after cell breakage by sonication, with the solutions described in the materials and methods chapter. After solubilisation in buffer containing 5 M urea, protein refolding was achieved by multi step dialysis at 4 °C. The SDS-PAGE (lane 3) and the HPLC reverse phase chromatographic profile of figure 2.11 show the result of the procedure. The refolding yield, for a starting protein concentration of 3.7 mg/ml, was about 50%. However, small changes in starting material or dialysis procedure can decrease this value. The purification yield can be very high, but there is still

variability in the final value: depending on the success of the refolding step, 15 to 25 mg of soluble purified protein can be obtained from 0.5 litre of culture. However, the sample is not stable over a long time and precipitation was observed after some days.

Comparable results were obtained with both TH 1-152 expressed in pET3 system (without tag) and pET28 system (with histidine tag).

TH 1-152 expressed in pPROEX system is less soluble and precipitates completely during refolding by dialysis. However, recent experiments show that TEV protease can cleave the histidine tag even in 1 M Urea solutions before the refolding step (data not shown), thus improve the protein solubility.

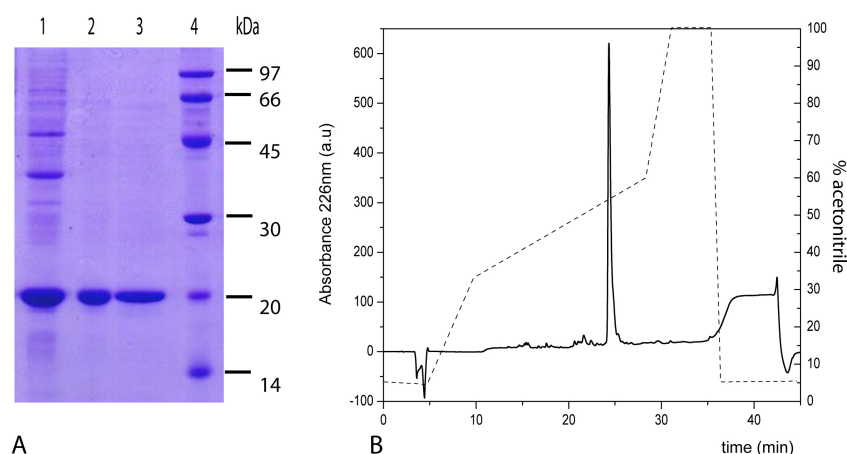


Figure 2.11_ A. 13% SDS-PAGE of TH 1-152 purification from inclusion bodies. Lane 1: total insoluble fraction; lane 2: soluble fraction after 5 M urea solubilisation; lane 3: sample after refolding by dialysis; lane 4: MM markers. **Figure 2_B.** Elution chromatographic profile of TH1-152 by HPLC reverse phase on a C4 column.

This purification protocol, successfully set up for the protein purified from bacteria grown in LB medium, is inefficient when the protein is produced in an ^{15}N -enriched minimal medium. Therefore, to prepare the NMR sample, we changed the purification strategy adding a chromatographic step after the isolation and urea solubilization of the inclusion bodies. The IMAC chromatography performed in denaturing conditions (8 M Urea) provides a good of purification (data not shown). Elution was performed with a pH gradient from 8 to 4.3. The last step is the usual refolding carried out by step dilution in Tris-HCl or phosphate based buffer.

TH 1-152 characterization

As shown in figure 2.12_B, the size exclusion chromatographic profile is characterized by a unique, well-defined, and quite sharp elution peak around 28.7 minutes. Using the calibration curve, we found that the elution time corresponds to a protein of about 40 kDa. The MM of the protein, determined by mass spectrometry, is 16453 daltons (the expected value minus one Met). This difference can be explained with the partially unfolded nature of the protein, which justifies a bigger hydrodynamic ratio, or with the formation of dimers in solution.

The FAR-UV circular dichroism spectrum is shown in figure 2.12_A. Deconvolution analysis gives a mixture of secondary structures estimated with a 25.6% of α -helix, 22.3% of β -sheet and the remainder as a non specified random, turn or loop contributions.

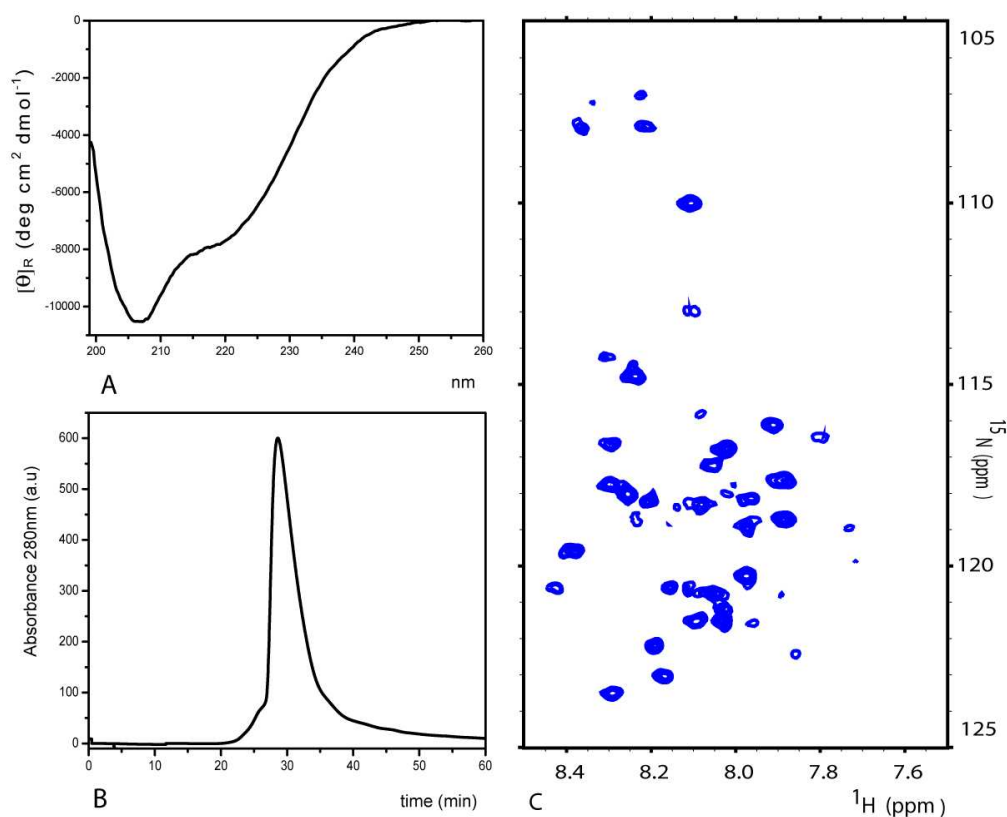


Figure 2.13_ Characterization of purified histidine tagged TH 1-152 **A.** FAR-UV Circular dichroism Spectrum. **B.** Size-Exclusion Chromatography profile on a Superdex 200 HR 10/30 column. **C.** ^1H - ^{15}N HSQC of 50 μM ^{15}N -labelled TH 1-152, recorded on a 600 MHz, at 283 K.

In figure 2.12_C, the ^1H - ^{15}N Heteronuclear Single Quantum Coherence (HSQC) spectrum of ^{15}N -labelled Histidine tagged TH1-152 is shown. It was recorded at 283 K in 3h 30min at 600 MHz, at a protein concentration of 50 μM . First of all, it is important to notice that only 40 peaks of the expected 160 (including the affinity tag amino acids and minus the 11 proline residues) are detectable. The narrow distribution of these resonances along the proton dimension (about 0.8 ppm) suggests the lack of a well-defined secondary structure. Because of the high flexibility of the backbone, the amide ^1H - ^{15}N signals of unfolded proteins are stronger and sharper than those of well-structured molecules. Possibly, in the experimental conditions used to acquire this spectrum, the only part of TH1-152 able to give detectable resonances is unstructured. The remainder of the protein could be involved in extended conformational exchange, thus preventing the acquisition of sharp and unique signals. The absence of the other signals can also be explained by the very low concentration used. It will be important to change conditions such as temperature, pH, protein concentration and acquisition parameters to detect the missing signals of the rest of the protein.

Finally, using the Bradford assay, by comparison with a BSA standard calibration curve, the histidine tagged TH 1-152 extinction coefficient in phosphate buffer (pH 7.4), was estimated as Abs 0.1%=0.215.

Table 2.1.1_ Principal features of the Tyrosine Hydroxylase deleted mutants produced

Cloned sequence	vector	Expression features	Protein domain
TH 1-152 TH 1-152A17R TH 1-152 6His (Thrombin) TH 1-152 6His(Tev) TH 1-152 A17R 6His(Tev)	pET3 pET3 pET28 pPROEX pPROEX	28° and 37°C: mostly insoluble Inclusion bodies	regulatory domain
TH 1-457	pET3	28° and 37°C: insoluble Inclusion bodies	regulatory domain + catalytic domain
TH 151-457 TH 151-457 6His (Thrombin)	pET3 pET28	28° and 37°C C: insoluble Inclusion bodies	catalytic domain
TH 151-497 6His (Tev)	pPROEX	28°C: soluble	catalytic domain + tetramerization domain
TH 1-497 6His (Tev)	pPROEX	28°C: partially soluble 37°C: insoluble Inclusion bodies	full length protein

2.2 Analysis of α -synuclein/14-3-3 interaction

Several lines of evidence, from proteomic (Zhou et al., 2004), immunological (Ostrerova et al., 1999) and biophysical (Sato et al., 2006) studies, indicate that α -synuclein interacts with 14-3-3 proteins both in physiological and pathological conditions (Xu et al., 2002).

The experiments described below are focused on the use of different biochemical and spectroscopic methods to analyse this protein-protein interaction in solution, at the molecular level, using the recombinant forms.

2.2.1 Biochemical characterization

The first experimental approach chosen to study the interaction of α -synuclein and 14-3-3 proteins in solution, combines both electrophoretic and chromatographic methods.

We performed NATIVE PAGE electrophoresis with samples of 14-3-3 ζ and α -synuclein incubated at different molar ratios: 1:1; 1:2; 1:3; 2:1. BSA was used as a standard. The result (figure 2.2.1), shows that the electrophoretic mobility of both 14-3-3 ζ and α -synuclein does not change comparing the mixture with the samples of the proteins alone. Moreover, there are no new bands that can be associated with the formation of the protein complex.

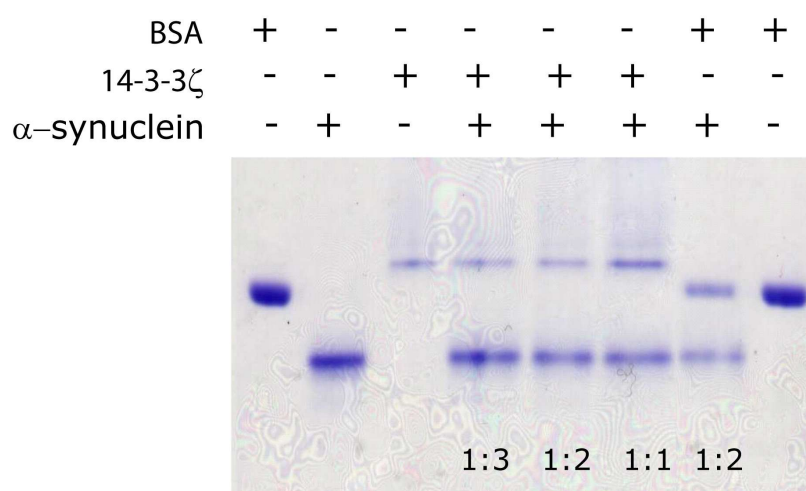


Figure 2.2.1 15% Native PAGE to study α -synuclein/14-3-3 ζ interaction. BSA was used as a standard. From left to right: BSA alone. α -Synuclein alone. 14-3-3 ζ alone. 14-3-3 ζ / α -synuclein mixtures at different molar ratios: 1:3; 1:2; 1:1. BSA/ α -synuclein mixture at a 1:2 molar ratio. BSA alone.

Size exclusion chromatography experiments were also performed, with samples of the two proteins incubated at different molar ratios (14-3-3 ζ / α -synuclein at 1:1; 1:2; 1:4; 2:1) and in different buffer conditions (PBS, Tris-HCl 20 mM, Tris-HCl 20 mM NaCl 150 mM, all at pH 7.4 and 298 K). The results of all these experiment are very similar, therefore only those of the experiments performed in PBS are shown (figure 2.2.2).

Since size exclusion elution times depend not only on the molecular weight but also on the molecular shape, α -synuclein, that is intrinsically disordered, elutes with an apparent

molecular mass of 50 kDa (19.3 minutes on the Superdex 75 H/R). The elution peak of α -synuclein is very close to that of 14-3-3 ζ that has a highly compact structure and elutes at the expected time for a stable dimer of 60 kDa (17.4 minutes).

For all the samples tested, there is no a significant change in the elution time of both 14-3-3 ζ and α -synuclein, comparing their combination at different molar ratios (red line) with the sample containing the protein alone (blue and green lines).

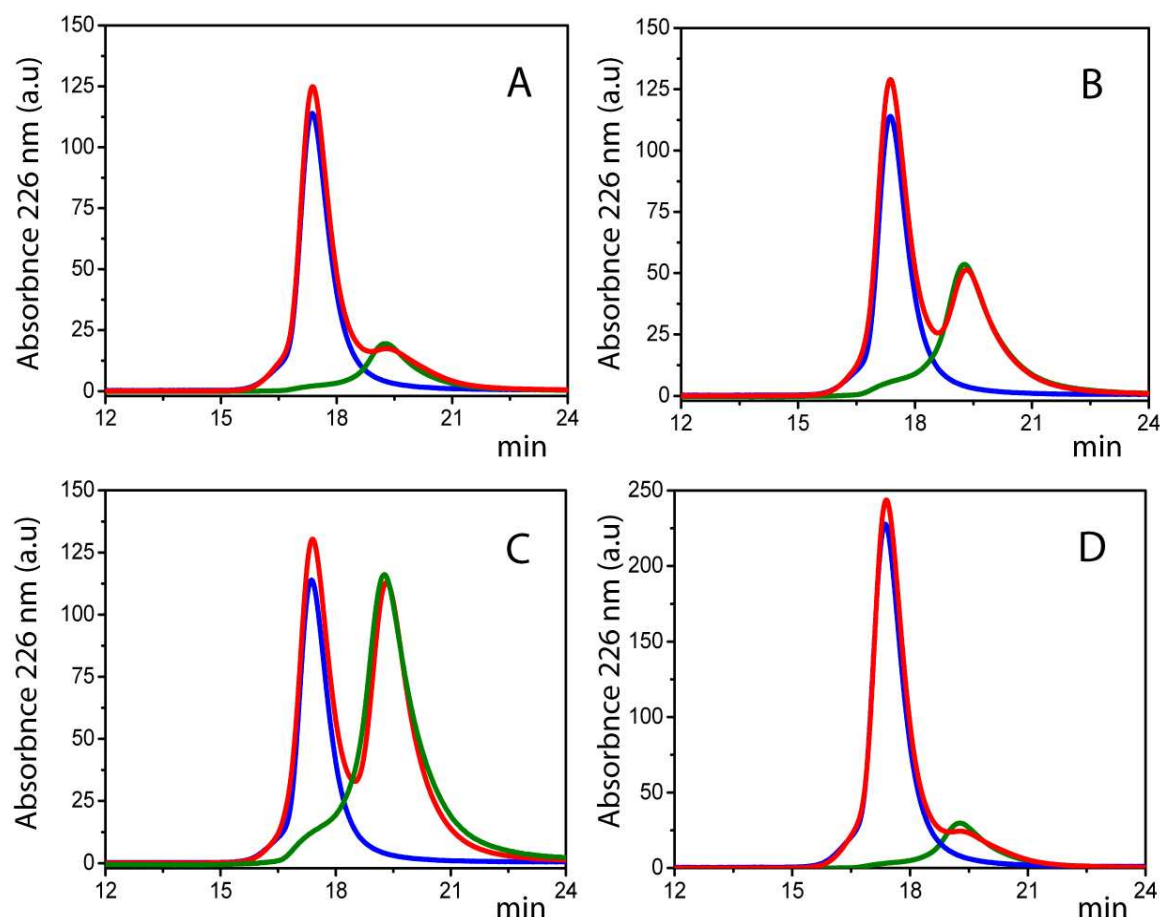


Figure 2.2.2_ Analytical size exclusion chromatography to study α -synuclein/14-3-3 ζ interaction. The experiments were carried out at room temperature, on a Superdex 75 H/R column equilibrated with Phosphate Buffer Saline (pH 7.4). The flow rate was 0.44 ml/min: only the central part of the chromatographic profile is shown, since from 0 to 12 min, and from 24 to 60 min, there is no protein elution.

The red line represents the elution profile of the protein mixture at different α -synuclein/14-3-3 ζ molar ratios **A.** 1:1 (15 μ M:15 μ M) **B.** 2:1 (30 μ M:15 μ M) **C.** 4:1 (60 μ M:15 μ M) **D.** 1:2 (15 μ M:30 μ M). The green line represents the elution profile of α -synuclein alone, normalized with respect to the concentration in the corresponding protein mixture; the blue line shows the elution profile of 14-3-3 ζ alone, normalized with respect to the concentration in the corresponding protein mixture.

Taken together, the results of these experiments indicate that the two recombinant proteins fail to form a stable complex in solution. Comparable results were obtained using the 14-3-3 η isoform.

Therefore, the formation of a transient complex was investigated by NMR.

2.2.2 NMR characterization with [^1H - ^{15}N] HSQC titration experiments.

Chemical shift and signal intensity perturbation analysis

Since with the biochemical approach we were not able to detect a stable complex of the two proteins in solution, we investigated the formation of a transient complex by NMR. Specifically, the titration of ^{15}N -labelled α -synuclein with 14-3-3 ζ and η isoforms was followed by [^1H - ^{15}N] HSQC (Heteronuclear Single Quantum Coherence) experiments. For an ^{15}N -labelled protein, every peak in this 2D NMR experiment correlates the ^{15}N chemical shift with that of the directly attached ^1H . The position of each peak is dependent on the chemical environment of each amide in the protein; hence, every different protein is characterized by a unique fingerprint [^1H - ^{15}N] HSQC spectrum.

Because the protein amide chemical shifts depend greatly on environmental changes, any alteration in the pH, temperature or binding to other molecules can induce variations in the spectra. Therefore, this type of experiment has become an effective tool to detect protein-protein interaction, even in the ultra-weak affinity range ($K_d > 10^{-4}$ M) (Vaynberg et al., 2006).

The [^1H - ^{15}N] HSQC spectrum of α -synuclein, recorded in PBS (pH 7.4) at 283 K, is shown in figure 2.2.3. The assignment came from the work of Dedmon et al., 2005 and Fernandez et al. 2004.

The interaction experiments were carried out by mixing the concentrated ^{15}N -labelled α -synuclein with protein 14-3-3 at different molar ratios.

In figures 2.2.4, the plots of backbone chemical shift differences (δ_{average}), as a function of residue number, for ^{15}N -labelled α -synuclein in the presence of 14-3-3 ζ and η proteins (molar ratio 1:3), relative to free α -synuclein, are shown.

These data demonstrate that there is no significant perturbation of the α -synuclein chemical shifts in the presence of the binding partners, since all the values are lower than 0.01 ppm. These changes are comparable or lower than the error bars (measured as standard deviation from the mean of the chemical shift of three independent [^1H - ^{15}N] HSQC spectra of α -synuclein alone). This result suggests that there is no interaction between the proteins.

To rule out a sensitivity issue as the origin of this negative result, for which the signals shifted derived from the complex are too weak and/or broadened to be detected, we performed a signal intensity analysis. In fact, besides modifications in the chemical environment, binding can cause changes in the dynamics of a protein, which can be reflected in the linewidth of the peaks.

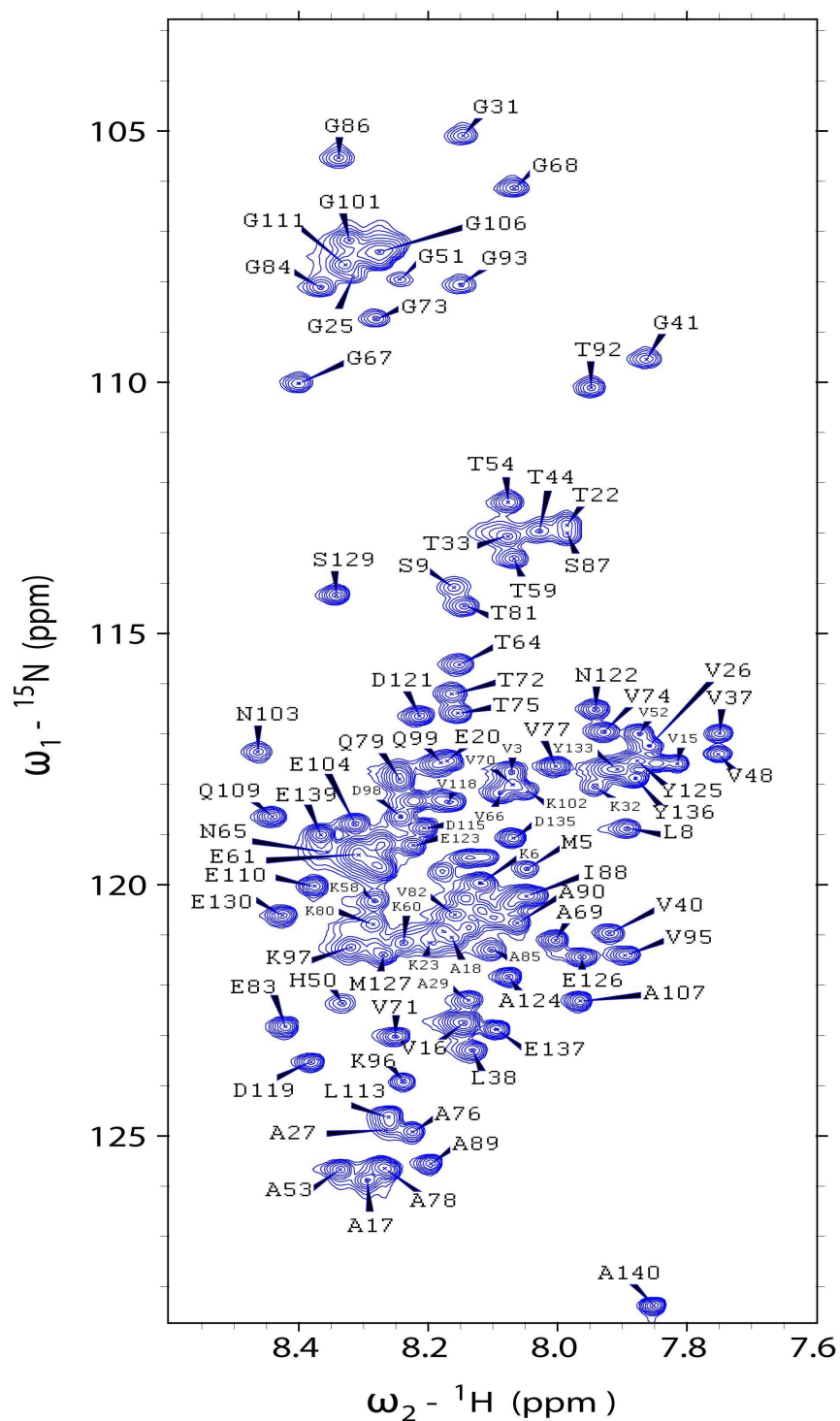


Figure 2.2.3_ [^1H - ^{15}N] HSQC spectrum of ^{15}N -labelled α -synuclein, 120 μM in PBS buffer (pH 7.4), recorded at 283 K on a 600 MHz instrument.

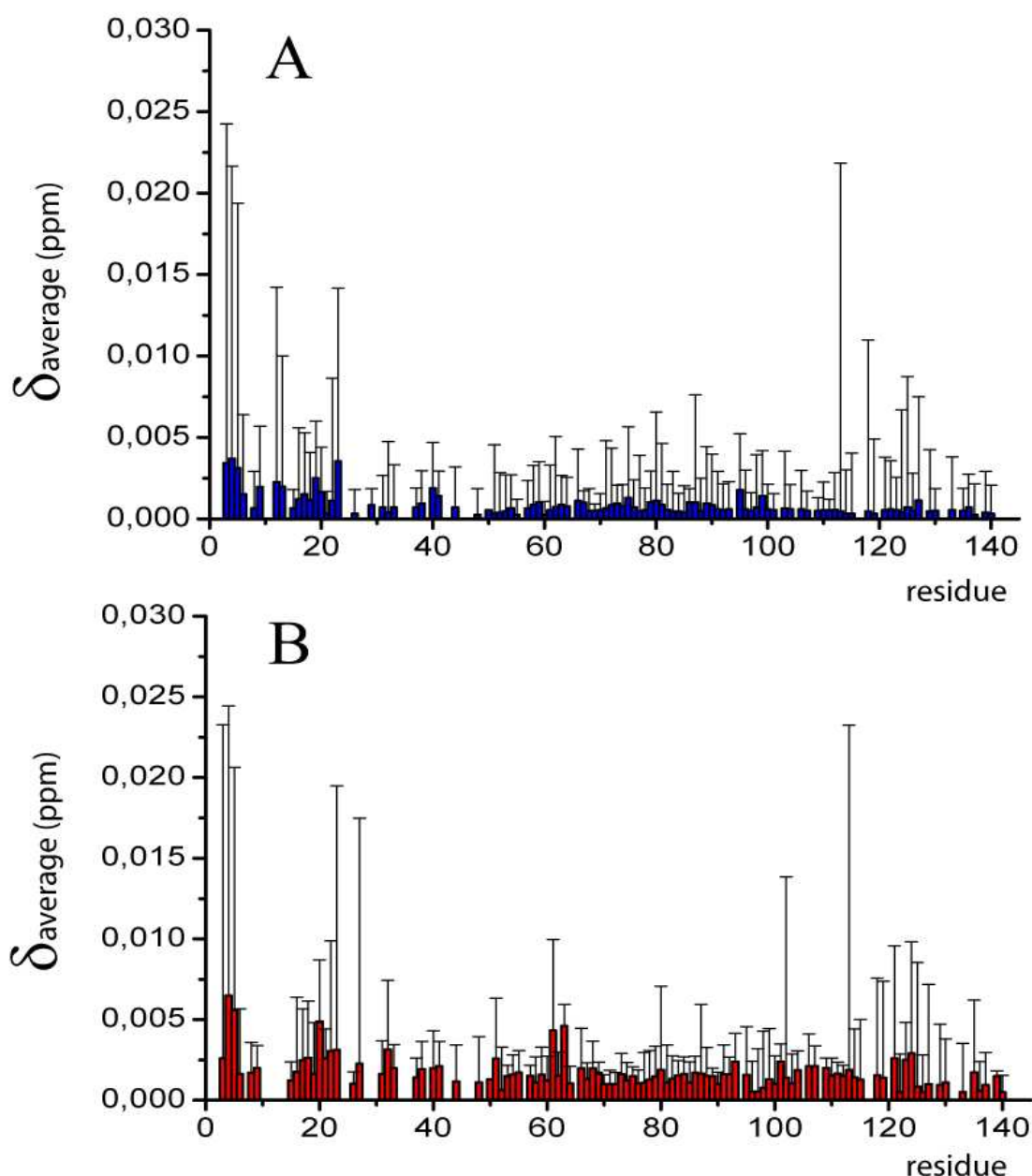


Figure 2.2.4_ Chemical shift perturbation analysis of $[^1\text{H}-^{15}\text{N}]$ HSQC α -synuclein/14-3-3 titration experiment. The backbone chemical shift differences (δ_{average}) as a function of residue number for ^{15}N labelled α -synuclein in the presence of 14-3-3 proteins, relative to free α -synuclein are shown. **A.** α -synuclein/14-3-3 ζ , 1:3 molar ratio; **B.** α -synuclein/14-3-3 η , 1:3 molar ratio.

In figure 2.2.5, the plots of the $[^1\text{H}-^{15}\text{N}]$ HSQC peak intensity changes relative to free α -synuclein (I/I_{free}) are shown for α -synuclein in the presence of 14-3-3 proteins as a function of residue number. The analysis performed on α -synuclein, in combination with either 14-3-3 ζ or η isoforms, shows different effects along the polypeptide chain.

In both experiments, the intensity of the resonances localized at the N-terminus (around the first 20 amino acids) decreases significantly with respect to the rest of the protein, with a more pronounced effect on the residues closest to the N-terminal end, for which there is a decrease of about 10%. This effect is specific, first because it concerns a localized portion

of the protein and second because any experimental artefacts are avoided by recording the spectra of the protein mixture and control in identical conditions of buffer, protein concentration, temperature and acquisition parameters.

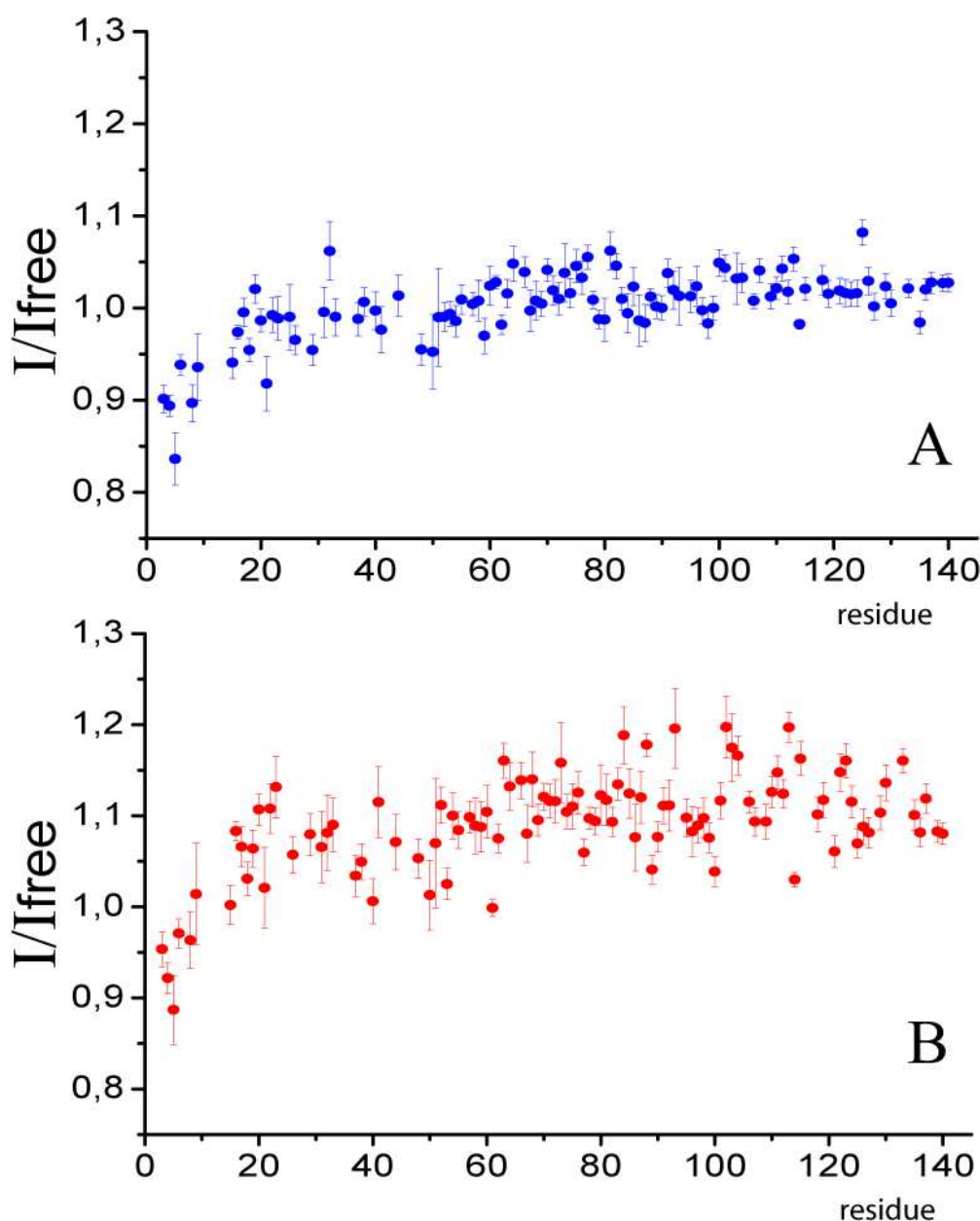


Figure 2.2.5_ Analysis of the signal intensity changes of $[^1\text{H}-^{15}\text{N}]$ HSQC α -synuclein/14-3-3 titration experiment. The intensity changes (I/I_{free}) as a function of residue number for ^{15}N labelled α -synuclein in the presence of 14-3-3 proteins, relative to free α -synuclein are shown.

A. α -synuclein/14-3-3 ζ , 1:3 molar ratio; **B.** α -synuclein/14-3-3 η , 1:3molar ratio.

The localized intensity decrease could be explained with an interaction that involves only the N-terminal residues of the protein, so restricting the backbone amide spins mobility and their correspondent signal intensity. Probably, the interaction is electrostatic in nature, as it involves the most basic region of α -synuclein and it is well known that the surface of all 14-3-3 isoforms is negatively charged (Fu et al., 2000). However, these results need to be confirmed by other techniques, since they are difficult to explain and cannot be associated to any functional effect.

The rest of the polypeptide chain, from residue 20 to the C-terminus, is not affected by the presence of 14-3-3 ζ , as the average signal intensity ratio is close to 1. This may be because it is not involved in the interaction, thus maintaining the dynamic properties of the free α -synuclein form.

In figure 2.2.6, the overlay between ^{15}N -labelled α -synuclein [^1H - ^{15}N] HSQC spectra recorded with (red) or without (blue) 3 molar excess of 14-3-3 ζ shows that there are no significant changes in the peak positions. Instead, the difference map reveals that some signals, relative to the first N-terminal residues of α -synuclein, decrease in intensity.

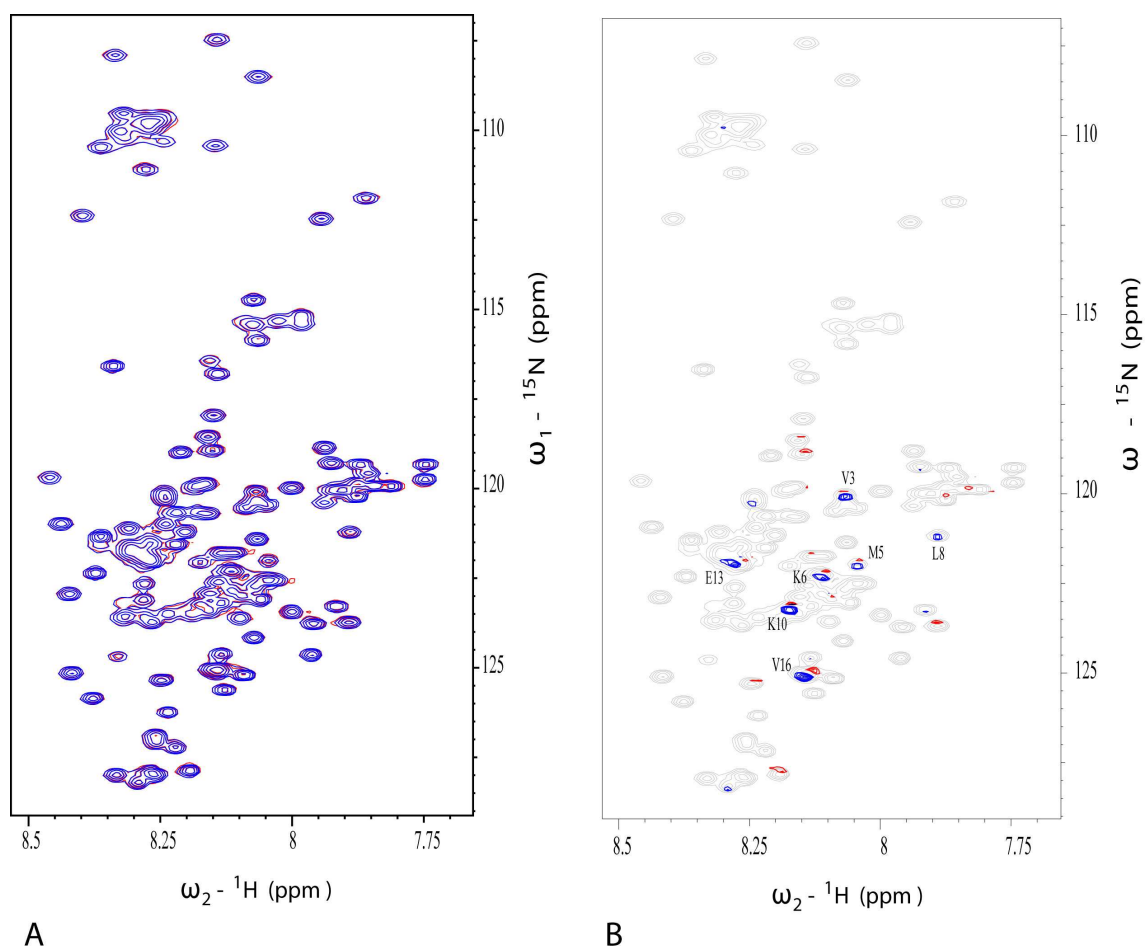


Figure 2.2.6_ Overlay (A) and difference map (B) of ^{15}N -labelled α -synuclein [^1H - ^{15}N] HSQC spectra recorded with (red) or without (blue) 3 molar excess of 14-3-3 ζ .

Although there is a common trend in the intensity change along the polypeptide, in the presence of 14-3-3 η , the average intensity of the α -synuclein signals is increased by about 10% relative to that in presence of ζ isoform. In the error analysis used, this seems a significant change that could be justified by the properties of 14-3-3 to act as a chaperone (Yano et al., 2006). In this speculative view, 14-3-3 η could dissolve the small portion of synuclein that is present in solution as aggregates, raising the monomer concentration and so the overall intensity of the signal with respect to the free form.

However, other non-predictable experimental artefacts can play a role in raising the signal. Therefore, we carried out aggregation assay experiments to have an answer about the possible function.

2.2.3 14-3-3 η interacts with α -synuclein in aggregation experiments

With the biochemical experiments described in 2.2.1, there is no experimental evidence of interaction between α -synuclein and 14-3-3 proteins in solution. The binding is not detected even using the NMR chemical shift perturbation analysis. Only the study of the peak intensity changes shows an effect in few peaks, all of which seem to be in the N-terminal region. These small variations can be a preliminary indication of a transient interaction between monomeric α -synuclein and 14-3-3 proteins, but we cannot say if this can be related to a function.

Based on these results, we decided to explore the possibility that 14-3-3 proteins interact with α -synuclein conformational states different from the monomeric one. Specifically, we asked whether, consistently with their chaperone function (Yano et al., 2006), 14-3-3 proteins might inhibit α -synuclein fibril formation, thus interacting with intermediate oligomeric species that lead to aggregation. Published data demonstrated that several molecular chaperons, such as Hsp70 and DJ1 (Dedmon et al., 2005; Zhou et al., 2006), can interact with α -synuclein oligomers, inhibiting the fibrillation process.

The Fluorescence polarization (FP) assay, described in 5.3.5, is the tool used to monitor the effect of 14-3-3 η protein on α -synuclein aggregation kinetics. Experiments and data elaboration were carried out by Laura Tosatto of our research group.

FP methods for the study of protein aggregation have been applied according to the protocol described by Luk et al., 2007. The central idea is that the free rotation of fluorescent moieties in solution is inversely proportional to the relative molecular mass. Therefore, any process that affects the tumbling of a fluorescently tagged protein, like its aggregation, can be effectively monitored using this method. α -Synuclein aggregation in the absence and in the presence of 14-3-3 η was monitored by fluorescence polarization of Oregon Green 488 labelled α -synuclein, mixed with non labelled α -synuclein at a final ratio of 1:100. Fluorescence was monitored every six hours at 535 nm, with excitation $\lambda = 490$ nm. To investigate the role of 14-3-3 oligomerization state on its potential chaperon function, we performed experiments with two different 14-3-3 samples, one with 14-3-3 dimer, the second with 14-3-3 monomer. The latter was obtained by mutagenesis of a key residue of the dimerization interface (Tyr 85 to Ala 85). Both 14-3-3 samples were provided by the crystallography group of H. L. Monaco.

Different α -synuclein:14-3-3 molar ratios (8:1; 4:1; 1:1) were employed to reveal whether 14-3-3 proteins have an effect on α -synuclein aggregation by a dose-dependence mode.

A control experiment with only 14-3-3 proteins and Oregon Green 488 labelled α -synuclein at a final ratio of 100:1, demonstrated that there is no FP signal increment due to possible 14-3-3 aggregation trapping labelled α -synuclein.

Each experiment was performed four times to obtain data with statistical significance: errors were determined as standard deviation of the four measurements.

A brief description of data elaboration is introduced as a premise to present the results obtained.

The variation of FP intensity, as a function of time, can be fitted with a sigmoid. The curve can be described with several parameters as the lower and upper asymptotes, lag phase,

time at which the signal reaches half of the highest FP value (t_{50}) and curve slope at t_{50} (Figure 2.2.7). The asymptotes define the baseline and the plateaux parts of the curve. The lag phase is the initial period of time during which it can not be observed FP increase. In this aggregation experiments, lag phase corresponds to the period of time required to form of α -synuclein oligomers, that lead to the increase in FP signals. Curve slope at t_{50} is a measure of the rate of aggregates growth. In our case, data interpolation do not allow to define accurately the lag phase. As an alternative, we opt for t_{50} as the parameter that define the relative times at which aggregation formation is triggered. t_{50} values has two contributions: the lag phase and the slope that define the aggregation rate. Whether slopes values do not vary, but there is an increase of the lag phase, the t_{50} value would reveal this change.

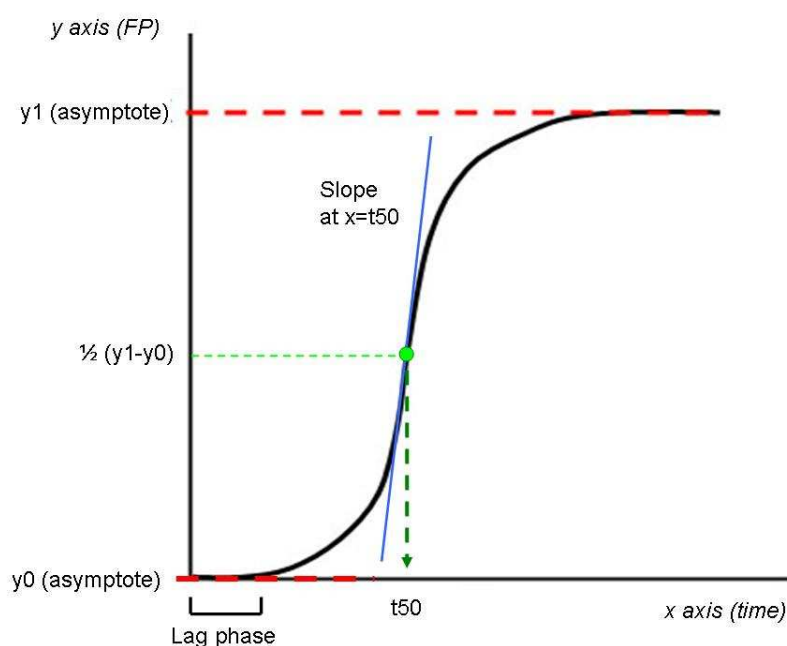


Figure 2.2.7_ Parameters that describes a sigmoidal trend. Asymptotes are reported with dashed red lines, while blue line indicate the straight line tangent to t_{50} value (green spot and green arrows) whose slope is indicative of oligomers enlargement rate.

Since asymptotes are equal for all the curves obtain, only t_{50} and slope parameters have been taken into account for the results analysis.

In figure 2.2.8, the results of the different conditions tested are shown. The curve fitting parameters (figure A) reveal a significant decrease on the aggregation rate of α -synuclein in the presence of 14-3-3 proteins, in comparison to α -synuclein alone. FP sigmoidal slope value decreases of 15-30% among the different molar ratio tested. However, due to the high errors, it is not possible to assign a dose-dependence to 14-3-3 chaperone function, although a linear trend is evident for monomeric 14-3-3. Furthermore, the data reveal that there is not a significant difference on the 14-3-3 protein action in monomeric or dimeric state. This suggest that probably the 14-3-3 chaperone effect does not require any structural determinants on the dimer interface.

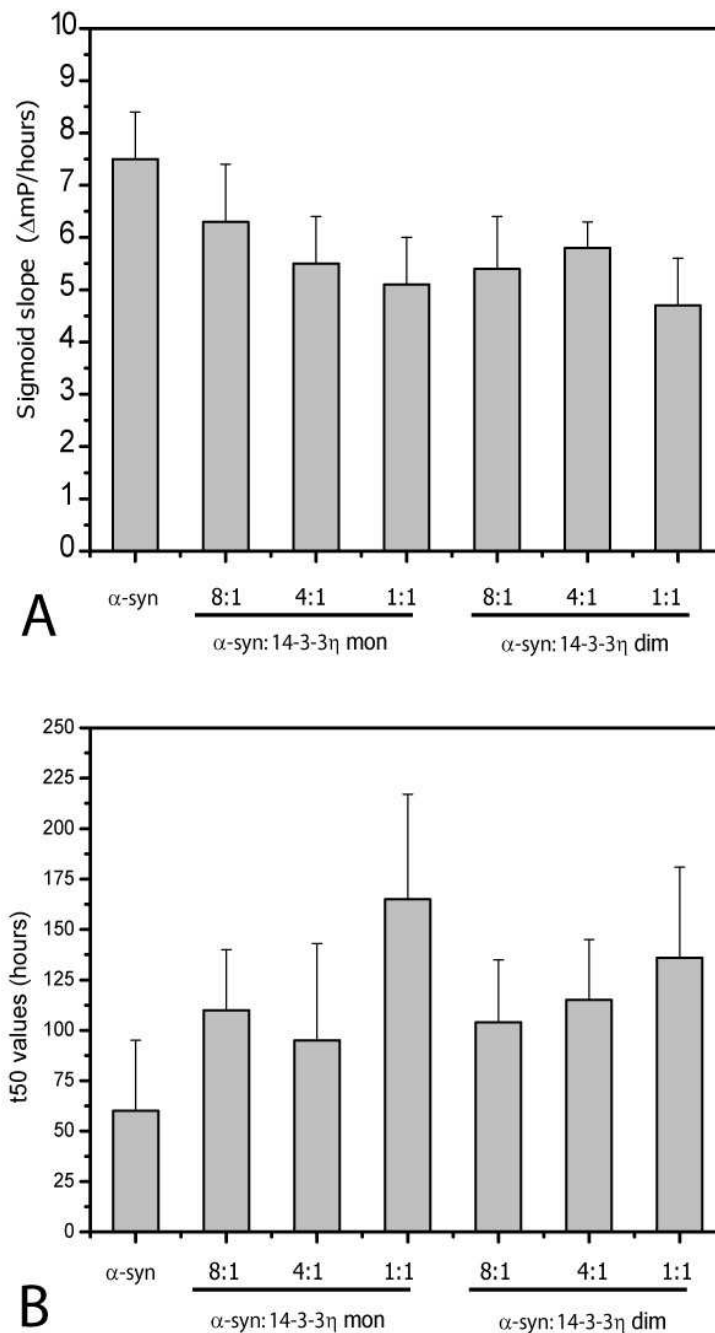


Figure 2.2.8_ Chaperon function of 14-3-3 proteins in α -synuclein aggregation experiments. Sigmoidal slope (A) and t50 (B) values are the parameters analysed to compare the different condition tested: α -synuclein alone; α -synuclein in the presence of monomeric 14-3-3 protein at 8:1; 4:1; 1:1 molar ratio; α -synuclein in the presence of dimeric 14-3-3 protein at 8:1; 4:1; 1:1 molar ratio.

Complementary data were obtained with the analysis of the t50 values (figure B). It reveals that, in the presence of 14-3-3 proteins, there is a significant decrease in the time required for α -synuclein aggregation trigger, with respect to α -synuclein alone. The effect is comparable with both monomeric and dimeric 14-3-3. Since t50 also include the lag phase information, a decrease of its value may suggest that there is an effect on both α -synuclein aggregation rate and time required to form oligomers whose growth triggers the nucleation-

dependent aggregation process. Other experiments are needed to verify a possible binding event between these oligomers and 14-3-3 proteins, and the effect on the overall process. Taken together, these data indicate that there is an interaction between the two proteins in solution, but only in particular conditions, related to a protein mis-folding context in which 14-3-3 proteins might bind to intermediates of α -synuclein aggregation to inhibit the fibrillation process. The investigation of the molecular mechanism of the effect of 14-3-3 binding on α -synuclein aggregation is the subsequent experimental aim of that research.

2.3 Analysis of α -synuclein/Tyrosine Hydroxylase interaction

2.3.1 Activity assay

Several published data (Perez et al., 2002; Zhou et al., 2004) suggest that the inhibition of Tyrosine Hydroxylase activity, in α -synuclein over-expressing culture cells, may be mediated by the binding of the two proteins. The hypothesis derived from different observations: the homology of α -synuclein with 14-3-3, the ability to co-immunoprecipitate TH with α -synuclein from brain and dopaminergic cells, and the co-localization of both proteins in neuronal cells.

To evaluate if α -synuclein can modulate Tyrosine Hydroxylase directly, we performed enzymatic activity analysis. The assay that was used is described in 5.2.19. Specifically, we explored two possibilities. In the first one, we investigated the role of α -synuclein as a classic inhibitor of TH activity. In the second one, we explored if α -synuclein can be a competitive substrate of Tyrosine Hydroxylase. This idea came from a recent published work where we demonstrated that, due to the high flexibility of the α -synuclein backbone, its endogenous tyrosine residues are exposed enough to be directly oxidised by the enzyme Tyrosinase (Tessari et al., 2008).

First, we verified that the purified recombinant enzyme was active: the measured K_M of 27 μM (data not shown), is in agreement with our previous results and with published data. (Nakashima et al., 1999; Fitzpatrick et al., 1991).

To study the possible inhibitory effect on TH activity, fluorescence assays were carried out in the presence of 45 μM α -synuclein. Since we wanted to work in saturated conditions, this concentration is in large excess with respect to that of the enzyme (0.6 μM). In spite of this, the measured value is comparable to that found in neurons (Uversy et al., 2001).

The results shown in figure 2.2.9 demonstrate that there is no experimental evidence of TH activity modulation by α -synuclein. The quantity of L-DOPA produced over time, at 50 μM tyrosine substrate, does not change significantly in the presence of 45 μM synuclein. TH activity tests were carried out also using α -synuclein as the only substrate. After incubation with the enzyme at 37°C for 30 minutes, α -synuclein was purified from the reaction mixture by HPLC reverse phase chromatography following the fluorescence signal at 330 nm, and the sample analysed by mass spectroscopy. As shown on the table 2.2.1, several parameters do not change with respect to the negative control (α -synuclein incubated in the same reaction mixture without TH): the elution time, the area values of the HPLC elution peak, and the MM of the two species found in solution. The protein with +16 dalton with respect to α -synuclein, found in both cases, reasonably derives from oxidation of one Met residue.

These data allow us to exclude that α -synuclein can be a competitive substrate of Tyrosine Hydroxylase.

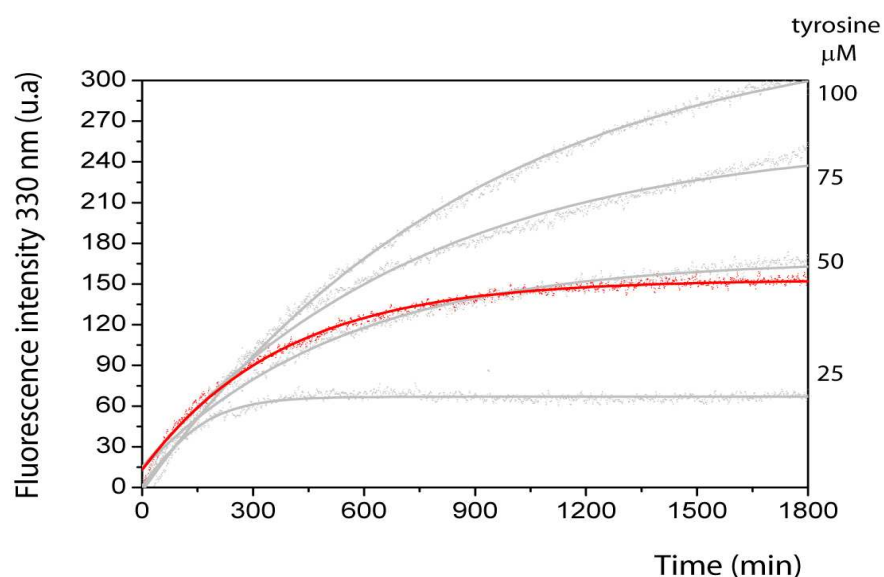


Figure 2.2.9_ Evaluation of Tyrosine Hydroxylase activity modulation by α -synuclein. TH activity was monitored by fluorescence emission at 330 nm using 25, 50, 75 and 100 μ M of tyrosine (grey lines). The red line represents the TH kinetic performed with 50 μ M of tyrosine, in the presence of 45 μ M of α -synuclein.

Sample	HPLC Elution time	HPLC elution peak area	MW
α -synuclein +TH	24.9 min	242.2	14461 $\pm$ 1
			14478 $\pm$ 1
α -synuclein -TH	24.9 min	236.2	14460 $\pm$ 1
			14476 $\pm$ 1

Table 2.2.1_ Evaluation of α -synuclein tyrosine oxidation by Tyrosine Hydroxylase. The elution times, the area of the elution peak in HPLC reverse phase chromatography, and the MW determined by Mass Spectroscopy are shown.

2.3.2 NMR characterization with [^1H - ^{15}N] HSQC titration experiments.

Chemical shift and signal intensity perturbation analysis

The same NMR strategy to study the α -synuclein/14-3-3 interaction was used with Tyrosine Hydroxylase. In figure 2.2.10, the plot of backbone chemical shift differences (δ_{average}) as a function of residue number for ^{15}N -labelled α -synuclein in the presence of the enzyme (molar ratio 5:1), relative to free α -synuclein, is shown. Also in this case, there is no significant perturbation of the α -synuclein chemical shifts in the presence of the binding partner.

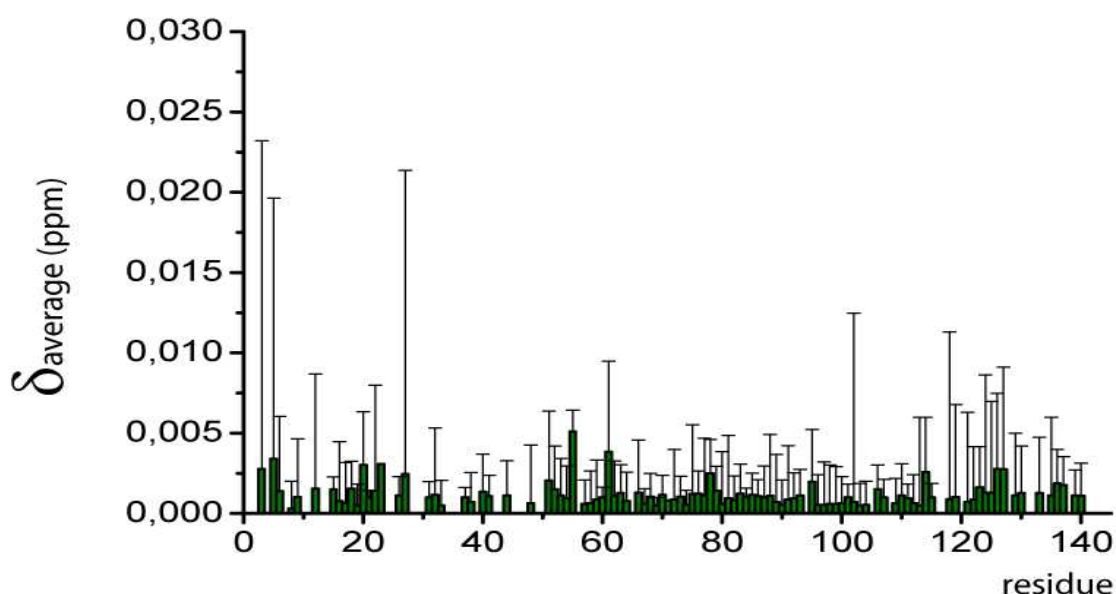


Figure 2.2.10_ Chemical shift perturbation analysis of [^1H - ^{15}N] HSQC α -synuclein/ wild type TH titration experiment. The backbone chemical shift differences (δ_{average}) as a function of residue number for ^{15}N labelled α -synuclein in the presence of TH, relative to free α -synuclein, are shown. The α -synuclein/TH molar ratio was 5:1.

In figure 2.2.11, the plot of the ^1H - ^{15}N HSQC peak intensity changes relative to free α -synuclein (I/I_{free}) is shown for α -synuclein in the presence of Tyrosine Hydroxylase as a function of residue number. The intensity decrease trend for the N-terminal part of α -synuclein, observed in the presence of 14-3-3 proteins, was not found in this case. We can only observe isolated residues, at the very terminal regions of the polypeptide chain, which show an intensity decrease relative to the control. Nevertheless, it will be necessary to improve the solubility of the enzyme and to repeat the experiment with a higher TH/ α -synuclein molar ratio to verify if this result is due to a problem of sensitivity.

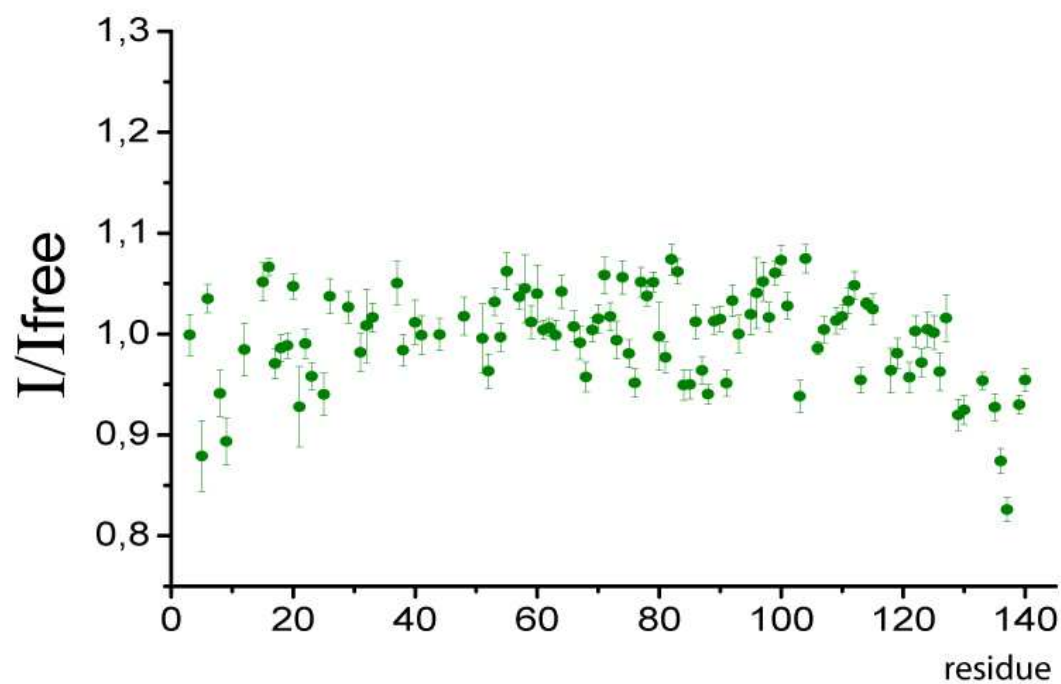


Figure 2.2.11_ Signal intensity changes analysis of [^1H - ^{15}N] HSQC α -synuclein/wild type TH titration experiment. The intensity changes (I/I_{free}) as a function of residue number for ^{15}N labelled α -synuclein in the presence of TH, relative to free α -synuclein, are shown. The α -synuclein/TH molar ratio was 5:1.

2.4 Characterization of the complex between 14-3-3 η and pTH1-152

2.4.1 Production and characterization for crystallographic screening

Since many published data confirmed that 14-3-3 proteins bind to the phosphorylated Tyrosine Hydroxylase N-terminal domain (pTH1-152) to regulate the activity of the enzyme (Itagaky et al., 1999; Kleppe et al., 2004), we planned to perform NMR titration experiments following the signals of the ^{15}N -labelled TH1-152 at different 14-3-3 molar ratios, to characterise the site of binding. However, as described in section 2.1.4, we were not able to find the conditions to improve the solubility of TH1-152, a necessary premise to have good $[\text{H}-^{15}\text{N}]$ HSQC spectra and, above all, to assign the backbone resonances. Improvement of the NMR sample quality is required before interaction experiments in the presence of 14-3-3 proteins can be performed.

One possibility to improve the low solubility of TH1-152, which will allow the backbone resonance assignment and the study of the details of the interaction with 14-3-3 proteins, is the high affinity binding with its partner. The K_d of the complexes between TH and 14-3-3 isoforms, determined by several techniques (Kleppe et al., 2004; Obsilova et al., 2008), is in the nM scale.

To make a strong complex with 14-3-3 proteins, phosphorylation at TH Ser 19 is required (Itagaky et al., 1999). Therefore, we carried out preliminary experiments to phosphorylate TH1-152 in vitro. As already published by Obsilova et al, 2008, to make residue Ser19 a substrate of the commercially available kinase PKA, residue Ala17 was mutated to Arg (A17R). The purification protocol is the same described for the “wild type” TH1-152. The purified protein was then phosphorylated by incubation with PKA as described in 5.2.18.

In contrast to the control represented by the same incubation mixture without PKA, the phosphorylated product showed a strong reduction in solubility and precipitation occurred. As shown in figure 2.2.12, the precipitated protein has a MM difference of +160 daltons with respect to the starting material. This difference is a strong indication of the incorporation of two phosphate groups as expected (at Ser 19 and 40, the two substrates of PKA).

To overcome the problem of insolubility of the phosphorylated form of TH1-152A17R, we repeated the same reaction in the presence of the binding partner 14-3-3 η . The phosphorylation reaction of purified TH1-152A17R was carried out with 14-3-3 η at a 2:1 molar ratio. In these conditions, phosphorylated TH1-152A17R did not precipitate, possibly because of its strong binding with 14-3-3 η .

The next step was to perform the experiment on a large scale, to purify a sufficient amount of the TH1-152A17R/14-3-3 η complex to characterize it by electrophoretic and chromatographic methods, to study it by NMR spectroscopy, and to set up the preliminary crystallization screening.

Six mg of purified TH1-152A17R were phosphorylated by PKA in the presence of six mg of purified histidine tagged 14-3-3 η (2:1 molar ratio). The molar ratio and the histidine

tagged 14-3-3 isoform were chosen to selectively purify the complex after phosphorylation by IMAC Chromatography. In this first attempt, we did not use ^{15}N -labelled protein.

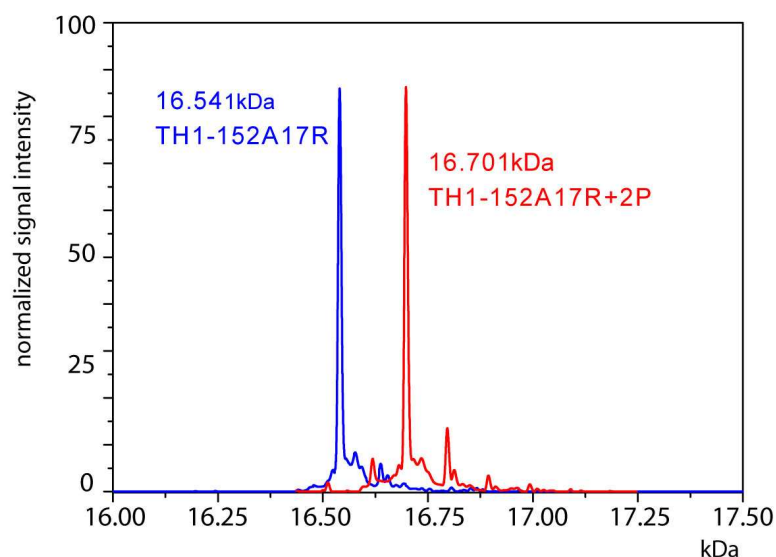


Figure 2.2.12_ Mass Analysis of phosphorylated TH1-152A17R. The control TH1-152A17R (not phosphorylated) has a MM of 16541 ± 1 daltons, as expected from the sequence. TH1-152A17R, after the phosphorylation reaction by PKA, has a MM of 16701 ± 1 daltons. The difference of +160 daltons corresponds to the incorporation of two phosphate groups in the polypeptide chain.

Both electrophoretic and chromatographic results indicate the formation of a strong complex between the two proteins.

Figure 2.2.13 shows the result of the Native PAGE electrophoresis. BSA (1st lane) was used as a standard; TH1-152 A17R (2nd lane) and 14-3-3 η (3rd lane) samples as controls.

TH1-152 A17R has an isoelectric point of 9.9, so it cannot run on this acidic native PAGE.

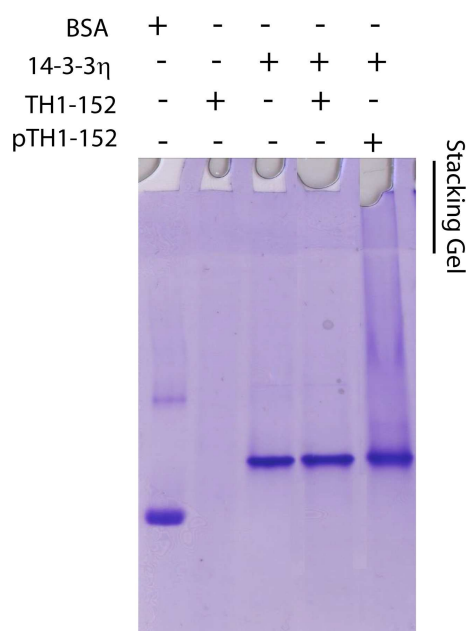


Figure 2.2.13_ 12% Native PAGE. Analysis of TH1-152A17R/14-3-3 η complex formation. BSA was used as a standard. From left to right: BSA alone. TH1-152A17R alone. 14-3-3 η alone. Not phosphorylated TH1-152A17R/14-3-3 η reaction mixture at a molar ratio of 2:1. Phosphorylated TH1-152A17R/14-3-3 η reaction mixture at a molar ratio of 2:1.

The sample of the two proteins, incubated at a 2:1 molar ratio overnight with the kinase PKA, was loaded on the last lane (5th). In the 4th lane there is the negative control, that is, the sample of the two proteins incubated overnight without kinase PKA.

The result shows that the combination of the two proteins, incubated with PKA, has a different electrophoretic mobility with respect to the same mixture incubated without the kinase. There is an evident smear and a protein band stalls on the edge of the stacking gel well. The mobility of 14-3-3 η does not change significantly.

These data are a strong indication that the kinase reaction worked successfully, determining a change in the electrophoretic behaviour of the sample. However, they are not sufficient to discriminate if this change is due only to TH1-152A17R phosphorylation, or to the complex formation. In both cases, the overall isoelectric point of TH1-152 would decrease, so the protein would have a different mobility with respect to the non-phosphorylated protein (2nd lane).

To verify the formation of the complex, we carried out size exclusion chromatography experiments on a Superdex 200 H/R column. The results are shown in figure 2.2.14. To compare the signals of the different chromatograms, the signal intensities were normalized and the same a.u scale was used. Thus, the signal of TH1-152A17R is not more visible, because of its very low extinction coefficient ($Abs_{280} 0.1\% = 0.1$, based on the sequence). Therefore, to visualize the chromatographic profile of TH1-152A17R loaded alone (panel A), the signal intensity was multiplied by a factor of 45, with respect to that of the chromatograms of panels B, C and D.

TH1-152A17R elutes at 30.1 min. Based on the calibration, the elution time corresponds to a protein of about 40 kDa, according to the chromatographic behaviour of not mutated TH1-152. 14-3-3 η elutes at 25.5 min (panel B) according to the molecular mass of its dimeric form (60 kDa).

Panel C shows the chromatographic profile of the combination of the two proteins (TH1-152A17R/14-3-3 η at a 2:1 molar ratio) incubated overnight in the phosphorylation buffer without the kinase. 14-3-3 η elutes at 25.5 min as in the control (panel B). Because of the low extinction coefficient, the 280 nm signal corresponding to the TH1-152A17R elution peak is too low to be revealed. The material eluted at 35.4 min includes the nucleotides ATP and cAMP added to the buffer.

Panel D shows the chromatographic profile of the combination of the two proteins (TH1-152A17R/14-3-3 η at a 2:1 molar ratio) incubated overnight in the phosphorylation buffer in the presence of the kinase. Comparing to panel C, (the same sample incubated without the kinase), we can see a significant change in the chromatogram. While nucleotides elute at 35.4 min as in the chromatogram of panel C, the elution peak relative to 14-3-3 η shifts from 25.5 min to 23.1 min and has a broader shape. The shift is a strong indication of the binding between TH1-152A17R and 14-3-3 η .

These data confirm that, when the two proteins are incubated with the kinase and TH1-152A17R is modified by phosphorylation, a stable complex is formed.

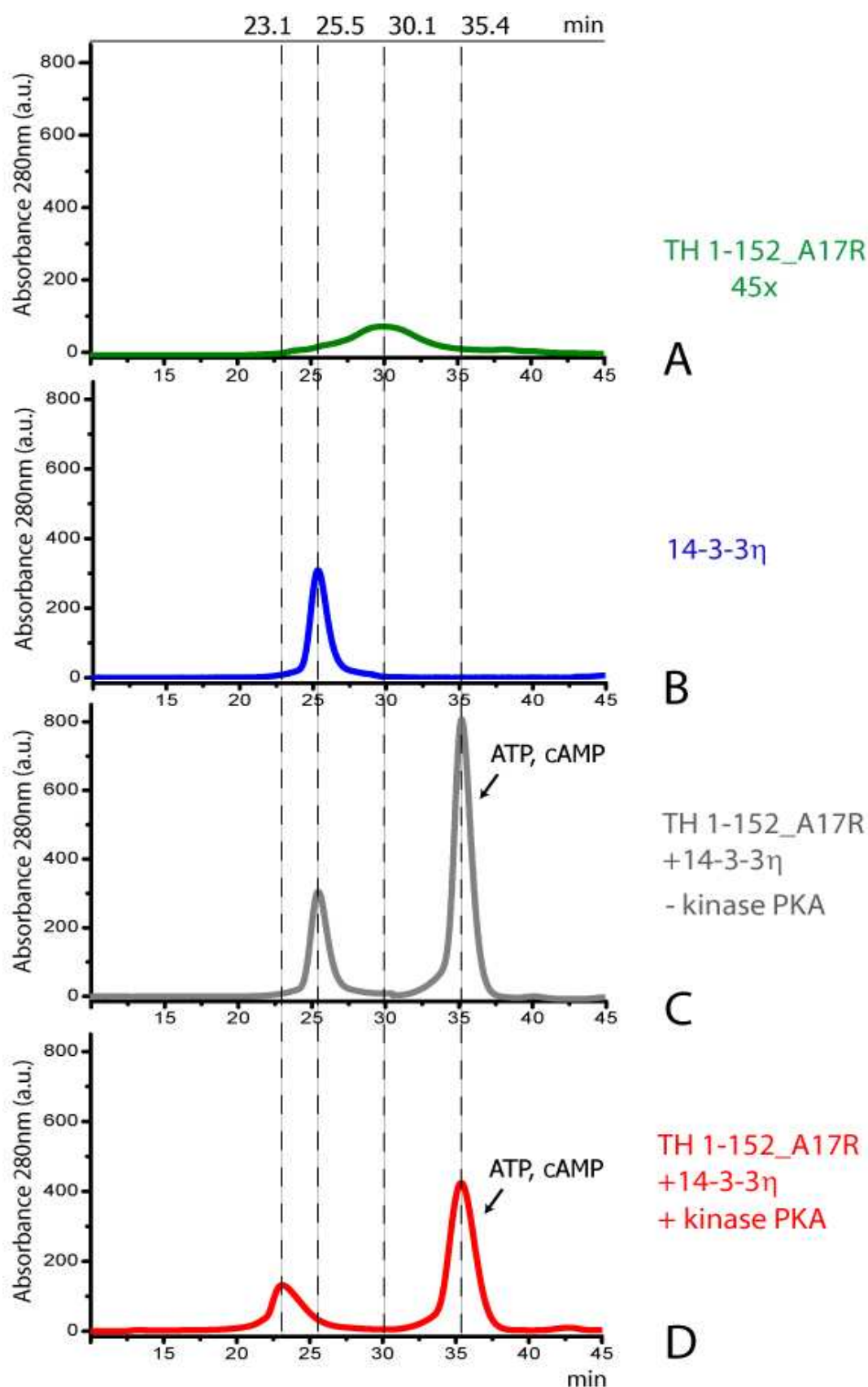


Figure 2.2.14_ Analytical size exclusion chromatography of the 14-3-3 η /TH1-152 interaction. The experiments were carried out at room temperature, on a Superdex 200 H/R column equilibrated with 20 mM Tris-HCl (pH 7.4), 150 mM NaCl. The flow rate was 0.5 ml/min.

A. TH1-152A17R alone (45X) **B.** 14-3-3 η alone **C.** TH1-152A17R/14-3-3 η mixture (2:1 molar ratio) incubated overnight at room temperature without the kinase. **D.** TH1-152A17R/14-3-3 η mixture (2:1 molar ratio) incubated overnight at room temperature with the kinase.

To selectively purify the complex between phosphorylated TH1-152A17R and 14-3-3 η protein and remove the free fractions of the two proteins and the contaminants, we performed an IMAC Chromatography after the kinase PKA reaction.

The idea is to trap the complex through the binding of histidine tagged 14-3-3 η to the matrix.

Briefly, a first dialysis step was performed to remove DTT from the solution, prior to loading the protein sample on the metal-charged matrix, equilibrated with the same protein buffer. Then, a washing step was performed with 10 mM imidazole and elution was carried out in two steps, of 50 and 250 mM imidazole.

As shown in figure 2.2.15, the two proteins remain associated in the high affinity complex, throughout all the purification steps. Phosphorylated TH1-152A17R is selectively trapped by IMAC Chromatography by the binding of its histidine tagged protein partner 14-3-3 η protein to the matrix.

Furthermore, comparing lane 7 to lane 2, most of all the protein contaminants were removed from the final sample.

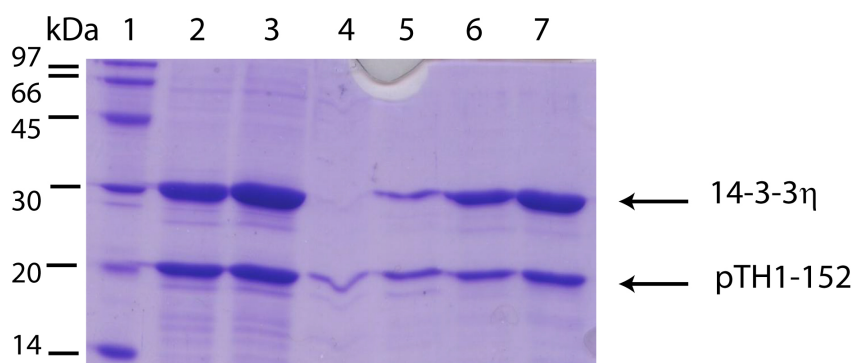


Figure 2.2.15_ A. 15% SDS-PAGE of the phosphorylated TH1-152A17R/14-3-3 η complex by IMAC chromatography. Lane 1: MM markers; lanes 2 and 3: sample after the phosphorylation reaction, before chromatography; lane 4: flow through; lane 5: washing step with 10 mM imidazole; lane 6: elution with 50 mM imidazole; lane 7: elution with 250 mM imidazole.

Elution fractions at 50 and 250 mM imidazole (lane 6 and 7) were combined and concentrated by ultrafiltration to 15 mg/ml after buffer exchange to 20 mM Tris-HCl (pH 7.4), 150 mM NaCl. SDS-PAGE analysis confirmed that no one of the two proteins was lost and precipitation did not occur in this step (data not shown).

Crystallization screening were carried out at the Biotechnology Department of the University of Verona, by Dott. Stefano Capaldi of the crystallography group of Prof. Hugo Monaco. Hanging drops consisting of 1:1 mixtures of the complex solution and well solution, were set up for 100 different conditions. (Structural Screening I and II, Molecular Dimension®). The crystallization work is now in progress.

Similar experiments will be performed with ^{15}N -labelled TH1-152 and 14-3-3 η for NMR analysis. The race is on...

Chapter III.

Results

Protein-protein interaction in the regulation of heterochromatin formation

3.1 Introduction

Hp1 β proteins is characterised by an N-terminal chromo domain (CD) connected to a slightly larger, C-terminal domain termed the chromo shadow domain (CSD). The hinge region linking the two motifs is unstructured in solution, enabling the chromo and chromo shadow domains to move independently in the intact protein (Brasher et al., 2000). NMR structures were deposited for the deleted mutants 10-80 and 102-171, corresponding to the two domains alone (PDB code: 1ap0, Ball et al., 1997; 1dz1, Brasher et al., 2000).

Although they are structurally related, with a core of three β -sheets packed against an α -helix, there are some important functional differences: while the chromo domain is monomeric and is responsible for the recognition of the tri-meK9 histone 3, the chromo shadow domain forms a tightly associated symmetric dimer and binds to non-histone proteins (Eissenberg et al., 2000).

The protein is purified as a stable dimer with an overall MW of 42000. With conventional NMR methods, two main problems arise when the object of the analysis is a large protein as Hp1 β . First, the large number of resonances causes signal overlap; second, the NMR signals relax faster, which leads to line broadening and poor sensitivity, and eventually to no signals at all. Since the major source of relaxation of the amide ^1H spins arise from dipolar interactions with proximal aliphatic ^1H nuclei, the replacement of most carbon-bound hydrogen atoms in the protein with deuterium results in increased resolution and sensitivity gain (Fernandez and Wider, 2003). Therefore, the sample used to acquire the 3D NMR experiments needed for the assignment is ^2H , ^{13}C and ^{15}N labelled, with a percentage of deuteration of 70%. Moreover, most of all the pulse sequences used take advantage of TROSY (transverse relaxation-optimized spectroscopy) elements to further reduce transverse relaxation, thus obtaining sharper and more intense signals (Pervushin et al., 1997).

The experiments to characterise the Hp1 β molecular tumbling and the tri-meK9 peptide recognition were carried out with a single ^{15}N -labelled sample.

All the labelled full length Hp1 β samples were prepared by Szabolcs Sörös of the group of Chromatin biochemistry in the Max Planck Institut in Göttingen.

In figure 3.1, the [^1H - ^{15}N] TROSY-HSQC spectrum of ^2H , ^{13}C and ^{15}N labelled Hp1 β is shown. 176 discrete cross-peaks can be detected, corresponding to the non-proline amino acids of Hp1 β .

The wide distribution of the signals in the proton dimension, from 11 to 6 ppm, confirms the well-defined structure of the protein. The more intense signals in the central part of the spectrum possibly belong to the more flexible, unstructured regions of the protein. The most disperse regions of the spectrum (above ~ 8.5 and below ~ 8 ppm) are characterised by two groups of signals with different intensity. This is a strong indication that they belong to the two protein domains characterised by a different tumbling. Possibly, the strongest signals come from the Hp1 β chromo domain, while the less intense come from the Hp1 β chromo shadow domain that is responsible for protein dimerization.

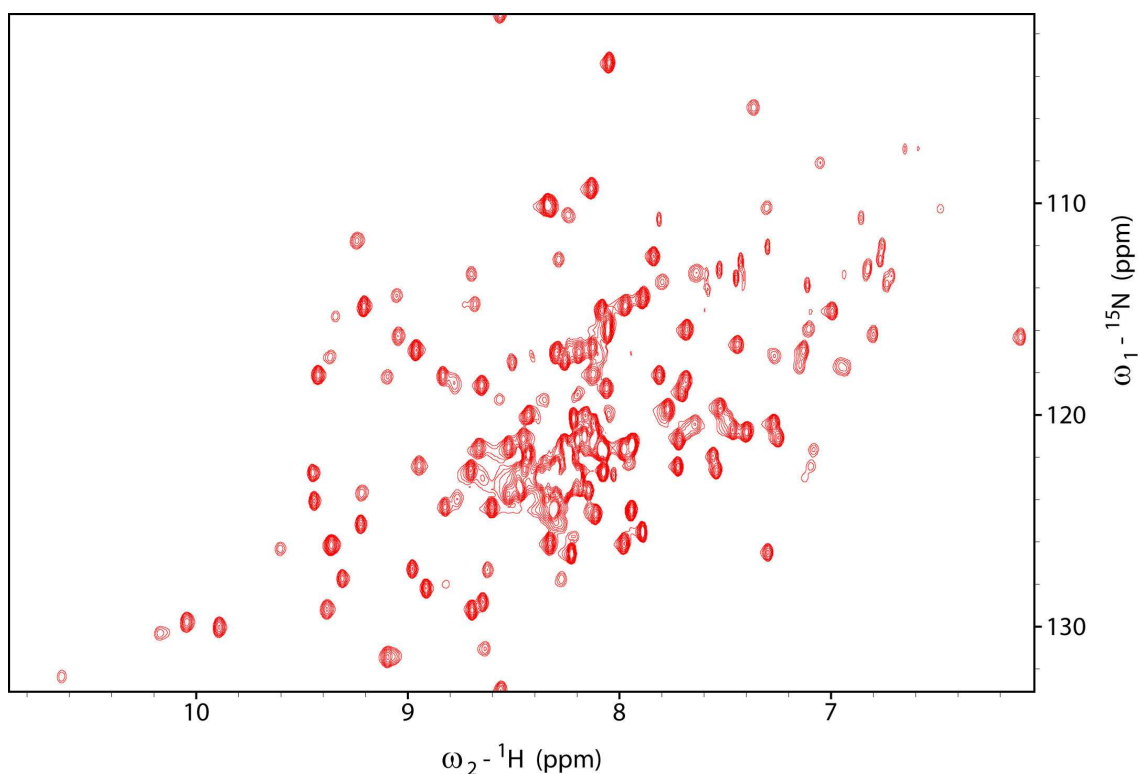


Figure 3.1 $[^1\text{H}-^{15}\text{N}]$ TROSY-HSQC spectrum of full length Hp1 β . The experiment was recorded with a ^2H , ^{13}C and ^{15}N -labelled 1.6 mM protein sample, on a 600MHz Bruker instrument, equipped with a cryo-probe. The spectrum was acquired at 303 K in 20 mM sodium phosphate buffer (pH 6.5), 20mM NaCl.

Due to the heterogeneous signal intensity observed, reflecting the motion properties of different parts of the protein, we also decided to carry out relaxation experiments to characterise the mobility of each $^1\text{H}-^{15}\text{N}$ inter-nuclear vector. The data obtained will be described in section 3.3.1.

As necessary premise to characterize by NMR the dynamic and structure properties of full length Hp1 β , and furthermore to study its interaction with the N-terminal tail of histone 3, we carried out 3D heteronuclear experiment to assign the ^1H , ^{15}N and ^{13}C backbone chemical shifts of the protein. The assignment strategy will be described in the next section.

3.2 Hp1 β : ^1H - ^{15}N - ^{13}C backbone resonance assignment

3.2.1 Resonance assignment strategy

The assignment strategy makes use of a combination of 3D heteronuclear experiments that correlate the backbone chemical shifts ($^1\text{H}(i)$, $\text{N}(i)$, $^1\text{H}(i+1)$, $\text{N}(i+1)$, $^1\text{H}(i-1)$, $\text{N}(i-1)$, $\text{CO}(i)$, $\text{CO}(i-1)$, $\text{C}\beta(i-1)$, $\text{C}\alpha(i-1)$) to establish the sequential connectivity between amino acids.

Specifically, we recorded HMQC-NOESY, HNN, [^1H - ^{15}N] TROSY-HNCO, [^1H - ^{15}N] TROSY-HN(CA)CO, and [^1H - ^{15}N] TROSY-CBCA(CO)NH experiments. Details on acquisition parameters and sample used can be found in 5.3.6.2.

A scheme of the magnetization transfer between the spins involved in the different experiments is shown in figure 3.2.

The HMQC-NOESY experiment, which combines both scalar and dipolar interactions to transfer the magnetization between spins, provided $\text{H}^{\text{N}}\text{-H}^{\text{N}}$ -NOEs between adjacent amide protons (Ying et al., 2007).

All the other experiments are heteronuclear triple resonance experiments that rely exclusively on scalar coupling to establish the sequential connectivity (Sattler et al., 1999).

The experiments are termed with the spins involved in the magnetization transfer pathway. Spins that are used only to relay the magnetization, without chemical shift evolution, are put in parenthesis (grey spheres in figure 3.2).

HNN (Panchal et al., 2001) provides correlation between sequential ^1H and ^{15}N resonance amide pairs, using the aliphatic $\text{C}\alpha$ spins to relay the magnetization along the polypeptide chain. Thus, there is a direct identification of ^1H and ^{15}N chemical shifts of neighbouring residues ($i-1$, and $i+1$).

The [^1H - ^{15}N] TROSY-HNCO (Saltzmann et al., 1998) spectrum correlates each amide proton resonance with the chemical shift of the carbonyl (CO) carbon of the proceeding residue ($i-1$). The complementary experiment [^1H - ^{15}N] TROSY-HN(CA)CO (Saltzmann et al., 1999), that provides the chemical shift correlation of each amide proton with the carbonyl (CO) carbon of the same (i) and with that of the proceeding residue ($i-1$), completes the information to have the sequential connectivity. As in the HNN experiment, $\text{C}\alpha$ spins are used only to relay the magnetization along the polypeptide chain.

In figure 3.3, an example of sequential assignment along the polypeptide chain is shown for the [^1H - ^{15}N] TROSY-HN(CA)CO experiment.

The [^1H - ^{15}N] TROSY-CBCA(CO)NH spectrum correlates each amide proton resonance with the chemical shift of the aliphatic ($\text{C}\alpha$ and $\text{C}\beta$) carbons of the proceeding residue ($i-1$). Carbonyl carbons (CO) are used as relay spins.

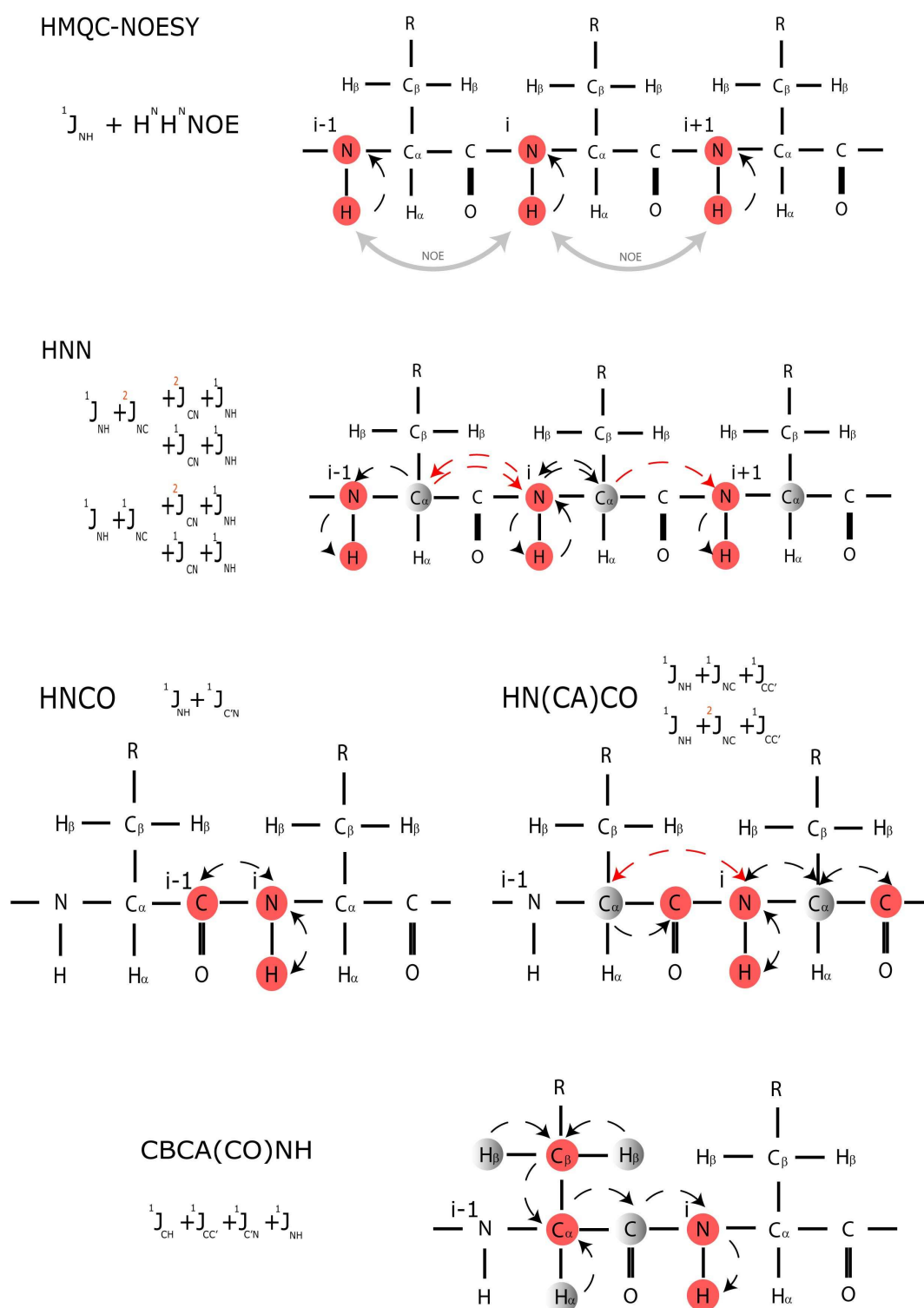


Figure 3.2_ Assignment strategy for $^1\text{H}/^2\text{H}\text{-}^{15}\text{N}\text{-}^{13}\text{C}$ labelled Hp1 β protein. Scheme of the triple resonance heteronuclear experiments carried out to establish the sequential connectivity of the protein backbone $^1\text{H}\text{-}^{15}\text{N}\text{-}^{13}\text{C}$ chemical shifts. The arrows indicate the magnetization flow between spins. The dashed ones are consistent with a coherent magnetization transfer mechanism. Orange spheres indicate the spins the chemical shifts of which are detected exploiting the connectivity established in the experiment. Grey spheres indicate that a spin is used as a relay spin, without chemical shift evolution. Close to the experiment title, are the J-correlations (scalar couplings) involved in magnetization transfer. When the scalar coupling interaction involves two spins separated by two covalent bonds (^2J), a red arrow was used. The light grey arrows are consistent with magnetization transfer due to dipolar interaction mechanism (NOE). Only in the HMQC-NOESY the sequential assignment relies on NOE crosspeaks between amide protons of adjacent amino acids.

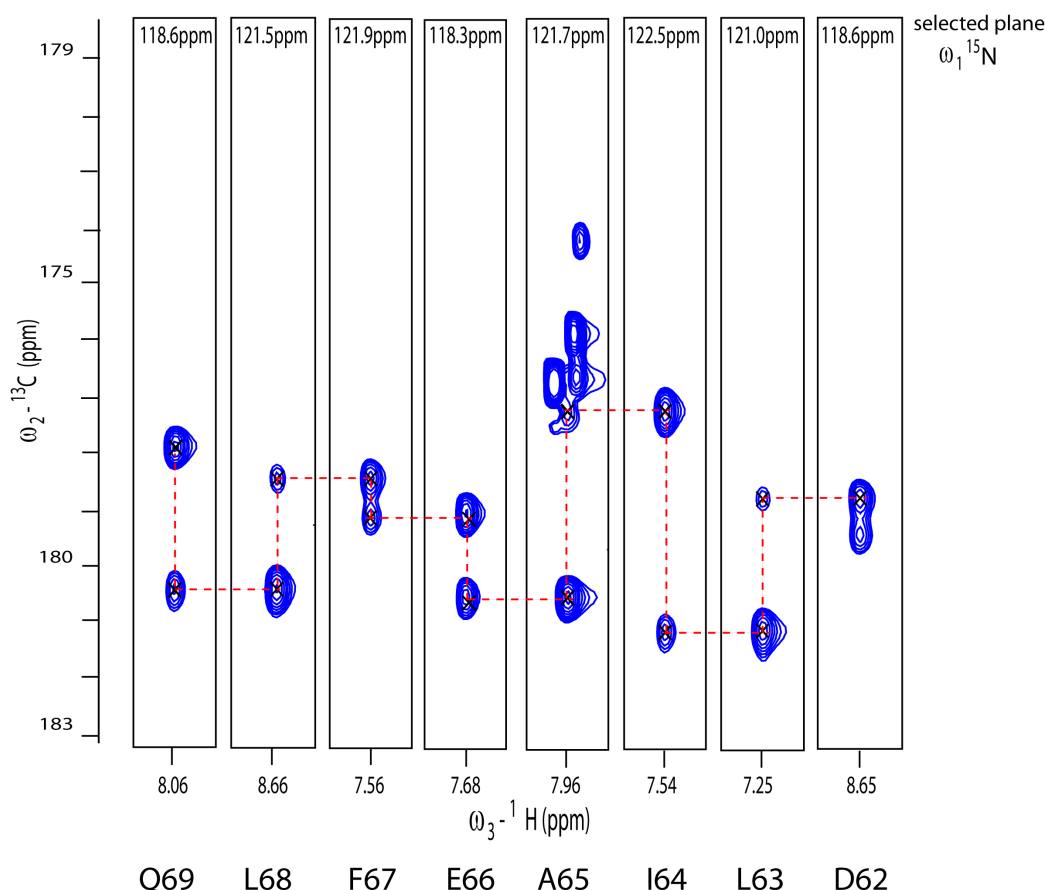


Figure 3.3_ $[\omega_2(^{13}\text{C}'), \omega_3(^1\text{H})]$ strips from a $[^1\text{H}, ^{15}\text{N}]$ -TROSY-HN(CA)CO experiment recorded with a 1.6 mM sample of $^2\text{H}/^{13}\text{C}/^{15}\text{N}$ -labelled Hp1 β at 303 K on a 600 MHz spectrometer with cryo-probe. The strips were taken at the ^{15}N chemical shift of residues 69-62 (selected planes of the 3D spectrum) and are centred about the corresponding $^1\text{H}^{\text{N}}$ chemical shift. Sequence specific assignment is defined by the one letter amino acid symbol and the number of residue in the amino acid sequence. Dashed line represents the sequential connectivities.

3.2.2 ^1H - ^{15}N - ^{13}C backbone resonance assignment of the chromo-domain

At present, the analysis of the HMQC-NOESY, HNN, $[^1\text{H}$ - $^{15}\text{N}]$ TROSY-HNCO, $[^1\text{H}$ - $^{15}\text{N}]$ TROSY-HN(CA)CO, and $[^1\text{H}$ - $^{15}\text{N}]$ TROSY-CBCA(CO)NH spectra allowed us to assign 50 of the 56 ^1H - ^{15}N - ^{13}C resonances of the Hp1 β chromo domain fragment 22-77.

Based on the published NMR structure (Ball et al, 1997) we know that this polypeptide chain fragment corresponds to the structured portion of the chromo domain, according to the good dispersion of the relative cross-peaks in the $[^1\text{H}$ - $^{15}\text{N}]$ TROSY-HSQC. The assignment of the chromo shadow domain is still in progress.

The most challenging target is the assignment of the unstructured regions, including the N-terminal part (1-21), the hinge region (79-117) and the C-terminal part (175-185). The signals of these protein segments, although very intense, have very poor dispersion in both ^1H and ^{13}C dimension. The consequence is that the carbon frequencies in the 3D spectra are accidentally degenerate, making the sequential assignment very hard. Therefore, we are

evaluating the use of other pulse sequences, with signal resolution improvement, in order to resolve these ambiguities.

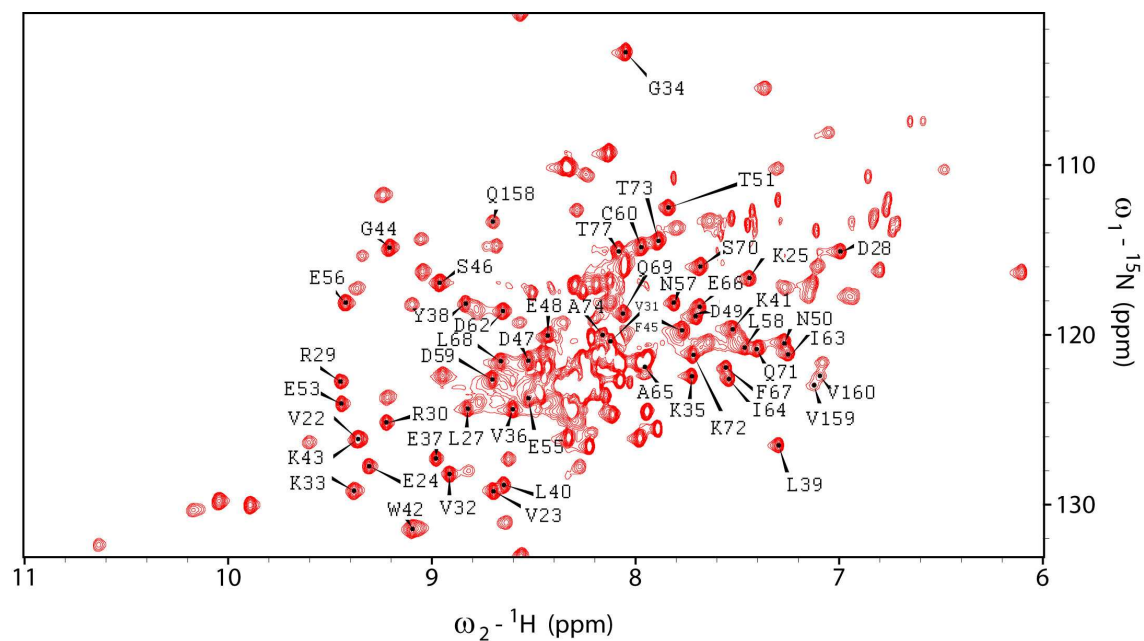


Figure 3.4_ Assignment of the chromo domain fragment 22-77 on the $[^1\text{H}-^{15}\text{N}]$ TROSY-HSQC spectrum of full length Hp1 β .

3.3 Hp1 β characterization

3.3.1 Characterization of the molecular tumbling

The experiments presented in this work required different protein concentrations from 0.1 mM to 1.6 mM. Therefore, to verify the sample homogeneity in the different experiments, we explored whether high protein concentrations could lead to aggregation.

The measurement of the rotational correlation time (τ_c), that is given by the average time taken for the molecule to rotate by one radian, provides a good estimation of the molecular weight in folded protein. Therefore, τ_c determination at different Hp1 β protein concentrations can indicate if aggregates are formed in the conditions tested.

Since molecular tumbling is intimately related to spin relaxation rate (Levitt, spin dynamics 2001, WILEY), we carried out experiments that permit the measurement of the effect of ^{15}N spin relaxation on the [^1H - ^{15}N] signal intensity.

Specifically, we recorded a series of one-dimensional amide protons (^1H) NMR spectra with variable relaxation periods on ^{15}N evolution time (from 2 to 200 ms). The relative intensity of the ^1H NMR signal (I_{rel}) decays exponentially with the increment of ^{15}N relaxation delays. I_{rel} is determined from integration of the 1D spectrum over the region 9.6-8.7 ppm, to avoid the underestimation effect from the flexible residues.

The pulse sequence used, named [^1H - ^{15}N] TRACT (TROSY for rotational correlation times), employs the TROSY approach. This permits the measurement of the effective τ_c of ^1H - ^{15}N backbone moieties, avoiding the artefacts due to the interference of relaxation mechanisms such dipolar coupling by remote protons and chemical exchange (Lee et al., 2006).

Figure 3.5 shows an example of 1D amide proton spectrum recorded with the [^1H - ^{15}N] TRACT pulse sequence on a 1mM ^{15}N -labelled Hp1 β sample, with 2 ms ^{15}N relaxation delay. The integration is performed only from 8.7 to 9.6 ppm where the signals from the structured part of the protein can be found. Below are the integration values of the 80 1D spectra recorded, plotted as a function of the ^{15}N relaxation delay used (from 2 to 200 ms). Relaxation of backbone ^{15}N nuclei is governed by chemical shift anisotropy (CSA) and dipolar interaction (D) between ^{15}N and its directly attached proton (Tjandra et al., 1996). The relaxation interference (or cross correlation) effect generated by the two mechanisms is different for a ^{15}N nucleus attached to a proton in the β state or in the α state (Levitt, spin dynamics 2001, WILEY). Therefore, the pulse sequence was designed to separately measure the relaxation of the two types of ^{15}N nuclei as signal intensity. The upper and lower curves correspond to the slowly relaxing β -spin state and the more rapidly relaxing α -spin state, respectively (Lee et al., 2006).

Data were fitted with a first order exponential function. The exponential decay rate constants obtained, R_α and R_β , are the starting point to calculate the τ_c value of the ^{15}N -

labelled Hp1 β protein. Data were elaborated as described in the Materials and Methods section of Lee et al., 2006. The equations used can be found in appendix II.

The [^1H - ^{15}N] TRACT experiment was carried out for ^{15}N -labelled Hp1 β samples at three different concentration values: 0.2, 0.6 and 1 mM. The same acquisition parameters were used to have the sample concentration as the only variable of the experiment.

Table 3.1 shows R_α , R_β , and final τ_c values of ^{15}N -labelled Hp1 β protein, obtained for the different sample concentrations tested: 14.4 ns for 1 mM, 12.5 ns for 0.6 mM, 13.4 ns for 0.2 mM.

Since these differences are not significant compared to the experimental variability (± 1.5 ns) observed, the result suggests that there is no change in molecular tumbling with increments in protein concentration. The data confirm that is it possible to use high protein concentrations without aggregation problems.

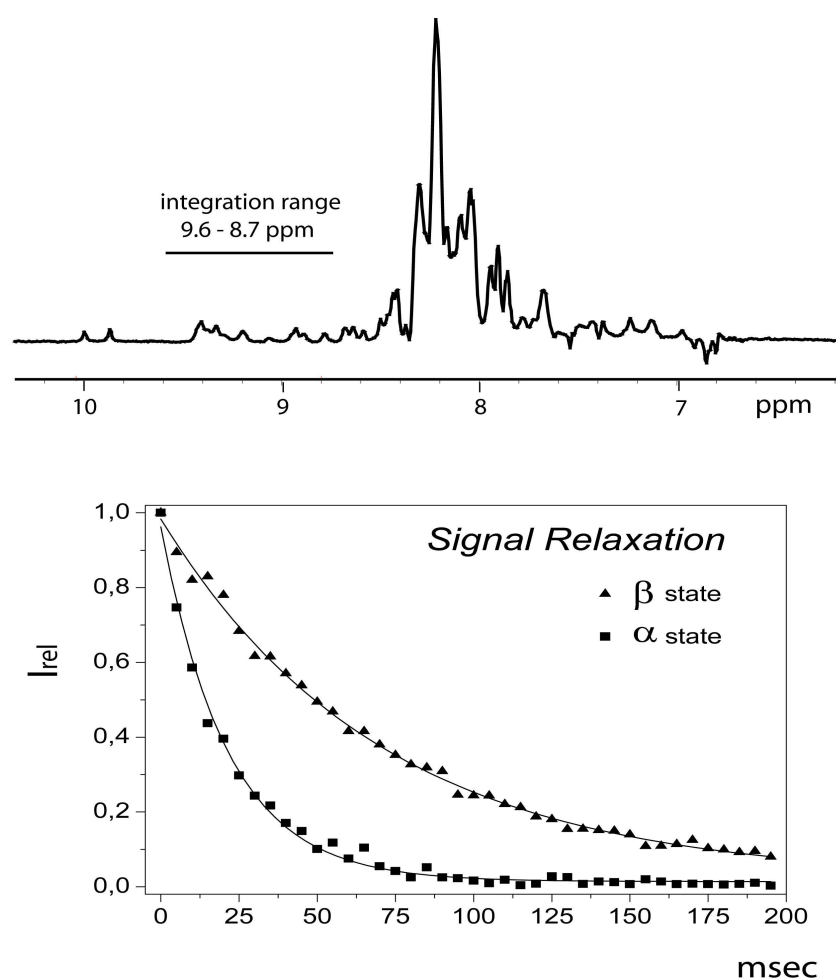


Figure 3.5_ Decay of the relative intensity of the ^1H NMR signal, I_{rel} , due to ^{15}N relaxation. I_{rel} was determined from integration of the 1D spectrum over the region 9.6-8.7 ppm (top). The 1D [^{15}N , ^1H] TRACT spectra were recorded with variable relaxation periods on ^{15}N evolution time (from 2 to 200 ms). Acquisition was performed with a 1 mM solution of ^1H - ^{15}N -labelled Hp1 β at 303 K at 700 MHz. The upper and lower curves correspond to the slowly relaxing β -spin state and the more rapidly relaxing α -spin state, respectively. Exponential fits provide R_α and R_β (relaxation rates) values to calculate the correlation time as described in the appendix II section.

Table 3.1_ [¹⁵N,¹H] TRACT experiments data

Sample concentration	R _β	R _α	Rotational correlation time (τ _c)
1 mM	14.2 Hz	46.8 Hz	14.4 ns
0.6 mM	13.6 Hz	42.3 Hz	12.5 ns
0.2 mM	12.3 Hz	42.9 Hz	13.4 ns

As introduced in 3.1, the [¹H-¹⁵N] TROSY-HSQC Hp1β (full length) spectrum is characterised by crosspeaks of different signal intensity that reflect the mobility of different parts of the protein.

The integration of 1D [¹H-¹⁵N] TRACT spectra provides the average of different amide protons τ_c value. It is therefore not possible to distinguish individual ¹H-¹⁵N moieties. To better characterise the Hp1β tumbling behaviour, we measured rotational correlation time values of each ¹H-¹⁵N inter-nuclear vector using a 2D version of a ¹⁵N spin relaxation experiment.

The experiment is essentially a modified [¹H-¹⁵N] HSQC with a relaxation period inserted before the ¹⁵N evolution period (Tjandra et al., 1996). The pulse sequence was designed to measure the cross correlation (between dipolar and CSA relaxation mechanism) effect quantitatively for each amide ¹⁵N nucleus. Cross correlation values, obtained from the cross-peaks intensity data as described in the Material and Methods section of Tjandra et al, 1996, represent the starting point to calculate τ_c value for each ¹H-¹⁵N inter-nuclear vector. The equations used can be found in appendix II.

The experiment was carried out on a 1 mM ¹⁵N-labelled Hp1β sample, at 298K.

Figure 3.6 shows τ_c values plotted as a function of the assigned residues of the 22-77 fragment of the Hp1β chromo domain. From residue 22 to 70 we find τ_c values distributed between 8 and 15 ns, comparable with the average result obtained with the [¹H-¹⁵N] TRACT experiment performed at 303 K. (14.4 ns obtained with signal integration over the structured part of the amide proton spectra). Three residues (Asp28, Thr51, Ile64) show values smaller than 3 ns: we are evaluating whether these differences are due to data elaboration errors or can be justified by a localised increment of backbone mobility.

Residues from 70 to 77 are characterised by a gradual decay of τ_c, due to increased backbone flexibility in the proximity of the unstructured hinge region (79-117).

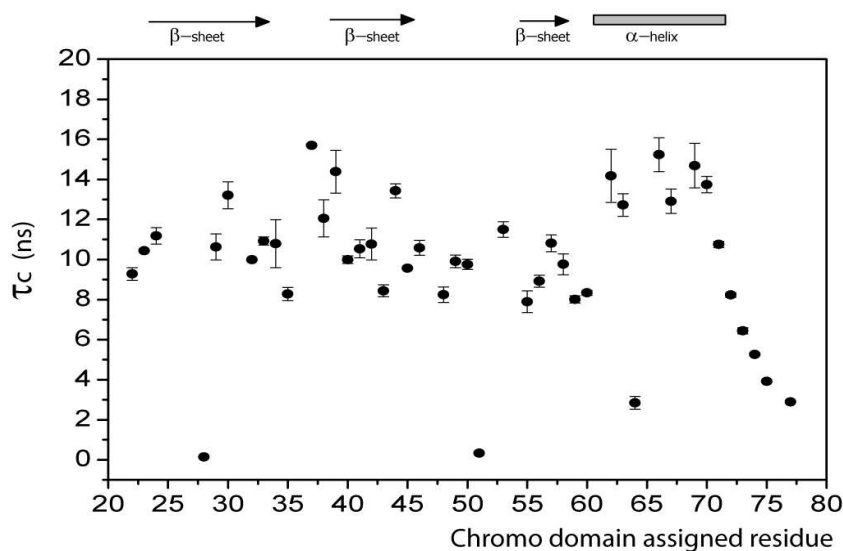


Figure 3.6_ Values of rotational correlation time as a function of CD assigned residue number in Hp1 β .

Figure 3.7 shows τ_c values plotted as a function of the non-assigned residues in Hp1 β unstructured regions, including the N-terminal part (1-21), the hinge region (79-117) and the C-terminal part (175-185). We find values between 0.7 and 3 ns, that reflect the high backbone flexibility in these unstructured parts.

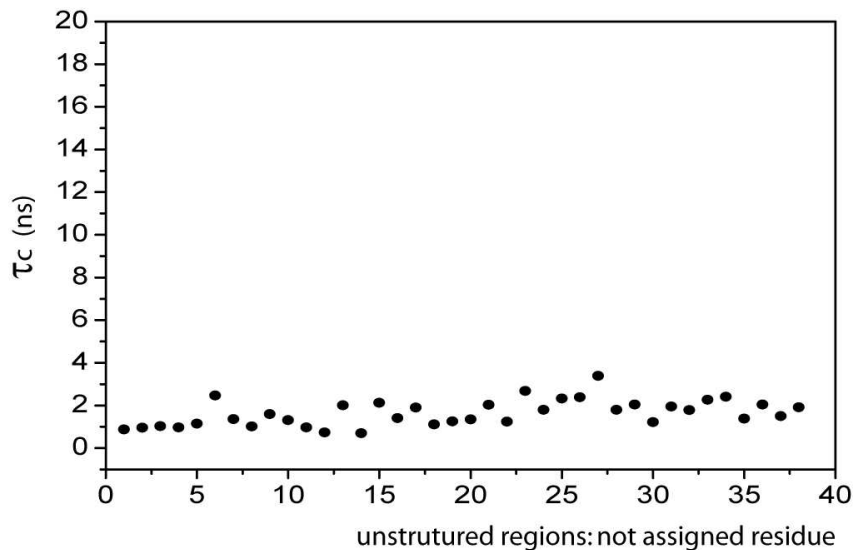


Figure 3.7_ Values of rotational correlation time as a function of non-assigned residues in Hp1 β unstructured regions.

Since the experiment provides a lower sensitivity (approximately by a factor of 5) compared to a regular [^1H - ^{15}N] HSQC, we did not obtain at all the less intense signals of the chromo shadow domain.

Acquisition of similar spectra at higher temperatures will allow us to obtain the complete information.

3.3.2 Residual dipolar couplings

Residual dipolar couplings (RDC) describe the orientation of inter-nuclear vectors with respect to the external magnetic field. They originate from the through-space dipole-dipole interaction between nuclei with a magnetic moment (Bax and Grishaev, 2005; Prestegard et al., 2005). While NOE effect, that is based on the same spin interaction, can give only short range restraints, RDC provide both short and long range orientational constraints, thus connecting remote parts of the backbone. Therefore, RDC became a widely applied method to obtain structural information. For example, they have been used to solve or validate structures (Cornilescu et al., 1998), determine the relative orientation between domains or between proteins in a complex (Fischer et al., 1999), or characterise intrinsically disordered proteins (Mohana-Borges et al., 2004; Cho et al., 2007). Recently, it has been shown that RDC are also sensitive to protein dynamics from ps to ms, thus providing information about a time scale not easily accessible by relaxation methods, as that of secondary structure motion (Lange, Lakomek et al., 2008).

For a pair of weakly coupled spin one-half nuclei this interaction can be represented as in Eq. 3.1. Here, r_{12} is the distance between nucleus 1 and 2, $\gamma_{1,2}$ are the magnetogyric ratios for the nuclei, μ_0 is the permeability of space, h is Planck's constant, and θ is the angle between the inter-nuclear vector and the magnetic field. For a directly bonded pair of nuclei, r is fixed and θ becomes the primary source of structural information (Prestegard et al., 2005).

$$\text{Eq. 3.1}_- \quad D_{12} = -\frac{\mu_0 \gamma_1 \gamma_2 h}{(r_{12} 2\pi)^3} \left\langle \frac{3 \cos^2 \theta - 1}{2} \right\rangle$$

When the molecule is free to explore all the possible directions in space during its tumbling, the average of the θ angles fall to zero, and no dipolar coupling is detected. However, the average can be made non-zero inducing a partial alignment of the molecule in the magnetic field thus allowing the observation of RDC in solution. To induce a slight but net alignment of a fraction of molecules (10^{-3} - 10^{-4}) with respect to the externally applied magnetic field, liquid crystalline media are employed. Weak alignment is required to obtain spectral simplicity and sensitivity comparable to that of conventional NMR spectra (Lipsitz and Tjandra, 2004; Bax & Grishaev, 2005). In anisotropic conditions, the observed splitting between two coupled nuclei will be the sum of scalar and dipole-dipole coupling. RDC are measured as differences in splitting of partially aligned ($J_{12} + D_{12}$) and isotropic (J_{12}) spectra. Several softwares can be used to convert RDC to structural information (RED CFRA FT, PALES), and a description of the theoretical background can be found in Lipsitz and Tjandra, 2004; Zweckstetter, 2008.

In the present work, we measured [^1H - ^{15}N] RDC on full length Hp1 β to characterise both the structured and the unstructured parts of the protein.

Analysis of RDC from the hinge region will provide insight on possible residual secondary structure or transient interactions of this unfolded part (Mohana-Borges et al., 2004; Cho et al., 2007).

Moreover, we can calculate the correlation between RDC measured for the well defined structured fragments (chromo and chromo-shadow domain) and the RDC calculated from

the respective known structures of the domains alone. (NMR or X-ray PDB files). Correlation parameters will indicate how the structure of the single domains in full length Hp1 β protein is similar to that of the PDB files, from which RDC were back calculated (Cornilescu et al., 1998; Zweckstetter, 2008).

Full length Hp1 β [^1H - ^{15}N] RDC were measured with two different alignment media, recording (IPAP) [^1H - ^{15}N] HSQC experiments (Ottinger et al., 1998) for each aligned sample and for the isotropic sample.

We employed Pf1 phages (Zweckstetter & Bax, 2001) and dodecyl-penta(ethylene glycol)(C12E5)/n-hexanol (Ruckert and Otting, 2000). While the first medium induces alignment mostly by electrostatic interactions, the latter has an alignment mechanism based on steric interaction. Details on sample preparation and data acquisition are in 5.3.6.1 and 5.3.6.2, respectively.

As an example, figure 3.8 shows how RDC values for G44 and E56 ^1H - ^{15}N pairs were measured using Pf1 phages as the alignment medium.

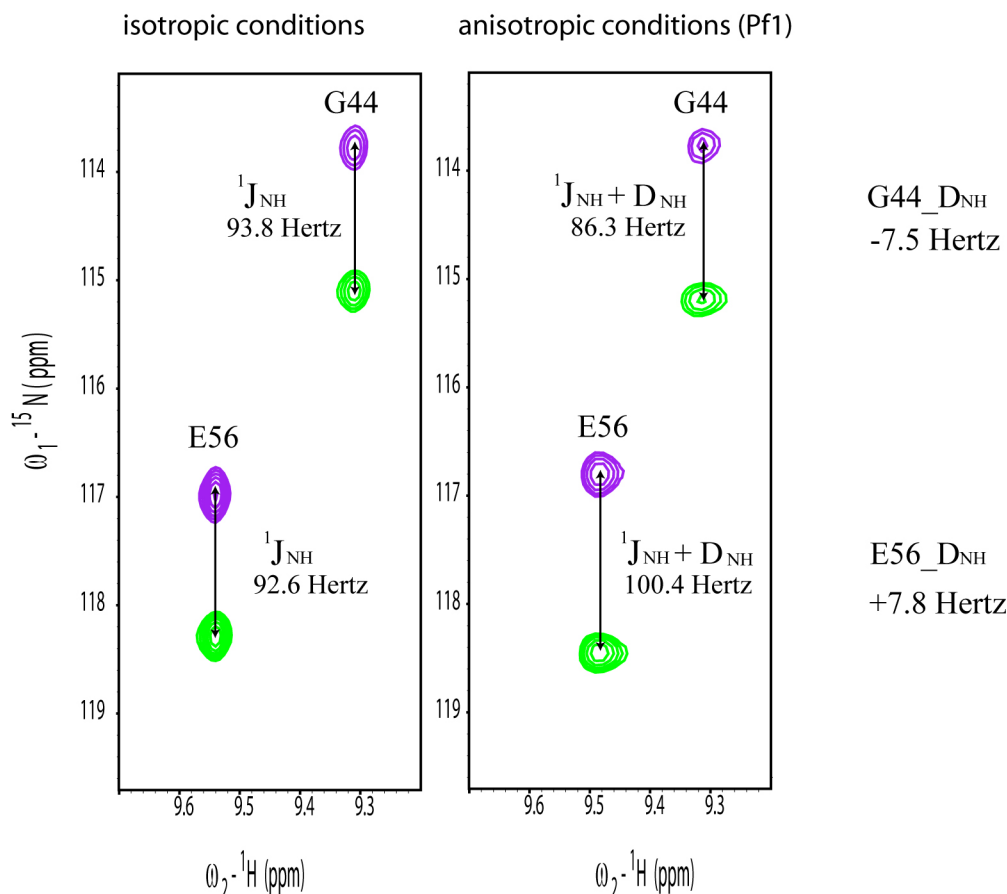


Figure 3.8_ Measurement of residual dipolar couplings from (IPAP) [^1H - ^{15}N] HSQC spectra. The one-bond $^1J_{\text{HN}}$ couplings are measured in isotropic solution, while $^1J_{\text{HN}} + D_{\text{NH}}$ values are obtained under slight anisotropic conditions (the example of Pf1 as the alignment medium is shown). D_{NH} is determined by subtraction of the $^1J_{\text{HN}}$ value determined in isotropic condition. A small part of the spectra was selected to better show the signal splitting, in the nitrogen dimension, for G44 and E56 ^1H - ^{15}N doublets. Downfield and upfield doublet components are coloured in green and violet, respectively.

At present, only the resonance assignment of the fragment 22-77 of Hp1 β was obtained. Therefore, the analysis of the ^1H - ^{15}N RDC is restricted to this part of the protein, corresponding to the chromo-domain.

The module –bestFit of the PALES software (Zweckstetter, 2008) was used to determine the correlation between experimental RDC, from residue 24 to 77, and the RDC back calculated from the NMR structure (PDB file 1ap0, Ball et al, 1997).

Specifically, we selected 28 experimental values corresponding to the α -helix and β -sheet secondary structural elements, and model 1 of the 1ap0 PDB file.

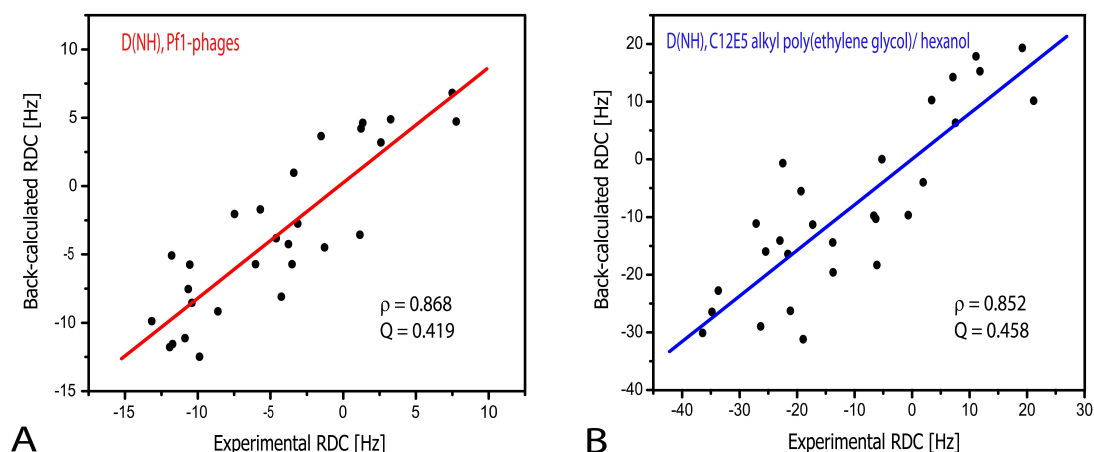


Figure 3.9_ Correlation of experimental residual dipolar couplings with back-calculated values from model 1 of Hp1 β chromo-domain NMR structure (PDB code: 1ap0) for two different alignment media. The alignment media employed are Pf1 phages (A) and n-dodecyl-penta(ethylene glycol)/n-hexanol (B). Q-values and ρ parameters indicate the correlation data quality.

To evaluate the quality of the data correlation two parameters were taken into account: the (ρ) Pearson's correlation coefficient (range [-1, 1]) and the RDC quality factor (Q) defined as:

$$\text{Eq 3.2_} \quad Q = \left\{ \sum_{i=1, \dots, N} [d_i^{\text{norm}}(\text{exp}) - d_i^{\text{norm}}(\text{calc})]^2 / N \right\}^{1/2} / D^{\text{r.m.s}}$$

with N being the number of measured and normalized couplings, $d_i^{\text{norm}}(\text{exp})$. $D^{\text{r.m.s}}$. refers to the root-mean-square value of RDC for randomly distributed internuclear vectors.

Typically good correlations have $\rho \geq 0.9$ and $Q < 0.4$ value (Bax & Grishaev, 2005).

Correlation results for both alignment media employed (Pf1-phages: $\rho=0.868$, $Q=0.419$; n-dodecyl-penta(ethylene glycol)/n-hexanol: $\rho= 0.852$, $Q= 0.458$) suggest that there is not a good correlation between experimental RDC and back-calculated RDC.

Comparable results (Pf1-phages: $\rho=0.823$, $Q=0.526$; n-dodecyl-penta(ethylene glycol)/n-hexanol: $\rho= 0.864$, $Q= 0.477$) were obtained using all the available RDC values (45 instead of 28, therefore considering the whole domain).

We are evaluating if this result can derive from experimental errors on RDC measurements, choice of PDB file or, possibly, can be justified by differences between the structure of the chromo-domain in the full-length Hp1 β , and that of the chromo domain alone (deleted mutant 10-77: PDB file).

3.4 Hp1 β /tri-mek9-histone tail interaction

3.4.1 [^1H - ^{15}N] TROSY-HSQC titration experiments

A previous study (Nielsen et al., 2002) characterised the interaction between the tri-mek9 histone 3 N-terminal tail (peptide 1-22) and the chromo domain of Hp1 β (10-80 residues). To investigate how the full length Hp1 β protein is involved in binding to its target, we added the unlabelled tri-mek9 peptide to the ^{15}N -labelled full length Hp1 β sample and monitored chemical shift changes for backbone residues in a [^1H - ^{15}N] TROSY-HSQC spectrum. The aim is to understand whether the hinge region and the chromo shadow domain can have a role in this interaction, or can have conformational changes upon the addition of the peptide to the chromo domain.

Figure 3.9 shows the overlay between the ^{15}N -labelled Hp1 β [^1H - ^{15}N] TROSY-HSQC spectra recorded with (blue) or without (red) the tri-mek9 peptide (1:1 molar ratio). In the most dispersed part of the spectrum (outside the 8-8.5 ppm strip) the position of a set of peaks is consistently perturbed. In the central part, where there are most of all the signals from the unstructured regions of the protein, there are no evident shifts.

The results obtained with the titration at different molar ratios (Hp1b/peptide 1:8, 1:1; 2.5:1; 5:1) reveal a slow exchange between Hp1 β free and in complex with the peptide (data not shown).

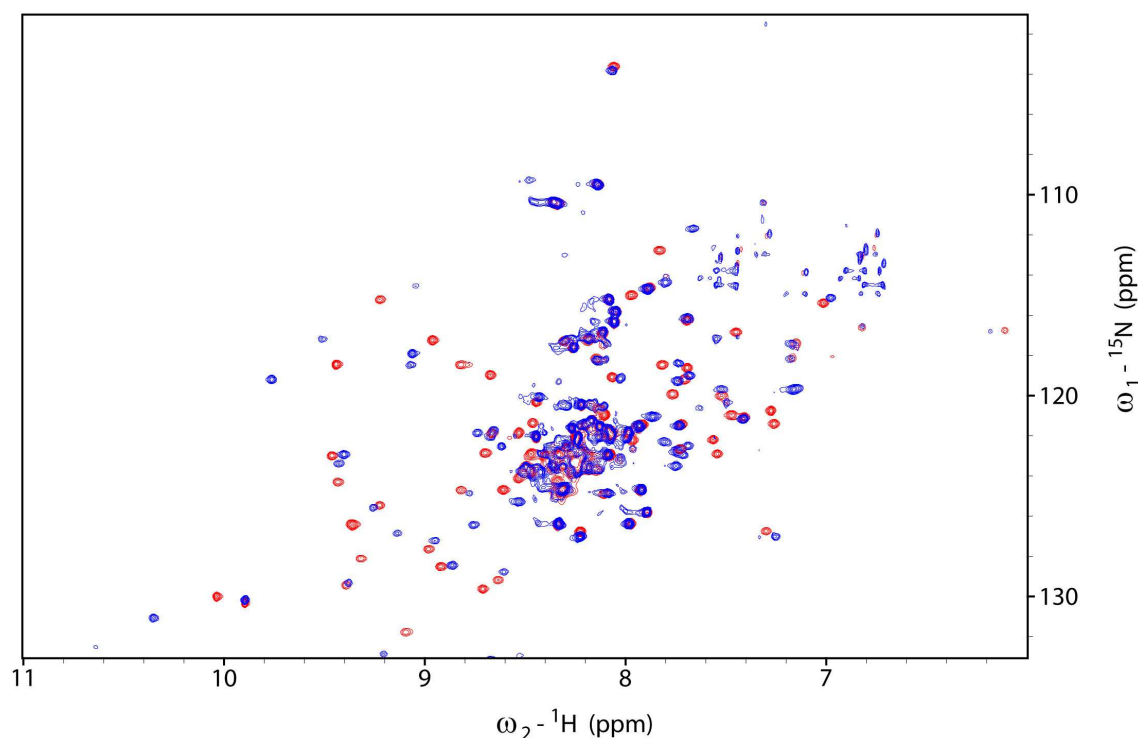


Figure 3.9_ Overlay of ^{15}N -labelled Hp1 β [^1H - ^{15}N] TROSY-HSQC spectra recorded with (blue) or without (red) the tri-mek9 peptide (1:1 molar ratio).

3.4.2 Chemical shift perturbation analysis

Since the resonance assignment obtained till now ranges from residue 22 to 77 of Hp1 β , the plot of chemical shift perturbation as a function of the assigned residue is restricted to the chromo domain. In figure 3.10, the plot of backbone chemical shift differences (δ_{average}) as a function of residue number for Hp1 β CD in the presence of the tri-meK9 peptide (molar ratio 1:1), relative to free Hp1 β CD, is shown.

Consistent with previous reports (Nielsen et al., 2002), data analysis confirms that specific residues are involved in recognition of the peptide: V22, V23, E24, K43, G44, E56, N57, L58, D59, L63 are characterised by $\delta_{\text{average}} > 0.15$ ppm. Y38, W42, N50, T51, C60 have $\delta_{\text{average}} > 0.1$ ppm. K25, V36, F45, S46, D47, E53, D62, I64 and F67 have $0.1 > \delta_{\text{average}} > 0.06$ ppm.

In figure 3.11, the [^1H - ^{15}N] TROSY-HSQC spectral perturbation data from figure 3.10 are plotted onto the surface of the Hp1 β chromo domain. Yellow corresponds to $0.1 > \delta_{\text{average}} > 0.06$ ppm, orange to $0.15 > \delta_{\text{average}} > 0.1$ ppm, and red to $\delta_{\text{average}} > 0.15$ ppm. The rest of the chromo domain, that has perturbation values from 0 to 0.06 ppm, is in grey.

The analysis performed on peaks not yet assigned, which include both the chromo shadow domain and the unstructured regions, suggests that there is not a perturbation comparable to that found in chromo-domain residues. Most of all the chemical shift differences values (δ_{average}) are smaller than 0.03 ppm. Few signals, belonging to the central part of the spectra, show values between 0.03 and 0.06 ppm. The complete assignment will possibly allow us to interpret these small differences.

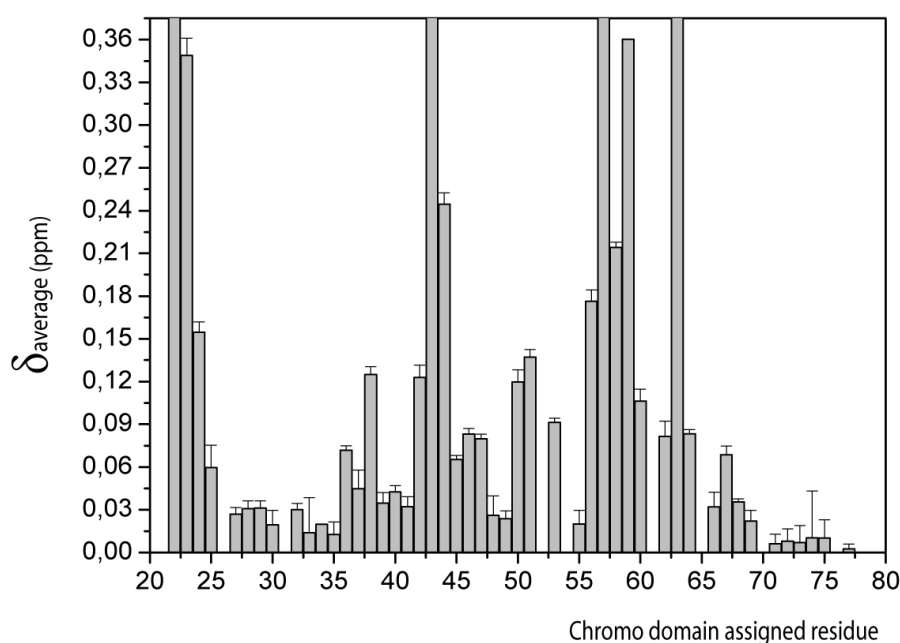


Figure 3.10 Plot of backbone chemical shift differences (δ_{average}) as a function of the residue number for ^{15}N labelled Hp1 β (1-185) in the presence of tri-meK9 peptide, ratio 1:1 relative to free Hp1 β . The analysis is restricted to the assigned signals of the chromo domain. δ_{average} values above 0.4 ppm have been cut.

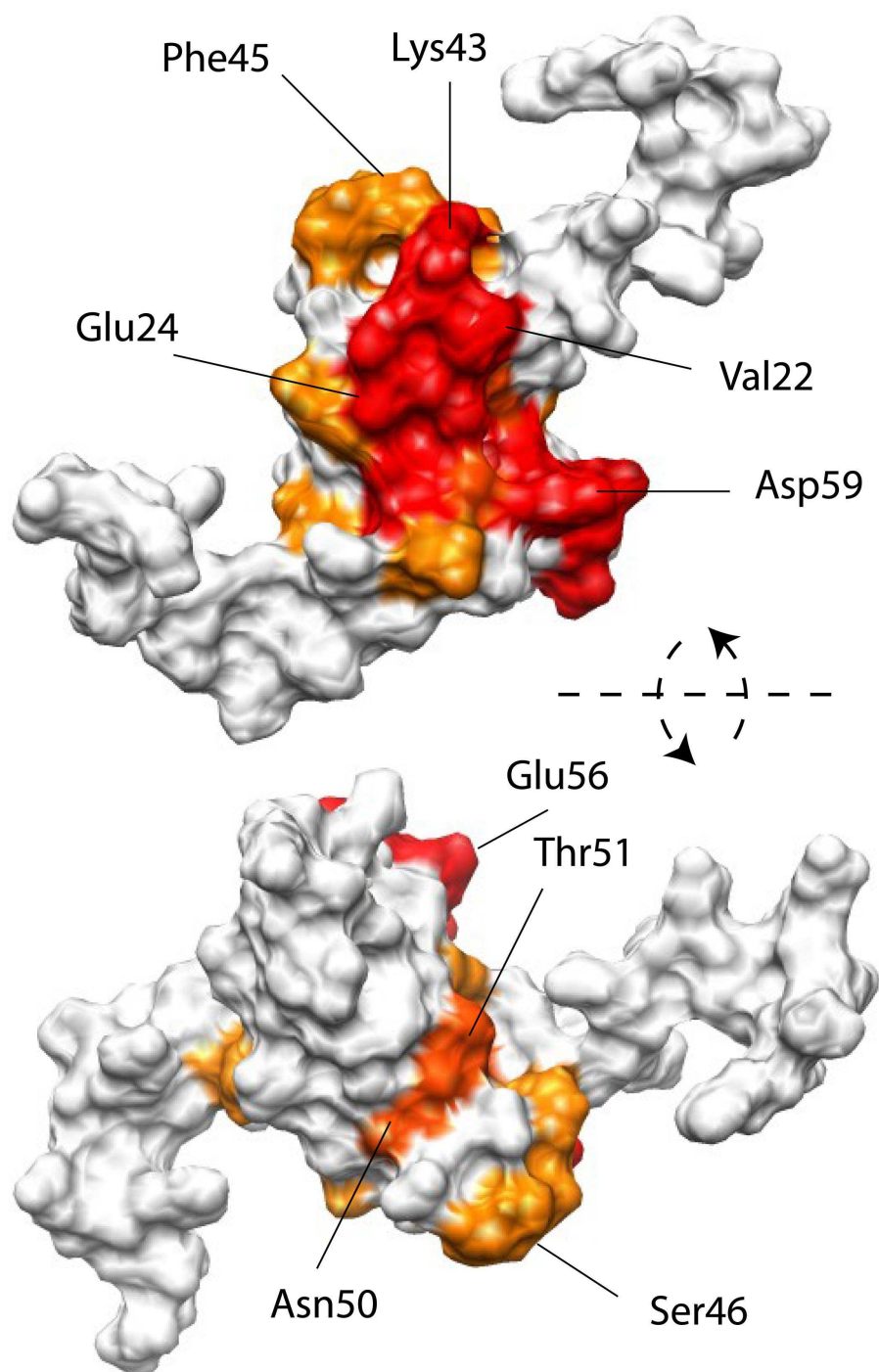


Figure 3.11_ Surface model of HP1 β chromo domain (PDB file: 1ap0. UCSF chimera software). The yellow to orange coloured surface represents the ensemble of residues characterised by chemical shift perturbation values between 0.06 and 0.4 ppm in the [^1H - ^{15}N] TROSY-HSQC interaction experiment with the tri-meK9 peptide (1:1). The grey surface defines the residues not significantly perturbed.

Chapter IV

Discussion

Discussion

The identification and characterization of protein-protein interactions is a key topic in structural biology and life science. The recognition between protein partners is a fundamental molecular mechanism by which proteins work out their role in the modulation of biological functions. As a result, many cellular processes are regulated by the cross-talk of different protein-protein interaction pathways.

My research is focused on the study of the molecular recognition mechanisms between proteins, using a combined biochemical and spectroscopic approach.

In particular, the project concerns two different examples of protein-protein interactions as molecular basis of biological functions.

The first part of the research aims at characterising the interactions between three key protein modulators of dopamine biosynthesis: α -synuclein, 14-3-3 proteins and Tyrosine Hydroxylase. This study is part of a wider research project in the group of Padova, aimed to unravel the molecular mechanism that leads to a failure of the regulation of dopamine metabolism that has been proposed to play a role in the pathogenesis of Parkinson Disease. The second part of the research is focused on the analysis of the molecular recognition between Heterochromatin protein 1 (Hp1 β) and a twenty-one residues peptide that mimics the histone 3 N-terminal tail of the nucleosome. This interaction is one of the regulation mechanisms of the heterochromatin synthesis that represents a tool used by eukaryotic cells to silence gene expression. The experiments were carry out during a six months visiting period in the structural biology department of Prof C. Griesinger, under the supervision of Prof M. Zweckstetter at the Max Planck Institute of Göttingen, Germany. To rationalize the results of the two research lines in their respective different biological contests, I will discuss the two subjects separately.

Protein-protein interactions in the regulation of the dopamine synthesis

L-Dopamine is a neurotransmitter with many regulatory roles both in the central nervous system and in the endocrine functions.

The modulation of the biosynthesis of its precursor, L-DOPA, by the reaction catalyzed by Tyrosine Hydroxylase (TH), is therefore tightly regulated. One fundamental regulation mechanism is the interaction of the enzyme with protein modulators (Itagaky et al., 1999; Perez et al., 2002). The first ones identified were 14-3-3 proteins (Ichimura et al., 1987), that are highly conserved and ubiquitously expressed molecules (Fu et al., 2000). Several data suggested that 14-3-3 proteins participate in the stimulus-coupled regulation of catecholamine synthesis that occurs in response to depolarization triggered Ca^{2+} phosphorylation of TH at Ser19 (Itagaky et al., 1999).

Biochemical and biophysical data (Kleppe et al., 2001; Yamauchi et al., 1981) confirmed that the interaction involves the N-terminal regulatory domain of the enzyme, and that this leads to the activity enhancement.

Further works suggest that also α -synuclein, a natively unfolded protein related to the pathogenesis of Parkinson Disease (Dev et al., 2003), interacts and modulates Tyrosine Hydroxylase activity (Perez et al. 2002 and 2004, Zhou et al., 2004). It has been suggested that α -synuclein could play a role in dopamine synthesis, through the inhibition of TH (Perez et al., 2002 and 2004; Peng et al., 2005).

Proteomic (Zhou et al., 2004), immunological (Ostrerova et al., 1999) and biophysical (Sato et al., 2006) studies demonstrated that there is an interaction also between α -synuclein and 14-3-3 proteins. This binding was suggested to be of functional significance in the context of Tyrosine Hydroxylase activity modulation.

As described in the introduction section, a large assortment of experimental data reveals these protein-protein interactions in neuronal extracts or in cell-cultures systems. However, few works characterise the complex with the recombinant proteins in solution, and there is not a structural description of the interaction.

The challenge of this research has been to evaluate and characterise the interaction between each of the possible protein pair, and furthermore to rationalise it in structural terms.

As necessary premise, an extensive part of this research work was invested to obtain, by molecular biology methods, the purified recombinant proteins for the interaction experiments. Moreover, the combination of biochemical and spectroscopic techniques allowed us to characterise the protein samples obtained.

As described in section 2.1.1 and 2.1.3, we optimized the purification protocol of both recombinant α -synuclein and Tyrosine Hydroxylase proteins. Furthermore, we successfully cloned, expressed and purified the TH1-152 and TH150-497 deleted mutants, which correspond to the N-terminal regulatory domain and the combination of this domain with the catalytic region, respectively. The production of single TH domains is part of an experimental strategy aimed to better define the possible binding site of the enzyme, in the interaction with its protein partners. Moreover, as both of them are more soluble than the full-length protein, they can be used in structural studies that require high protein concentrations. Of particular relevance is the production of TH1-152 mutant, that is the regulatory N-terminal domain of Tyrosine Hydroxylase. We set up an efficient protocol to purify the protein by refolding from inclusion bodies, where the molecule was confined as insoluble aggregates. Section 2.1.4 describes the procedure to obtain an average yield of 20 mg of purified soluble protein per litre of culture.

Chromatographic and spectroscopic studies were subsequently used to characterise the protein: biochemical analysis confirmed that the preparation is homogeneous, while circular dichroism experiments provide information on its secondary structure. In agreement with recently published data (Obsilova et al., 2008), we found that the TH regulatory domain is mostly unstructured with a minor contribution of α -helical and β -sheet secondary structure elements. The mainly unfolded nature of this domain can justify the particular behaviour in size exclusion experiments, for which TH1-152 has an estimated molecular mass (calculated ~ 40 kDa) greater than that expected for a compact molecule (~ 20 kDa). Moreover, the highly flexibility character of the regulatory domain might

explain the reason why several crystallization trials of full length human Tyrosine Hydroxylase, carried out by our group, failed. Interestingly, the published crystal structures of the rat protein were obtained only after the removal of this domain (Goodwill et al., 1997 and 1998).

Furthermore, we carried out 2D heteronuclear NMR experiments on ^{15}N -labelled TH1-152 to evaluate the possibility to assign its backbone ^1H , ^{15}N and ^{13}C resonances. The relevance of this experimental aim is justified by the perspective to follow on $[^1\text{H}-^{15}\text{N}]$ HSQC spectra the effect of 14-3-3 proteins binding on TH1-152 structure.

The preliminary results show that only one fourth of the expected peaks can be detected. Their narrow distribution along the proton dimension suggests that they derived from the part of the protein that lacks a well-defined secondary structure. The rest of the protein may be involved in extended conformational exchange, thus preventing the acquisition of sharp, single signals. It is therefore important to change conditions such as temperature, pH, protein concentration and acquisition parameters to detect the missing signals of the rest of the protein.

The samples of 14-3-3 protein, both in dimeric and monomeric forms, were provided by the bio-crystallography group of Prof. H. L. Monaco of the Verona University.

The subsequent step of the research is the analysis of the binding between each of the possible pair of the protein partners Tyrosine Hydroxylase, α -synuclein and 14-3-3 proteins. Biochemical and NMR methods were employed to both evaluate and characterise the protein-protein interaction.

As a preliminary work, we explored the possibility that α -synuclein and 14-3-3 proteins form a stable complex in solution, using electrophoretic and chromatographic methods. The results of both native PAGE electrophoresis and size exclusion chromatography experiments, performed in different conditions and protein molar ratio, indicate that recombinant α -synuclein and 14-3-3 proteins fail to form a high affinity complex in solution. Comparable results were obtained using 14-3-3 ζ and η isoforms and are in agreement with the surface plasmon resonance analysis carried out by Sato et al., 2006. The dissociation constant for α -synuclein and 14-3-3 η was calculated to be 1 μM .

On these premises, we decided to change experimental strategy. The formation of a weak-transient complex was investigated by NMR. Specifically, the titration of ^{15}N -labelled α -synuclein with 14-3-3 ζ and η isoforms was followed by $[^1\text{H}-^{15}\text{N}]$ HSQC (Heteronuclear Single Quantum Coherence) experiments. For an ^{15}N -labelled protein, every peak in this 2D NMR experiment correlates the ^{15}N chemical shift with that of the directly attached ^1H . Since chemical shifts of protein amides are very sensitive to environmental changes, the binding of a protein partner can induce spectral changes on this two-dimensional spectrum. For protein-protein interactions, changes in peak position are usually associated with a particular set of residues that are either directly involved in the interaction or involved in binding-induced conformational rearrangements. The experiment is therefore an effective tool to detect protein-protein interaction, even in the ultra-weak affinity range ($K_d > 10^{-4}$ M) (Vaynberg et al., 2006).

The analysis of the results obtained from experiments conducted at different α -synuclein and 14-3-3 ζ/η molar ratios does not reveal an evident interaction between the two

proteins. No binding was detected by chemical shift perturbation analysis. On the other hand, the study of the peak intensity changes shows an intensity decrease in few peaks, all of which seem to be clustered in the N-terminal region when α -synuclein spectra are acquired in the presence of 14-3-3 proteins. The localized intensity decrease could be explained with an interaction that involves only the N-terminal residues of the protein, so restricting the backbone amide spins mobility and lowering the corresponding signal intensity. The interaction is probably electrostatic in nature, as it involves the most basic region of α -synuclein and it is well known that the surface of all 14-3-3 isoforms is negatively charged (Fu et al., 2000). These small variations can be a preliminary indication of a transient interaction between monomeric α -synuclein and 14-3-3 proteins, but we cannot say if this can be related to a function. Based on these results, we decided to explore the possibility that 14-3-3 proteins interact with conformational states of α -synuclein different from the monomeric one. Specifically, we asked whether, consistently with their chaperone function (Yano et al., 2006), 14-3-3 proteins might inhibit α -synuclein fibril formation, thus interacting with partially folded intermediate species that lead to aggregation. Molecular chaperones have an essential protective role in preventing the misfolding of protein in stress conditions. They function by mediating the flux between different protein conformations, with a common mechanism that involves the binding of unfolded or partially folded regions of proteins characterised by an extensive hydrophobic stretch (Dobson et al., 2003).

Published data demonstrated that several molecular chaperons, such as Hsp70 and DJ1 (Dedmon et al., 2005; Zhou et al., 2006), can interact with α -synuclein oligomers, inhibiting the fibrillation process.

The spectroscopic technique employed to explore a possible 14-3-3 chaperone function on α -synuclein aggregation is fluorescence polarization. The technical principle and data elaboration are described in 2.2.3. Surprisingly, the results reveal a significant decrease on the aggregation rate of α -synuclein in the presence of 14-3-3 η proteins and suggest an inhibitory effect on the formation of oligomers whose growth triggers the nucleation-dependent aggregation process. These effects were confirmed with both native dimeric 14-3-3 protein and with a point mutant (Y85A) unable to dimerize, suggesting that the chaperone effect of 14-3-3 does not require any structural determinants at the dimer interface. Moreover, the experiments performed with the different molar ratios tested (α -synuclein:14-3-3 molar ratios 8:1; 4:1; 1:1) gave comparable results, implying that the critical 14-3-3 concentration needed to inhibit α -synuclein aggregation is below the values used.

Taken together, these data indicate that there is an interaction between the two proteins in solution, but only in particular conditions, related to a protein mis-folding context in which 14-3-3 proteins might bind to intermediate α -synuclein aggregated species to inhibit the fibrillation process.

Since α -synuclein forms amyloid fibrils in vitro that closely resemble those found in Lewy bodies of Parkinson Disease (Spillantini et al., 1997; Serpell et al., 2000), the investigation of the molecular mechanism of the effect of 14-3-3 binding on α -synuclein aggregation is of particular relevance and it represents the subsequent experimental aim of this research.

To summarise the findings obtained: biochemical and NMR studies revealed that there is no evident interaction between 14-3-3 proteins and α -synuclein in its “native”-monomeric state. Fluorescence polarization aggregation assay demonstrated that 14-3-3 proteins are able to inhibit α -synuclein fibrillation. It has to be verified whether this effect is governed by the binding of the pre-fibrillar oligomeric species that could expose the hydrophobic NAC region, a possible recognition motif for the molecular chaperone.

In this speculative view, we maybe failed to reveal the interaction between monomeric α -synuclein and 14-3-3 proteins probably because, in “native” conditions, the NAC region is shielded by the C-terminal tail of α -synuclein itself, as proposed by Dedmon et al., 2005 and Bertoncini et al., 2005.

Contrary to our results, many reports demonstrate the interaction of the two proteins in brain homogenates and cell-cultures. It is possible that, in *in vivo* conditions, other cellular factors promote the interaction between 14-3-3 proteins and α -synuclein, and that the absence of such components *in vitro* may prevent the formation of the complex. Another hypothesis is that the biochemical experimental procedures of the published works can promote, as a side effect, the formation of a small fraction of aggregated α -synuclein, and that the oligomeric species are recognised by 14-3-3 proteins.

The other protein pair object of this research is Tyrosine Hydroxylase and α -synuclein.

First of all, we wanted to confirm published data (Perez et al., 2002; Zhou et al., 2004), which support the interaction and the inhibition of TH activity by α -synuclein. Therefore, we investigated the role of α -synuclein as a classic inhibitor or as a competitive substrate of TH activity using fluorescence spectroscopy. This idea came from a recently published work where we demonstrated that, due to the high flexibility of the α -synuclein backbone, its endogenous tyrosine residues are exposed enough to be directly oxidised by the enzyme Tyrosinase (Tessari et al., 2008).

We found no experimental evidence of TH activity modulation by α -synuclein neither as modulator or competitive substrate. The result can be rationalised considering that the inhibitory effect of α -synuclein in cell culture experiments (Perez et al., 2002) can be mediated by different mechanisms besides a direct Tyrosine Hydroxylase/ α -synuclein interaction. In fact, several data demonstrate that α -synuclein is able to interact, in cell cultures experiments, with Calmodulin (Lee et al., 2002; Martinez et al., 2003) and 14-3-3 proteins (Ostrerova et al., 1999; Sato et al., 2006), to inhibit Protein kinase C (Ostrerova et al., 1999), and to activate phosphatase PPA2 (Peng et al., 2005). All of these interactions could lead to Tyrosine Hydroxylase inhibition.

Established that in our experimental conditions we do not reveal any inhibitory effect of α -synuclein on TH activity, we used the [^1H - ^{15}N] HSQC experiments strategy described previously to evaluate whether the two proteins can interact in solution anyway.

In addition to the absence of chemical shift changes, no line broadening was observed for ^{15}N -labelled α -synuclein in the presence of Tyrosine Hydroxylase, suggesting that no significant interaction occurs. Nevertheless, it will be necessary to improve the solubility of the recombinant enzyme and to repeat the experiment at a higher TH/ α -synuclein molar

ratio to verify if this result is due to a problem of sensitivity. An alternative strategy is the use of the more soluble TH deleted mutants 151-497.

The third protein-protein interaction of the dopaminergic system, investigated in this work, involved Tyrosine Hydroxylase and 14-3-3 proteins.

Since many published data confirmed that 14-3-3 proteins bind to the phosphorylated N-terminal domain of TH (pTH1-152) and enhance the activity of the enzyme (Itagaky et al., 1999; Kleppe et al., 2004), we planned to perform NMR titration experiments following the signals of the ^{15}N -labelled TH1-152 at different 14-3-3 molar ratios, to characterise the binding site. However, as described in section 2.1.4, we were not able to find the conditions to improve the solubility of TH1-152, a necessary premise to have good [^1H - ^{15}N] HSQC spectra and, above all, to assign the backbone resonances. Improvement of the NMR sample quality is required before interaction experiments in the presence of 14-3-3 proteins could be performed. Therefore, a different strategy was conceived to improve the solubility of TH1-152. Since the dissociation constant of the complexes between TH and 14-3-3 isoforms, determined by several techniques (Kleppe et al., 2004; Obsilova et al., 2008), was found in the nM range, we aimed at purifying the high affinity complex for subsequent structural studies of the TH regulatory domain in complex with the chaperon.

For a strong complex with 14-3-3 proteins, phosphorylation at TH Ser 19 is required (Itagaky et al., 1999). As already published by Obsilova et al, 2008, to make residue Ser19 a substrate of the commercially available kinase PKA, residue Ala17 was mutated to Arg (A17R). The phosphorylation reaction of purified TH1-152A17R worked efficiently in the presence of the binding partner 14-3-3 η . The next step was to perform the experiment on a large scale, to purify a sufficient amount of the TH1-152A17R/14-3-3 η complex to characterize it by electrophoretic and chromatographic methods, to study it by NMR spectroscopy, and to set up a preliminary crystallization screening. Biochemical experiments confirmed the formation of a high affinity complex between the two proteins. We also carried out a preliminary crystallisation screening and the optimisation of the crystallisation conditions is now in progress.

To combine both dynamic and structural information, we are preparing the same sample with labelled TH1-152A17R for NMR analysis. The availability of Tyr85Ala 14-3-3, the mutant unable to dimerize, opens the possibility to gain more information about the structure of the protein complex. We can explore whether the TH regulatory domain is able to interact also with monomeric 14-3-3 proteins, or if it requires to recognise structural determinants on the 14-3-3 dimerization interface to make a high affinity complex.

Furthermore, due to the smaller dimension, we will also produce ^{15}N -labelled-monomeric 14-3-3 to study, by NMR, the structural effect of the interaction with TH1-152.

Protein-protein interactions in the heterochromatin formation

The formation of Heterochromatin, a tightly packed form of DNA, is a regulatory mechanism to inhibit gene expression in specific regions of the chromatin. Specifically, the packaging involves post-translational modifications of the nucleosomes that represent the fundamental repeating units of eukaryotic chromatin. (Eissenberg et al., 2000).

Methylation of histone 3 at lysine 9 (H3K9) is one of the most significant examples of the effect of a covalent modification on the chromatin remodelling. Di- or tri-methylation of H3K9 generates a binding site for the chromo domain of Heterochromatin protein 1 (Hp1). Through the binding to chromatin, Hp1 stabilizes the packaging of DNA in a structure not readily accessible to transcription, with a final silencing effect on gene expression (Jacobs et al., 2001).

The structure of Hp1 proteins includes an N-terminal chromo domain (CD) connected to a slightly larger, C-terminal domain termed chromo shadow domain (CSD). The hinge region linking the two motifs is unstructured in solution, enabling the chromo and chromo shadow domains to move independently in the intact protein (Brasher et al., 2000). NMR structures were deposited for the deleted mutants 10-80 and 102-171, corresponding to the two domains alone (PDB code: 1ap0, Ball et al., 1997; 1dz1, Brasher et al., 2000).

This part of the experimental research aimed at assigning the ^1H - ^{15}N - ^{13}C backbone chemical shifts of full length Hp1 β protein to study its interaction with a peptide, trimethylated in K9 (tri-meK9), that mimics the tail (1-22) of histone 3. Hp1 β is one of the three human Hp1 isoforms and it is widely distributed on the chromosome (Thiru et al., 2004). Although there are already published data on the interaction between this peptide and the chromo domain alone (Nielsen et al., 2002), we are interested in studying the binding with the full length protein to see if also the remainder of the polypeptide chain can mediate the interaction. In particular, we want to understand whether the hinge region and the chromo shadow domain can have a role in this interaction, or can undergo conformational changes upon addition of the peptide.

The assignment strategy employed makes use of a combination of 3D heteronuclear experiments that correlate the backbone chemical shifts ($^{\text{N}}\text{H}(i)$, $\text{N}(i)$, $^{\text{N}}\text{H}(i+1)$, $\text{N}(i+1)$, $^{\text{N}}\text{H}(i-1)$, $\text{N}(i-1)$, $\text{CO}(i)$, $\text{CO}(i-1)$, $\text{C}\beta(i-1)$, $\text{C}\alpha(i-1)$) to establish the sequential connectivity between amino acids.

Specifically, we recorded HMQC-NOESY, HNN, [^1H - ^{15}N] TROSY-HNCO, [^1H - ^{15}N] TROSY-HN(CA)CO, and [^1H - ^{15}N] TROSY-CBCA(CO)NH experiments.

The analysis of the 3D spectra allowed us to assign 50 of the 56 ^1H - ^{15}N - ^{13}C resonances of the Hp1 β chromo domain fragment 22-77.

While the assignment of the chromo shadow domain is in progress with the available data, the most challenging target is the assignment of the unstructured regions, including the N-terminal part (1-21), the hinge region (79-117) and the C-terminal part (175-185). The signals of these protein segments, although very intense, have very poor dispersion in both ^1H and ^{13}C dimension. The consequence is that the carbon frequencies in the 3D spectra are accidentally degenerate, making the sequential assignment very hard. Therefore, we are

evaluating the use of other pulse sequences, with signal resolution improvement, to resolve these ambiguities. In particular, we plan to take advantage of pulse programs that employ constant time evolution in the ^{13}C dimension (Salzmann et al., 1989). This will provide better resolution to resolve the degeneracy in the carbon chemical shifts of the unstructured Hp1 β regions. Moreover, another valid alternative is the APSY (automated projection spectroscopy) experiment which takes advantage of the short effective rotational correlation times in unfolded polypeptides to record five- to seven-dimensional NMR data sets, which reliably resolves chemical shift degeneracies (Hiller et al., 2007).

Furthermore, to characterise the tumbling of the different parts Hp1 β protein, we measured rotational correlation time (τ_c) values for each ^1H - ^{15}N inter-nuclear vector using a 2D version of an ^{15}N spin relaxation experiment based on cross correlation (Tjandra et al., 1996).

The results indicate that the assigned region, from residue 22 to 70, is characterised by τ_c values distributed between 8 and 15 ns, in agreement with the expected value for a globular protein of that dimension. On the other hand, the non-assigned residues in Hp1 β unstructured regions, including the N-terminal part (1-21), the hinge region (79-117) and the C-terminal part (175-185), are characterised by τ_c values between 0.7 and 3 ns, which reflect the high backbone flexibility in these unfolded parts.

Since the experiment provides lower sensitivity (approximately by a factor of 5) compared to a regular [^1H - ^{15}N] HSQC, we did not obtain at all the less intense signals of the chromo shadow domain. Acquisition of similar spectra at higher temperatures will allow us to obtain the complete information.

We also measured [^1H - ^{15}N] residual dipolar couplings (RDC) on full length Hp1 β to obtain structural information of both the structured and the unstructured parts of the protein.

The analysis of RDC from the hinge region aims at providing insights on possible residual secondary structure or transient interactions of this unfolded part (Cho et al., 2007).

The determination of correlation parameters between RDC measured for the well defined structured fragments (chromo and chromo-shadow domain) and the RDC calculated from the respective known structures of the domains alone (NMR or X-ray PDB files), provides important information. It indicates how the structure of the single domains in full length Hp1 β protein is similar to that of the PDB files, from which RDC were back calculated (Zweckstetter, 2008).

For this purpose, full-length Hp1 β [^1H - ^{15}N] RDC were measured with two different alignment media: Pf1 phages (Zweckstetter & Bax, 2001) and dodecyl-penta(ethylene glycol)(C12E5)/n-hexanol (Ruckert and Otting, 2000). While the first medium induces alignment mostly by electrostatic interactions, the latter has an alignment mechanism based on steric interaction.

Since at present we obtained the resonance assignment of the fragment 22-77 of Hp1 β , the analysis of the [^1H - ^{15}N] RDC obtained was restricted to this part of the protein, corresponding to the chromo-domain. Correlation results for both alignment media employed (Pf1-phages: $\rho=0.868$, $Q=0.419$; n-dodecyl-penta(ethylene glycol)/n-hexanol:

$\rho = 0.852$, $Q = 0.458$) suggest that there is not a good correlation between experimental RDC and back-calculated RDC.

We are evaluating whether this result can derive from experimental errors on RDC measurements, choice of PDB file or, possibly, can be justified by differences between the structure of the chromo-domain in the full-length Hp1 β and that of the chromo domain alone (deleted mutant 10-77: PDB file).

The final study is focused on the characterisation of the interaction between Hp1 β and tri-mK9 peptide that mimics the histone 3 N-terminal tail.

Since the resonance assignment obtained till now ranges from residue 22 to 77 of Hp1 β , the analysis of the chemical shift perturbation is restricted to the chromo domain. Consistent with previous reports (Nielsen et al., 2002), data analysis confirms that specific residues are involved in recognition of the peptide: V22, V23, E24, K43, G44, E56, N57, L58, D59, L63 are characterised by $\delta_{\text{average}} > 0.15$ ppm. Y38, W42, N50, T51, C60 have $\delta_{\text{average}} > 0.1$ ppm. K25, V36, F45, S46, D47, E53, D62, I64 and F67 have $0.1 > \delta_{\text{average}} > 0.06$ ppm.

The analysis performed on peaks not yet assigned, which include both the chromo shadow domain and the unstructured regions, suggests that there is not a perturbation comparable to that found in the chromo-domain residues. Most of all the chemical shift differences values (δ_{average}) are smaller than 0.03 ppm. Few signals in the central part of the spectra show values between 0.03 and 0.06 ppm. The complete assignment will possibly allow us to interpret these small differences.

Chapter V

Materials and Methods

5.1 Molecular Biology

Materials

5.1.1 Bacterial strains

<i>E. coli</i> strain	Genotype	Application
DH5α	endA1, recA1, hsdR17 (r_k^- , m_k^+), supE44, thi-1, gyrA96, relA1, lacZ Δ M15, Δ (lacZYA-argF), U169, deoR.	Cloning
BL21(DE3)	F ⁻ , ompT, hsdSB (r_B^- , m_B^+), gal dcm (DE3).	Recombinant protein expression

5.1.2 Protein expression vectors

Vector type	Antibiotic resistance	Promoter	Inductor	Fusion tag	Protease for tag cleavage
pET3a	ampicillin	T7	IPTG	/	/
pET28b	kanamycin	T7lac	IPTG	His-tag	Thrombin
pPROEX	ampicillin	Trc	IPTG	His-tag	TEV

5.1.3 Culture Media

These are the recipes of bacterial culture media. LB Medium was used for the growth of both BL21(DE3) and DH5 α strains. M9 Medium was used for labelled recombinant protein expression. SOC Medium was used for both BL21 (DER) and DH5 α strains growth during transformation protocol.

LB Medium (Luria-Bertani)	10 g Bacto tryptone; 5 g yeast extract; 5 g NaCl; 1 ml of 1 M NaOH; deionized water to 1 litre. Sterilize by autoclaving. For agar plating solution, add 15 g agar.
M9 Medium (Minimal)	100 ml Autoclave sterilized 10xM9 salts*; 1 ml autoclave sterilized 0.1 M CaCl ₂ ; 2 ml filter sterilized 1M MgSO ₄ ; 40 ml filter sterilized 10% glucose; autoclave sterilized deionized H ₂ O, to 1 litre.
10xM9 salts*	17.1 g Na ₂ HPO ₄ · 12 H ₂ O; 3 g KH ₂ PO ₄ ; 0.5 g NaCl; 1 g (NH ₄)Cl. For ¹⁵ N labelled protein production, use ¹⁵ (NH ₄)Cl. Deionized H ₂ O to 0.1 litre. Sterilize by autoclaving.
SOC Medium	20 g Bacto tryptone; 5 g yeast extract; 0.5 g NaCl; 1.875 g KCl; deionized H ₂ O to 1 litre. Sterilize by autoclaving, add 20 ml of sterile 1 M glucose and 10 ml of sterile 1 M MgCl ₂ before use.

5.1.4 Agarose Gel Electrophoresis Buffers

TAE Buffer 50X (Tris-Acetate EDTA)	242 g Tris base salt; 57 ml Glacial acetic acid; 100 ml of 500 mM Na ₂ EDTA (pH 8.0). H ₂ O to 1 litre (adjust the pH to 8).
Loading Buffer 6X	0.4% (w/v) Orange G ; 0.03% (w/v) Bromophenol Blue ; 0.03% (w/v) Xylene Cyanol FF; 15% (w/v) Ficoll 400; 100 mM Tris-HCl (pH 7.5); 50 mM EDTA (pH 8.0).

5.1.5 DNA Oligonucleotides

Universal primers

Primer name	destination vector	sequence
T7 Forward	pET	5'-TAATACGACTCACTATAGGG-3'
T7 Reverse	pET	5'-GCTAGTTAT'TGCTCAGCGG-3'
M13/pUC Reverse	pPROEX	5'- AGCGGATAACAATT'TCACACAGG-3'
pBAD Reverse	pPROEX	5'-CTGATTTAATCTGTATCAGGCTG-3'

Cloning of Tyrosine Hydroxylase in pET expression vectors

Primer name	T _m	Sequence
Forward TH 1- 152	74 °C	5'-GGAGATATAC <u>CATATG</u> CCCACCCCC- 3' <i>NdeI</i>
Forward TH Δ 1-150	74 °C	5'-AGTGGTGTGCGCC <u>CATATG</u> TCAGAG-3' <i>NdeI</i>
Reverse TH 1-152	76 °C	5'-CGGGGATCCGCACGTCCTATGAC-3' <i>BamHI</i>
Reverse TH Δ 458-497	76 °C	5'-CGGATCCA <u>ACTAC</u> ACGGAGAAGGG-3' <i>BamHI</i>

Cloning of Tyrosine Hydroxylase in pPROEX expression vectors

Primer name	T _m	Sequence
<i>Forward</i> TH 1-497	88 °C	5'-AATTC <u>GGCGCC</u> ATGCCCACCCCGAC-3' <i>Nar I</i>
<i>Forward</i> TH 151-497	86 °C	5'-AATTC <u>GGCGCC</u> ATGTCAGAGGACGTGC-3' <i>Nar I</i>
<i>Reverse</i> TH 1-497	86 °C	5'-GGACGCCG <u>GGTACCT</u> AGCCAATGGCA-3' <i>Kpn I</i>

Tyrosine Hydroxylase site directed mutagenesis

Primer name	Sequence
TH152stop <i>Forward</i>	5'- CGCCAGGTGTCATAGGACGTGCGCAGC - 3'
TH152stop <i>Reverse</i>	5'- GCTGCGCACGTCCTATGACACCTGGCG-3'
THA17R <i>Forward</i>	5'- GGCTTCCGCAGGCGCGTGTCTGAGCTC-3'
THA17R <i>Reverse</i>	5'- GAGCTCAGACACGCGCCTGCGGAAGCC -3'

5.1.6 Restriction/modifying enzymes and Standard

Enzyme	Application
NdeI (Promega)	CA↓TA TG GT AT↑AC
BamHI (Promega)	G↓GATC C C CTAG↑G
Turbo NarI (Promega)	GG↓CG CC CC GC↑GG
KpnI (Promega)	G GTAC↓C C↑CATG G
Pfu DNA Polymerase (Promega)	High fidelity PCR
Taq Polymerase (home made)	Screening colony PCR
Calf Intestinal Phosphatase CIP (Biolabs)	Preventing re-ligation of linearized DNA vectors by removing phosphate groups from both 5' termini.
T4 DNA ligase (Promega)	Joining double-stranded DNA molecules with cohesive or blunt ends
Dpn I (NEB)	Digestion of template DNA vector in mutagenesis protocol
1 Kb DNA ladder (Promega)	250 to 10.000 bp reference
100 bp DNA ladder (Promega)	100 to 1500 bp reference
dNTPs mix (Promega)	PCR

Methods

5.1.7 Isolation of Plasmid DNA from *E. coli*

Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Quiagen, Hilden) according to manufacturing instructions. Briefly, 10 ml of the overnight culture were subjected to alkaline lysis, the cellular RNA was digested by addition of RNase A, and plasmid DNA was bound to a silica matrix under high-salt conditions. Elution of plasmid DNA with concentrations of 100-200 ng/μl was performed with 50 μl of Milli-Q water.

5.1.8 Transformation of *E. coli*

100 μl of competent *E. coli* cells were defrosted on ice, supplemented with 0.2-0.4 μg of DNA from a ligation reaction or 0.01-0.1 μg of circular plasmid DNA and incubated for 20 min on ice. Cells were then heat shocked for 90 sec at 42 °C in a water bath, and incubated on ice for another 2 min. After addition of 900 μl of SOC medium, the reaction mixture was incubated for 1 h at 37 °C with shaking and subsequently plated onto LB-agar plates containing the corresponding antibiotics for selection of positive transformants.

5.1.9 Polymerase chain reaction

Preparative PCR reactions were performed using 2-5 pg of template DNA, added in 50 μl of a mixture containing 0.2 mM of mix dNTPs, 0.5 μM of each primer and 1.5 U of Pfu DNA polymerase. MgCl₂ was included in the Polymerase buffer. DNA was amplified with 27 cycles of denaturation at 95 °C for 30 sec, annealing at 72 °C for 30 sec, extension at 72 °C for 1 min per kilobase. The final extension was performed at 72 °C for 10 min. When required, a “mutagenesis step” was included at the beginning of the PCR scheme: DNA was amplified with 5 cycles of denaturation at 95 °C for 30 sec, annealing below the melting temperature of primers for 30 sec, extension at 72 °C for 1 min per kilobase. Finally, amplified DNA was purified with PCR purification columns (GenElute™ PCR Clean-Up Kit, Sigma).

For Screening PCR, a Taq DNA Polymerase was used.

5.1.10 Cloning

Vectors and DNA inserts were digested with appropriate restriction enzymes and separated by agarose gel electrophoresis followed by DNA band extraction from gel (Agarose NA, Amersham) with Wizard® SV Gel and PCR Clean-Up System (Promega). Alternatively, without extracting the DNA from gel, Calf Intestinal Phosphatase (CIP) was used to de-phosphorylate the cut ends of both insert and vector. In this case, after restriction reaction, the DNA was purified with PCR purification columns (GenElute™ PCR Clean-Up Kit,

Sigma) and mixed with Phosphatase buffer and CIP according to manufacturing instructions.

For cloning of PCR fragments, restriction sites were introduced at the ends of primers. After PCR amplification, products were purified by a PCR purification system, digested with appropriate restriction enzymes and further purified by gel purification or using again the PCR purification system.

Fragments were quantified and ligated using 1 U of T4 DNA ligase in 20 µl reaction using 100 µg of vector and a corresponding stoichiometric amount of insert to give vector: insert ratios of 1:0 (control), 1:3, and 1:5.

Ligations were incubated for 3 h at 25 °C and 10 µl were used to transform 100 µl of competent DH5α cells. Some transformed clones were chosen for plasmid extraction in order to sequence their insert and verify the nucleotide sequence.. A single colony, positive at the sequencing test, was grown in LB medium at 37 °C overnight. Final clones (1 ml of culture with 0.5 ml of 100% glycerol) were stocked at –80 °C for storage.

5.1.11 Mutagenesis

For Mutagenesis, 20-40 ng of template DNA were used, added in 50 µl of a mixture containing 0.2 mM of dNTPs, 0.5 µM of each primer and 1.5 U of Pfu DNA polymerase. MgCl₂ was provided with Pfu buffer.

DNA was amplified with 12 or 18 cycles of denaturing at 95 °C for 30 sec, annealing at 55 °C for 1 min, extension at 72 °C for 1 min per kilobase. Depending on the desired mutation, the number of cycles can vary from 12 (point mutation), 16 (single amino acid change) or 18 (insertion, deletion). Mutagenic primers contain the desired mutation.

For digestion of parental (non mutated) DNA, 1 µl of *DpnI* restriction enzyme was added to the amplification reaction and incubated for 1 h at 37 °C. 5 µl were used to transform 100 µl of competent DH5α cells. Clones selection and stocks preparation were done as described in 5.1.10.

5.1.12 Agarose Gel Electrophoresis

Analytical and preparative separation of DNA fragments, according to their molecular weight, was obtained by electrophoresis using 1% (w/v) agarose gels (0.5-10 kb). For smaller DNA fragments separation, a 2% (w/v) agarose gel was used. To prepare 1% (w/v) gels, 1 g of agarose was dissolved in 100 ml of TAE buffer by heating. After cooling to about 60 °C, agarose solution was supplemented with 0.5 µg/ml ethidium bromide.

DNA samples were supplemented with 6× loading sample buffer and separated by electrophoresis under constant voltage (50-100 V). DNA bands were identified by ethidium bromide fluorescence emission in the visible spectral region.

5.1.13 Recombinant protein expression

For recombinant protein production, competent BL21(DE3) cells were used. Following transformation, some colonies were grown overnight in 20-50 ml of LB medium supplemented with the proper antibiotic. The culture was diluted 1/100 in the final volume of liquid medium containing the antibiotic and incubated with shaking at 37 °C until an O.D. of 0.5-0.6 was reached.

Expression of the desired gene construct was then induced with 0.1-0.2 mM IPTG. After 3-4 hours of incubation at room temperature, 28 °C or 37 °C, cells were harvested by centrifugation at 5000g for 10 minutes at 4 °C.

The bacterial pellet was then washed with PBS, centrifuged again and stocked at –80 °C. For recombinant proteins localised into the periplasm, the last step is not included and a special protocol starts immediately after cell harvesting as described in 5.2.11.

5.2 Biochemistry

Materials

5.2.1 Buffer Solutions

General Buffers

PBS (Phosphate-buffered Saline)	137 mM NaCl; 2.7 mM KCl; 10 mM Na ₂ HPO ₄ ; 2 mM KH ₂ PO ₄ . Adjust the pH to 7.4. Sterilize by autoclaving.
TBS (Tris-buffered Saline)	150 mM NaCl; 20 mM Tris-HCl. Adjust the pH to 7.4. Sterilize by autoclaving.

SDS-PAGE Gel Electrophoresis Buffers

Running Buffer	25 mM Tris base; 250 mM Glycine; 0.1% (w/v) SDS.
Resolving Gel Buffer	375 mM Tris-HCl (pH 8.8); 0.1% (w/v) SDS; 10-12-13 or 15% (w/v) acrylamide*; 0.1 % (w/v) APS; 0.01% (w/v) TEMED.
Stacking Gel Buffer	125 mM Tris-HCl (pH 6.8); 0.1% (w/v) SDS; 5% (w/v) acrylamide*; 0.1 % (w/v) APS; 0.01% (w/v) TEMED.
Coomassie Solution	1 g Coomassie brilliant blue R-250; 50 ml glacial acetic acid; 500 ml Methanol. H ₂ O up to 1 litre.
Destain Solution	10% Isopropanol; 10% acetic acid.
Sample loading Buffer 4x	200 mM Tris-HCl (pH 6.8); 400 mM DTT; 8% (w/v) SDS; 0.4% (w/v) Bromophenol Blue; 40% (w/v) glycerol.

*diluite 40% acrylamide/bisacrylamide mix solution

Native Gel Electrophoresis Buffers

Resolving Gel Buffer 375 mM Tris-HCl (pH 8.8); 10-12-13 or 15% (w/v) acrylamide*; 0.1 % (w/v) APS; 0.01% (w/v) TEMED.

Stacking Gel Buffer 125 mM Tris-HCl (pH 6.8); 5% (w/v) acrylamide*; 0.1 % (w/v) APS; 0.01% (w/v) TEMED.

Running Buffer 25 mM Tris-HCl (pH 8.3); 192 mM Glycine.

*diluite 40% acrylamide/bisacrylamide mix solution

5.2.2 Protein purification Buffers

α -Synuclein 140 purification

Osmotic Shock Buffer 30 mM Tris-HCl (pH 7.2); 2 mM EDTA; 40% Sucrose.

Anionic Exchange Binding Buffer 20 mM Tris-HCl (pH 8.0).

Anionic Exchange Elution Buffer 20 mM Tris-HCl (pH 8.0); 500 mM NaCl.

Wild type Tyrosine Hydroxylase purification

Sonication Buffer 50 mM Hepes (pH 7.4); 100 mM KCl; 75 μ M EDTA; 10% glycerol; PMSF, bacteria protease inhibitors cocktail.

Heparin Binding Buffer 50 mM Hepes (pH 7.4); 100 mM KCl; 75 μ M EDTA; 10% glycerol.

Heparin Elution Buffer 50 mM Hepes (pH 7.4); 800 mM KCl; 75 μ M EDTA; 10% glycerol.

Recombinant protein purification from inclusion bodies

Sonication Buffer	20 mM Tris-HCl (pH 7.4); 150 mM NaCl; PMSF and bacteria protease inhibitors cocktail.
DNase-RNase Buffer	20 mM Tris-HCl (pH 7.4); 150 mM NaCl; 2 mM MgCl ₂ ; 0.5 mM CaCl ₂ .
IBB1 (Inclusion bodies Buffer 1)	20 mM Tris-HCl (pH 8.0); 200 mM NaCl; 1% Sodium deoxycholate; 2 mM EDTA.
IBB2 (Inclusion bodies Buffer 2)	20 mM Tris-HCl (pH 8.0); 1 mM EDTA; 0.25% Sodium deoxycholate.
USB1 (Urea solubilization Buffer 1)	20 mM NaH ₂ PO ₄ /Na ₂ HPO ₄ (pH 7.4); 500 mM NaCl; 5 M Urea.
IMAC USB2 (Urea solubilization Buffer 2)	50 mM Tris-HCl; 50 mM NaH ₂ PO ₄ (pH 8.0); 8 M Urea

IMAC chromatography of urea-solubilized recombinant proteins

UIWB (Urea IMAC Washing Buffer)	50 mM Tris-HCl; 50 mM NaH ₂ PO ₄ (pH 6.3); 8 M Urea.
UIEB (Urea IMAC Elution Buffer)	50 mM Tris-HCl, 50 mM NaH ₂ PO ₄ (pH 4.3); 8 M Urea.

Histidine tagged recombinant proteins purification

Sonication Buffer	20 mM NaH ₂ PO ₄ /Na ₂ HPO ₄ (pH 7.4); 500 mM NaCl; 5 mM imidazole; PMSF bacteria protease inhibitors cocktail.
IMAC Binding Buffer	20 mM NaH ₂ PO ₄ /Na ₂ HPO ₄ (pH 7.4); 500 mM NaCl; 5 mM imidazole.
IMAC Elution Buffer	20 mM NaH ₂ PO ₄ /Na ₂ HPO ₄ (pH 7.4); 500 mM NaCl; 500 mM imidazole.

5.2.3 Biochemical tools

Bovine Thrombin (Amersham Bioscience) Tobacco Etch Virus TEV (recombinant)	Histidine tag cleavage
Bovine Protein Kinase A (Sigma)	Protein phosphorylation
BSA (Promega)	Native Page Standard
Markers Size Exclusion Chromatography (Pharmacia)	MW standards
Low molecular weight (Amersham Bioscience)	MW SDS-PAGE standards
Bovine DNase (Ambion) Bovine RNase (Sigma)	Nucleic acid digestion
Bovine Catalase (Sigma) BH4 (Sigma)	TH enzymatic assay

5.2.4 Enzymatic Reaction Buffers

Protein Kinase A	20 mM Tris-HCl (pH 7.5); 5 mM DTT; 250 mM NaCl; 15 mM MgCl ₂ ; 0.006 mM cAMP; 0.75 mM ATP.
TEV Proteolysis	20 mM NaH ₂ PO ₄ /Na ₂ HPO ₄ (pH 7.4); 100 mM NaCl; 1 mM DTT; 0.5 mM EDTA.
Tyrosine Hydroxylase	50 mM Hepes (pH 7.0); 200 mM (NH ₄) ₂ SO ₄ ; 7 mM DTT; 25 µg/ml Catalase; 25 µM (NH ₄) ₂ Fe(SO ₄) ₂ · 6H ₂ O; BH4 30 µM.

5.2.5 Equipment

HiTrap™ IMAC FF 1 ml (GE Healthcare)	Affinity chromatography column
HiTrap™ Heparin HP 1 ml (Amersham Bioscience)	Affinity chromatography column
HiTrap™ Desalting 5 ml (Amersham Bioscience)	Desalting column
RESOURCE™ Q 6 ml (Pharmacia Biotech)	Anionic Exchange column
Reversed Phase C4™ (phenomenex)	Reversed Phase column
Superdex 75™ HR 10/30 (Amersham Bioscience)	Size Exclusion column
Superdex 200™ HR 10/30 (Amersham Bioscience)	Size Exclusion column
FPLC ÄKTApurifier system™ (GE Healthcare)	Protein purification
HPLC Agilent Technology	Protein/peptide analysis
Ultrafiltration Membranes (Millipore)	Protein concentration/ Buffer Exchange
Ultrafiltration Spin columns (Sartorius)	Protein concentration/Buffer Exchange
Stirred Ultrafiltration Cell_Amicon (Millipore)	Protein concentration/ Buffer Exchange
Dialysis Membrane (Spectrum Laboratories)	Dialysis
Spectrafuge (Labnet International) Mikro 22R (Hettch Zentrifugen) TJ-25 Centrifuge (Beckman Coulter)	Sample centrifugation

Methods

5.2.6 SDS-PAGE Electrophoresis

To resolve proteins between 45-70 kDa, a 10-12% acrylamide resolving gel was prepared. For small proteins (10-30 kDa), 13-15% acrylamide was used.

The method requires protein denaturation: samples were mixed with 4× loading buffer and boiled at 100 °C for 10 min before loading. Electrophoretic run was carried out at room temperature applying 100-150 V. Loading of low molecular weight-SDS markers provides the references. Following electrophoresis, gels were stained with Coomassie Solution and finally washed in Destain solution.

5.2.7 Native-PAGE Electrophoresis

Since the run is performed in native conditions, no denaturing agents were used to prepare both samples and polyacrylamide gels. Therefore, none of the buffers contained either SDS or DTT and protein samples were not boiled before loading.

Proteins were separated in a 12 or 15% acrylamide resolving gel, according to their charge and shape. Runs were performed at 4 °C applying 100-150 V. BSA was used as a marker.

Following electrophoresis, the gels were stained with Coomassie Solution and finally washed in Destain solution.

5.2.8 Preparation of Cell Extracts from Bacteria Cells

The following procedure was used as starting step in the purification of most of the recombinant proteins.

The bacteria pellet, collected at 5000g for 10 minutes at 4 °C, was washed with PBS and centrifuged again. The pellet was then resuspended in 50 ml of ice-cold buffer per litre of culture. The solution used is usually the buffer of the first chromatographic step, supplemented with 1 mM PMSF and Bacterial protease Inhibitors Cocktail (Sigma).

Cell disruption was achieved by sonication on ice, with short bursts until clearing of the suspension. Centrifugation at 10000g at 4 °C for 30 minutes allowed the insoluble fraction to separate from the soluble cell components.

The supernatant, kept on ice, was 0.22 µm filtered before loading on chromatographic column. When required, the sample was incubated with DNase and RNase at room temperature, to digest nucleic acid contaminants.

In the inclusion bodies purification protocol, the insoluble fraction is further treated as described in 5.2.9.

5.2.9 Purification of Inclusion Bodies

This procedure allowed insoluble recombinant proteins to be isolated with high yield and high purity. The level of inclusion bodies purification plays a fundamental role in the success of the subsequent protein refolding.

After cell breakage, the insoluble fraction was washed with different solutions to purify the inclusion bodies from cell debris, including membranes, nucleic acids and membrane proteins.

In the first step, the insoluble material was resuspended in 50 ml RNase-DNase Buffer per litre of culture, and incubated at room temperature, under stirring, with 50 U of DNase and RNase per litre of culture.

After centrifugation for 20 min at 10000g at 4 °C, the pellet was resuspended in 50 ml of IBB1 solution and the suspension stirred for 30 minutes at room temperature.

The pellet, collected by centrifugation, was then taken up in 50 ml IBB2 and the suspension stirred for another 30 minutes. This step was performed twice. The insoluble fraction was then solubilised in 5 M Urea (in 32 ml USB1 buffer per litre of culture), the suspension stirred at room temperature for 30 minutes, and finally centrifuged at 4 °C for 20 min at 28000g. The soluble fraction obtained in the last step consists of purified inclusion bodies of the recombinant protein

5.2.10 Protein Refolding

To allow recombinant protein refolding, the denaturing agent was removed by step dialysis over 48 hours: the procedure was carried out at 4 °C, dialysing the supernatant obtained, against 4 M, 2 M, 1 M, 0.5 M, 0 M Urea Buffer. The successfully folded protein was separated by centrifugation (28000g, 30 min at 4 °C) of the dialysed solution and concentrated with Amicon device (Millipore) for further experiments.

To improve the purification yield, an IMAC chromatographic step can be added before in vitro refolding. The purified inclusion bodies are solubilised in 8 M Urea (USB2 buffer), the solution centrifuged and filtered, and applied to a Ni²⁺-column, equilibrated with the same buffer. Elution with a pH gradient (UIEB buffer) gives a highly purified preparation to start the refolding by dialysis as described above.

5.2.11 Periplasmic fraction purification

For recombinant proteins secreted in the bacteria periplasm, a protocol based on periplasmic fraction isolation allowed fast and high-yield purification. This method was used for over-expressed α -synuclein that contains an endogenous signal translocation sequence at its N-terminus (Ren et al., 2007). The osmotic shock to release the α -synuclein contained in the periplasm was carried out according to Huang et al., 2005.

The cell pellet from a 1 l culture was resuspended in 100 ml osmotic shock buffer and incubated for 10 min at room temperature. The pellet collected by centrifugation at 10000g for 20 min was resuspended quickly with 90 ml cold water followed by addition of 37.5 μ l

of saturated MgCl_2 , and kept on ice for 3 min. During this step, the periplasmic proteins are released into the MgCl_2 solution.

The supernatant containing the periplasmic proteins was collected by centrifugation at 10000g for 20 min and dialyzed against the desired buffer overnight. Only for α -synuclein, that is a natively unfolded protein, dialysis can be performed against water to improve the purification yield.

5.2.12 Protein quantification

The concentration of proteins in solution was determined, according to the Lambert-Beer law, by measuring the absorption at 280 nm (A_{280}).

$$A_{\lambda} = \epsilon_{\lambda} \cdot c \cdot d$$

A_{λ} : absorption at wavelength λ

ϵ_{λ} : molar absorptivity at wavelength λ in $\text{M}^{-1} \text{cm}^{-1}$

c: protein concentration (M)

d: thickness of the cell (cm)

When the molar absorptivity was not available, protein concentration, in a purified sample, was determined with the Bradford assay (Bradford, 1976). Briefly, 0.1 ml of protein sample was mixed with 3 ml of Bradford Reagent (Sigma) and the absorbance measured at 595 nm. The protein concentration was determined by comparison with a standard curve, prepared with BSA samples of known concentration ranging from 0.1 to 1.4 mg/ml.

5.2.13 Preparative Chromatography

Protein purification in the milligram scale was carried out with an FPLC ÄKTA-purifier system. All the samples were passed through a 0.22 μm or a 0.45 μm filter and/or centrifuged immediately before loading into the column. Protein elution was followed monitoring the Absorbance at 280 nm. All the chromatographies were performed at room temperature.

IMAC Chromatography

Most of the recombinant proteins studied were cloned and purified as Histidine-tagged forms because of their highly selective affinity for metals immobilized on chromatographic media that allowed high purification yields. This chromatographic technique is termed immobilized metal ion affinity chromatography (IMAC).

We used prepacked 1-ml HiTrap IMAC HP columns with sepharose matrix functionalized with chelating groups charged with Ni^{2+} .

The system was equilibrated with sodium phosphate buffer (pH 7.4), supplemented with NaCl 500mM, was used. To prevent non specific binding, 5 M imidazole was included both in sample and in binding buffer.

In the purification procedure performed in native conditions, Histidine-tagged proteins elution was achieved with a linear imidazole gradient (5-500 mM), typically in 40 minutes.

In the protocol under denaturing conditions (8 M urea), as an alternative to elution with imidazole, a pH gradient from pH 8 to approximately pH 4.5 was used. All the chromatographic steps were carried out at 1 ml/min flow rate.

Anionic Exchange Chromatography

IEX (Ionic Exchange) Chromatography was used to separate molecules on the basis of differences in their net surface charge. Recombinant proteins with acidic pI were purified with a pre-packed column RESOURCE™ Q made of polystyrene/divinyl benzene matrix to which $-\text{CH}_2-\text{N}^+(\text{CH}_3)_3$ groups have been covalently bonded.

Tris-HCl buffers with 2 pH units above the pI of the target protein were used. Elution was carried out increasing the ionic strength with a linear 0-500 mM NaCl gradient in 40 minutes.

Column equilibration and chromatographic steps were carried out at 1 ml/min flow rate.

Desalting

Desalting columns were used as a faster method to remove $(\text{NH}_4)_2\text{SO}_4$ from Tyrosine Hydroxylase samples before Heparin chromatography.

We used 5 ml Amersham HiTrap Desalting columns, packed with Sephadex™ G-25 Superfine. The medium is based on cross-linked dextran with an exclusion limit of approximately Mr 5000.

Samples were loaded in 1 ml volume and the desalting was performed at 5 ml/min flow rate.

Heparin Chromatography

Immobilized matrix heparin can operate both as an affinity ligand and as a high capacity cation exchanger, due to its anionic sulphate groups. It has been used to selectively purify Tyrosine Hydroxylase, which has a Heparin binding epitope at its N-terminal. The buffer used is 50 mM Hepes, 10% glycerol.

Protein elution was achieved applying a linear 0-500 mM KCl gradient in 40 minutes. All the chromatographic steps were carried out at 1 ml/min flow rate.

Size Exclusion Chromatography

Size Exclusion Chromatography separates molecules according to differences in size as they pass through a gel filtration medium packed in a column.

For purification purposes, Superdex 75 HR 10/30 or Superdex 200 HR 10/30 columns were used, depending on the fractionation range required (respectively 3-70 kDa and 10-600 kDa). The medium is based on highly cross-linked porous agarose particles with dextran covalently bonded.

The choice of the equilibrating buffer varied according to the sample or the requirements for subsequent purification, analysis or storage. Typically, samples were loaded in a 0.5 ml volume and the chromatography was carried out at a 0.5 ml/min flow rate.

5.2.14 Analytical Chromatography

Chromatography for analytical purpose was carried out with an Agilent Technology HPLC system.

All the samples were passed through a 0.22 μm filter and/or centrifuged immediately before applying them to the column. Protein-peptide elution was followed monitoring the Absorbance at 280 nm and 226 nm. All chromatographies were performed at room temperature.

Size Exclusion Chromatography

As an analytical method, Size Exclusion Chromatography was used to estimate the molecular weight of target proteins and to probe the formation of stable complexes in solution between putative protein partners.

For molecular weight determination, the retention time of the protein of interest was compared with the values obtained for several calibration standards, in the same experimental conditions.

The calibration curve allowed also the determination of the protein oligomerization status. Both Superdex 75 HR 10/30 and 200 HR 10/30 columns, connected with the HPLC system, were used with 0.44-0.5 ml/min flow rate, in 20 mM Tris-HCl or sodium phosphate buffer (pH 7.4) or PBS (pH 7.4).

Analysis of protein-protein interaction by Size Exclusion Chromatography was carried out after mixing the two protein partners at different molar ratios and incubation at room temperature for 1 hour. 20 μl samples were applied on Superdex 75 or 200, and the chromatography performed at a 0.44-0.5 ml/min flow rate. Different buffer and ionic strength systems (PBS pH 7.4, Tris-HCl 20 mM pH 7.5, Tris-HCl 20 mM pH 7.5 NaCl 150 mM) were used.

Reversed phase

Reversed Phase Chromatography was performed on a C4 column connected to an HPLC system, to prepare highly purified proteins before mass spectrometry analysis. Moreover, it has been used as a general method to check the quality of the protein samples.

The solvents used were water (A) and acetonitrile 95% (B), both supplemented with TFA, 0.1% and 0.085% respectively. Elution was achieved with acetonitrile 0-95% gradient, optimized based on the sample features. The flow rate was 0.6 ml/min.

5.2.15 Protein Concentration

This step allowed the sample volume to be reduced before Size Exclusion Chromatography, therefore maximizing the purification efficiency. Moreover, it represents the final step of sample preparation for experiments that required high protein concentrations.

Two different devices were used for this purpose: both are based on ultrafiltration processes.

The first are Vivaspin 500 centrifugal filter units (Sartorius) that contain a polyethersulfone membrane with a 3000 molecular weight cut off (MWCO). After washing, they can be loaded with a 600 µl sample. Concentration down to 100 µl was carried out at 4 °C or room temperature (depending on the sample) in a fixed angle rotor at 10000g.

The second device are 10 ml Amicon centrifugal cells (Millipore) supplemented with Regenerated Cellulose YM membranes with 3000 or 10000 MWCO.

After membrane washing, the concentration down to 1 ml is performed under gentle stirring at 4 °C with 3 bar operating pressure.

These devices were also used for buffer exchange.

5.2.16 Buffer Exchange

Buffer exchange or desalting, during protein sample preparation, was achieved with different methods depending on the required time and the protein features. Dialysis was always performed at 4 °C with washed regenerated cellulose tubing with 3000 (Spectrum) or 14000 (Sigma) MWCO. Ultrafiltration process was carried out at 4 °C or at room temperature using centrifugal filter columns (Vivaspin 500, Sartorius; 3000 MWCO) or Amicon centrifugal cell with YM membranes 3000 and 10000 MWCO (Millipore). The operating procedure is the same as described in 4.2.15, repeating the step several times with concentrated sample diluted in the new buffer for buffer exchange.

5.2.17 Cleavage of histidine-tag

For recombinant proteins cloned in fusion with an N-terminal histidine-tag, the final step of sample preparation was the removal of the affinity tag by proteolysis.

Specific proteases cleave a recognition sequence introduced between the polyhistidine stretch and the natural N-terminal of the target protein.

In pPROEX expression vectors the recognition sequence is specific for TEV (Tobacco etch virus) protease. Dialysis at 4 °C was necessary before proteolysis in order to remove the imidazole used for IMAC elution. Alternatively, Size Exclusion Chromatography was used. Proteolysis was conducted for 2-3 hours at 30 °C. A W/W ratio of 1:20 between TEV and substrate was used. Finally, to remove the protease from the sample another IMAC Chromatographic step was carried out, after DTT and EDTA clearance by dialysis. This because an histidine-tagged TEV was used.

Histidine-tag removal from recombinant proteins expressed in pET28 vectors, was carried out with bovine Thrombin. Only small scale proteolysis tests were conducted using 10 units of enzyme per mg of substrate, in PBS (pH 7.4), at room temperature.

5.2.18 Protein Phosphorylation

Protein phosphorylation was carried out for the mutant TH1-152A17R, to obtain a strong complex with 14-3-3 η protein.

The purified protein was incubated with 0.7 units of Protein Kinase A per μ g of substrate in the presence of 15 mM MgCl₂, 0.006 mM cAMP, 0.75 mM ATP and 5 mM DTT. The reaction was carried out at room temperature, overnight. The success of the phosphorylation reaction was then verified by mass spectrometry analysis.

5.2.19 Tyrosine Hydroxylase fluorimetric assay

The assay takes advantage from the different fluorescence properties of the substrate and the product of Tyrosine Hydroxylase (L-tyrosine and L-DOPA).

To probe the enzymatic activity, the purified recombinant enzyme was first incubated, to a final concentration of 500 nM, in a solution composed of 200 mM (NH₄)₂SO₄, 7 mM DTT, 25 μ g/ml catalase, 25 μ M (NH₄)₂Fe(SO₄)₂ · 6H₂O, 50 mM Hepes (pH 7.0). After 15 min equilibration at room temperature, the reaction solution was completed adding the cofactor BH₄ to 30 μ M. The reaction started with the final addition of the substrate tyrosine, from a 2.5 mM stock solution.

The assay was carried out, under stirring, in a HELMA cell with Suprasil® windows and 10 mm optical path length, thermostated at 37 °C.

The product formation was followed exciting the sample at 294 nm and monitoring the emission intensity at 330 nm.

A standard curve, made with known L-DOPA concentrations in the buffer condition described above, allow the calculation of the yield of product formation from the intensity of fluorescence emission in the enzymatic reaction.

5.2.20 Protein aggregation assay

α -Synuclein labelling with Oregon Green 488 and aggregation tests were carried out by Laura Tosatto of our Padova research group, according to the procedure described in Luk et al. Biochemistry 2007.

Aggregation tests were performed on 96-well polycarbonate plates (0.2 ml per reaction) at 37°C in an orbital mixer providing constant agitation at 1000 rpm. The assay was carried out in PBS (pH 7.4) supplemented with 5mM DTT, 0.02% NaN₃ using 1 mg/ml of unlabelled α -synuclein and 1:100 of Oregon Green 488 labelled α -synuclein. The purified protein solution was filtered with a 0.22 μ m cellulose acetate filter to remove any aggregate before the experiment.

Aggregation was monitored following the Fluorescence polarization of Oregon Green 488 labelled α -synuclein, as described in 5.3.5.

5.3. Spectroscopy

Materials

5.3.1 Equipment

UV-VIS	UV-Vis spectrophotometer Agilent 8453 diode-array HELLMA Suprasil cells
Circular Dichroism	UV-Vis CD spectrophotometer Jasco J-715 HELLMA Suprasil cells
Fluorescence	Fluorescence spectrophotometer Perkin-Elmer LS 50 HELLMA Suprasil cells
Nuclear Magnetic Resonance	Bruker Avance 600 MHz, triple axis gradient (Padova) Bruker 400 MHz, triple axis gradient (MPI) Bruker DRX 600 MHz, triple axis gradient (MPI) Bruker Avance 600 MHz, with cryo-probe, z-axis gradient (MPI) Bruker Avance 700 MHz, triple axis gradient (MPI) Bruker DRX 800 MHz, triple axis gradient (MPI)

5.3.2 Software

UV-VIS	Chemstation Software
Circular Dichroism	J-700 program, CDNN for deconvolution
Fluorescence	FL-WinLab program
NMR	X-WinNMR 2.6 (Bruker BioSpin) Topspin 1.3 and 2 (Bruker BioSpin) Gifa (M.A.Delsuc, CBS, France) NMRPipe (Delaglio, NIH, USA) Sparky (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco)
Data Analysis	Origin 7.5, Mathematica

Methods

5.3.3 UV-Visible

UV-Visible absorption measurements (at selected wavelengths or as 190-1100 nm spectra) were recorded on a diode-array Agilent 8453 UV-visible spectrophotometer interfaced with a personal computer using the Chemstation Software for Windows®. Optical measurement were performed at 25 °C using HELLMA quartz cells with Suprasil® windows and an optical path length of 1 cm. To avoid linear baseline artefacts, the absorption average over the range 700 to 900 nm was subtracted from the entire spectrum

5.3.4 Circular Dichroism

CD measurements were carried out on a JASCO J-715 spectropolarimeter interfaced with a personal computer and the spectra were acquired and processed using the J-700 program for Windows®. All experiments were performed at room temperature using HELLMA quartz cells with Suprasil® windows and 0.02 or 0.1 cm (FAR-UV) optical path length. FAR-UV spectra were recorded in the wavelength range of 190-260 nm, using a bandwidth of 2 nm and a time constant of 2 s at a scan speed of 50 nm/min. The signal:noise ratio was improved by accumulating at least four scans. The choice of buffer and protein concentration value depended on the path length used and on the experiment requirements.

All spectra are reported in terms of mean residue molar ellipticity ($\text{deg cm}^2 \text{dmol}^{-1}$).

5.3.5 Fluorescence

Fluorescence measurements were recorded on a Perkin-Elmer Life Sciences LS 50 spectrofluorimeter equipped with a thermostated cell compartment and interfaced with a personal computer using the FL-WinLab program for Windows®.

Emission spectra (305 to 450 nm) were obtained at 25 °C, using excitation wavelengths of 280 or 295 nm and recording at a scan rate of 120 nm/min with a 10 nm bandwidth and 10 scans. Sample measurements were carried out using a HELLMA ultra-micro cell with Suprasil® windows and an optical path length of 10×2 mm.

Fluorescence Tyrosine Hydroxylase Kinetics were carried out at 37 °C, under stirring in a HELLMA cell with Suprasil® windows and an optical path length of 10×4 mm.

Emission measurements were recorded every second for 30 min at 330 nm, using 294 nm wavelength for sample excitation and a 6 nm bandwidth.

Fluorescence polarization measurements were recorded on a Plate reader DTX 880 Multimode Detector Beckman Coulter, every 6 hours at 535nm. Excitation wave length was 490nm.

5.3.6 Nuclear Magnetic Resonance

The NMR spectra of α -synuclein and TH1-152 samples were recorded on a 600 MHz Bruker spectrometer equipped with a triple resonance probe, at the Chemical Sciences Department of the University of Padova. The spectra were acquired with the X-WIN NMR software and processed using GIFA or NMRPipe. The analysis was performed using GIFA and Sparky.

The NMR experiments on Hp1 β samples were carried out on 400, 600, 700 and 800 Bruker spectrometers equipped with triple resonance probes, at the Max Planck Institute of Göttingen (Germany). The spectra were acquired with Topspin 1.3 or 2.0 software and processed using NMRPipe. The analysis was performed using Sparky.

5.3.6.1 NMR Samples

α -Synuclein samples were prepared in 50 mM PBS (pH 7.4) or Hepes buffer (pH 7.0), with 10% D₂O (v/v) and 0.02% NaN₃ (w/v). For HSQC spectra acquisition, the samples contained 0.12 mM ¹⁵N-labelled protein.

TH1-152 spectra were recorded with 0.05 mM ¹⁵N-labelled protein sample in 20 mM sodium phosphate buffer (pH 7.4), 100 mM NaCl, with 10%D₂O and 0.02% NaN₃.

Hp1 β samples were prepared in sodium phosphate buffer (pH 6.5), 20 mM NaCl, with 10% D₂O and 0.02% NaN₃.

For 2D experiments and for the HMQC-NOESY spectrum, a ¹⁵N-labelled sample, at different protein concentrations, was used. For the other 3D experiments, a ¹⁵N-¹³C-²H- (70% deuteration) labelled protein sample, at 1.6 mM concentration, was used. These protein samples were provided by the Chromatin biochemistry group of the Max Planck Institute in Göttingen.

The measurement of ¹H-¹⁵N residual dipolar couplings on Hp1 β sample required the partial alignment of the protein with respect to the magnetic field. For this purpose, two alignment media were used: Pf1 filamentous phages (Hansen et al., 1998) and ether/alcohol liquid crystalline phases (Ruckert and Otting, 2000).

In the first case Pf1 phages from a 50 mg/ml stock solution were collected by ultracentrifugation (100000 rpm, 4 °C for 60 min), washed on the buffer used for the experiment, and finally resuspended on a ¹⁵N-labelled sample of Hp1 β 0.2 mM, to the final concentration of 18 mg/ml. Washing and protein buffers contained sodium phosphate buffer (pH 6.5), 300 mM NaCl, with 10% D₂O and 0.02% NaN₃. In these conditions, the quadrupolar splitting of the deuterium signal was 10 Hz.

The sample aligned in ether/alcohol liquid crystalline phases was prepared as described by Ruckert et al Otting using dodecyl-penta(ethylene glycol) C12E5 and hexanol. A 14%

(w/v) C12E5 stock solution was mixed, by vortexing, with a concentrated protein sample to the final concentration of 5% (w/v) C12E5 and 0.36 mM ^{15}N -labelled Hp1 β . Hexanol was added in 1-2 μl increments, with vortexing after each addition, until the solution went from clear to milky, turbid, then to translucent and viscous and clear again. The buffer used to prepare the C12E5 stock solution was the same of the protein sample: sodium phosphate buffer (pH 6.5), 20 mM NaCl, with 10% D_2O and 0.02% NaN_3 . The measured deuterium splitting was 25 Hz.

5.3.6.2 Data Acquisition

2D Heteronuclear experiments

$[^1\text{H}-^{15}\text{N}]$ - HSQC

α -Synuclein and TH1-152 spectra were collected at 283 K, using 256×512 complex incremental data points in the F1 (^{15}N) and the F2 (^1H) dimensions, with 32 scans per increment and a 1 s relaxation delay. Spectral widths of 26 ppm (centred at 115.7 ppm) and 3 ppm (centred at 7.5 ppm) were used for the ^{15}N and the ^1H dimension, respectively.

The HSQC spectra of 1 mM Hp1 β ^{15}N -labelled sample were recorded at different temperature, using 256×2048 complex incremental data points in the F1 (^{15}N) and the F2 (^1H) dimensions, with 32 scans per increment, and a 1 s relaxation delay. Spectral widths of 32 ppm (centred at 117 ppm) and 14 ppm (centred at 4.7 ppm) were used for the ^{15}N and the ^1H dimension, respectively.

In the TROSY version, the same spectral widths were used. Spectra were acquired at 303 K with 256 (or 512) $\times 2048$ complex incremental data points in the F1 (^{15}N) and the F2 (^1H) dimensions, and a 1 s relaxation delay. The number of scans varied depending on the protein sample concentration: typically 32 with 1 mM and 96 with 0.2 to 0.3 mM.

$[^1\text{H}-^{15}\text{N}]$ - ipap HSQC

The pulse sequence used to measure $^1\text{H}-^{15}\text{N}$ J and J+D coupling values for Hp1 β RDCs experiment is a modified version of the HSQC called In-Phase-Anti-Phase (IPAP) (Ottiger et al., 1998).

As maximum resolution was required, spectra were recorded with 1024×1024 complex data points in both dimensions. 64 scans and a 1 s relaxation delay were used. The choice of the working temperature depended on the alignment medium stability: 298 K for the C12E5/hexanol system, 303 K for Pf1 phages system.

Estimation of the rotational correlation time

[¹H-¹⁵N]-TRACT experiment

1D [¹⁵N,¹H]-TRACT experiments (Lee et al., 2006) were recorded at 303 K on a Bruker 700 MHz spectrometer with Hp1 β samples at different concentrations: 0.2, 0.6 and 1 mM. 2048 complex points in the ¹H dimension and 256 scans were used; the ¹H spectral width was set at 12 ppm and the incrementing delay in the ¹⁵N dimension was 2 ms: with 100 points, the relaxation time ranges from 0 to 200 ms.

[¹H-¹⁵N]- HSQC cross correlation experiment

2D cross correlation experiments were carried out at 298 K with a 1 mM Hp1 β sample, on a Bruker 600 MHz equipped with cryo-probe.

Spectra were acquired with 2048 (t₂) $\times$ 256 (t₁) complex data points; a total of 52 scans per complex t₁ increment was collected. Spectral widths were the same used in the classic ¹H-¹⁵N HSQC experiment.

Two experiment were recorded, one with the relaxation delay d23 (inserted before the ¹⁵N evolution period) set at 10 msec, the other with d23 = 20 s. As described in appendix Tjandra et al., 1997, for every d23 delay chosen, the experiment is performed twice, once applying the shaded ¹H 90° and composite ¹H 180° pulses, and once without applying these pulses. The receiver gain must have the same value in each pair of experiment.

3D Heteronuclear experiments

¹⁵N-edited HMQC-NOESY

The 3D HMQC-NOESY experiment, supplemented with the mixed-time parallel evolution approach (MT-PARE) to improve both sensitivity and resolution (Ying et al., 2007) was acquired on a Bruker 700 MHz, at 303 K. The sample contained 0.36 mM ¹⁵N-labelled Hp1 β .

The spectrum was acquired as a 1024 (¹H) $\times$ 50 (¹⁵N) $\times$ 170 (¹H) data matrix with 26 transient per fid and a 1 s delay between scans. The spectral width in the F2 (¹⁵N) dimension was 30 ppm, with the carrier centred at 117 ppm, and in the F1 (¹H) dimension peaks were folded into a region of 4.0 ppm, centred at 4.7 ppm. The ¹H spectral width in acquisition was 14 ppm. The mixing time of the NOESY block was set to 120 ms.

[¹⁵N-¹H]-TROSY-HNCO/HN(CA)CO

The ¹⁵N-¹H TROSY version of HNCO and HNCACO experiments (Saltzmann et al., 1998 and 1999) were recorded on a Bruker 600 MHz equipped with cryo-probe at 303 K. The sample used contained 1.6 mM ²H-¹⁵N-¹³C-labelled Hp1 β protein (70% deuterated). The following parameters were used for the HNCO experiment: data size 46 (¹³C) $\times$ 42 (¹⁵N) $\times$ 2048 (¹H); spectral width in ω_1 (¹⁵N), ω_2 (¹³C), ω_3 (¹H): 13, 16 and 13 ppm, respectively.

The spectral widths were centred at 174, 117 and 4.7 ppm. 48 scans per increment were acquired. For the HN(CA)CO experiment: data size $48 (^{13}\text{C}) \times 44 (^{15}\text{N}) \times 2048 (^1\text{H})$ with the same spectral widths as in the HNCO; 48 scans per increment were acquired. Only for the latter experiment, deuterium was decoupled during carbon carbonyl evolution.

HNN experiments (Panchal et al., 2001) were recorded with 1.6 mM ^2H - ^{15}N - ^{13}C -labelled Hp1 β protein sample, on a Bruker 700 MHz at 310 K. Spectral widths comprised 32 ppm (^{15}N , F1 and ^{15}N , F2), centred at 118 ppm, and 14 ppm (^1H , F3), centred at 4.7 ppm. Time domain data consisted of $68 \times 68 \times 1024$ complex points along t1, t2 and t3, respectively. 40 scans were accumulated per transient.

[^{15}N - ^1H]-TROSY-CBCA(CO)NH

The ^{15}N - ^1H TROSY version of the CBCA(CO)NH experiment was recorded on a Bruker 800 MHz equipped with cryo-probe at 303 K. The sample contained 1.6 mM ^2H - ^{15}N - ^{13}C -labelled Hp1 β protein. The following parameters were used: data size $68 (^{13}\text{C}) \times 40 (^{15}\text{N}) \times 2048 (^1\text{H})$; spectral width in ω_1 ($^{13}\text{Ca}/^{13}\text{Cb}$), ω_2 (^{15}N), ω_3 (^1H): 52, 32 and 13 ppm, respectively centred at 42, 117 and 4.7 ppm. Carbonyl carbon offset was set at 174 ppm; aliphatic carbon offset was at 54 ppm. 64 scans per increment were acquired.

5.3.6.3 Chemical Shift Perturbation experiments

α -Synuclein-protein interaction NMR experiments were carried out on a Bruker 600 MHz, at 283 K. [^1H - ^{15}N]-HSQC spectra of 0.12 mM ^{15}N -labelled α -synuclein were recorded with and without the unlabelled putative protein partner, dissolved in the same buffer to avoid artefacts in the chemical shift perturbation analysis.

The molar ratio used was 1:3 for the α -synuclein/14-3-3 proteins experiment, and 5:1 for the α -synuclein/Tyrosine Hydroxylase experiment.

For Hp1 β NMR titration, several sets of [^1H - ^{15}N]-TROSY-HSQC experiments were recorded on a Bruker 600 MHz equipped with a cryo-probe, at different Hp1 β /tri-meK9 peptide molar ratios, keeping all the acquisition parameter unaltered. The working temperature was 303 K. Hp1 β and the peptide were dissolved in the same buffer to avoid artefacts in the chemical shift perturbation analysis. Successive additions of unlabelled peptide stock (11.7 mM) were made to the protein solution containing 0.3 mM ^{15}N -labelled Hp1 β ; TROSY-HSQC spectra were recorded at peptide to protein ratios of 0:1; 1:5; 1:2.5; 1:1; 8:1.

In all of these experiments, chemical shift values were measured with the program SPARKY and the shift displacement was evaluated as the weighted average of the proton and nitrogen chemical shift changes (ppm) between ^{15}N -labelled protein alone and in combination with the protein partner, according to the formula:

$$\Delta\delta_{average} = 1/2\sqrt{\Delta\delta H^2 + (\Delta\delta N/5)^2}$$

For each peak, the shift displacement error was calculated as the standard deviation on the chemical shift value derived from three HSQC experiments, acquired in the same conditions, of ^{15}N -labelled protein alone.

To detect signal intensity changes in the α -synuclein experiments, a list of all the peak intensity values was obtained for each HSQC spectrum recorded with or without the protein partner. The ratio of these value I/I_{free} is the parameter chosen for the analysis. The associated error was calculated by the error propagation method of the noise value provided for each spectrum by the program SPARKY.

APPENDIX I

Data elaboration for cross correlation experiments

A comprehensive description of cross-correlation can be found in the book “Spin dynamics, Basics of Nuclear Magnetic Resonance” written by Malcom H. Levitt. (Wiley). Some concepts are reported here to introduce the basic equations required to elaborate $[^1\text{H}-^{15}\text{N}]$ -TRACT and $[^1\text{H}-^{15}\text{N}]$ - HSQC cross correlation experiments data.

The ^{15}N nuclei of $^{15}\text{N}-^1\text{H}$ spin systems in a protein backbone are subjected to two sources of local fields: the magnetic field emanating from the neighbouring proton, and the chemical shift anisotropy (CSA) interaction from the induced currents in the surrounding electron clouds. The two fields are correlated as both originate from sources within the molecule. With molecular tumbling, the local fields are modulated and lead to ^{15}N relaxation.

Relaxation of peptide backbone ^{15}N nuclei is therefore governed by the cross-correlation (or relaxation interference) between the dipolar interaction (D) between ^{15}N and the directly attached proton, and the CSA.

In the case of ^{15}N coupled to a proton in the β spin state, the ^{15}N local field is reduced by the interference between the CSA and the dipolar interaction. When the ^1H is in the α state, the ^{15}N local field is instead increased by the interference of the two mechanisms.

As a result, the two types of ^{15}N nuclei relax at very different rates.

Since CSA is proportional to the external magnetic field, at certain B_0 value CSA and dipolar interaction cancel each other for the β proton spin state. The relaxation rate difference between ^{15}N coupled to ^1H in α or β states is therefore amplified. The TROSY technique exploits this differential line width effect to study large molecules by NMR.

From the measurement of the cross correlation for ^{15}N nuclei in $^{15}\text{N}-^1\text{H}$ systems, it is possible to derive the rotational correlation time of the molecule. This is because spin relaxation mediated by dipolar interaction and CSA depend strictly on the tumbling properties of the molecule.

The elaboration of the experimental data, to obtain cross correlation values, is different for $[^1\text{H}-^{15}\text{N}]$ -TRACT (Lee et al., 2006) and for $[^1\text{H}-^{15}\text{N}]$ - HSQC cross correlation experiment

(Tjandra et al., 1996). In both publications, elaboration of the data is described in the materials and methods section.

In the first case, exponential fitting of the intensity data is employed to obtain spin relaxation rates, R_α and R_β . The transverse cross-correlated relaxation rate η is calculated as the difference between the two values:

$$R_\alpha - R_\beta = 2\eta$$

In the work of Tjandra et al., a hyperbolic tangent function is used to fit the experimental data I_A/I_B (A and B refer to the value obtained with two different schemes of the pulse sequence, 2Δ is the relaxation delay value inserted before ^{15}N evolution):

$$I_A/I_B = \tanh(2\Delta\eta)$$

Cross correlation values are then used to derive τ_c , with the following relations:

$$\eta = 2D\delta_N(4J(0)+3J(\omega_N))3\cos^2\theta -1)/2$$

D is the Dipolar coupling term between ^1H and ^{15}N of the ^{15}N - ^1H moiety:

$$D=\mu_0\gamma_H\gamma_N h/(16\sqrt{2}\pi^2 r_{\text{HN}}^3)$$

and δ_N is the Chemical shift anisotropy term (CSA) of ^{15}N :

$$\delta_N=\gamma_N B_0 \Delta\delta_N/(3\sqrt{2})$$

where γ_H and γ_N are the gyromagnetic ratios of ^1H and ^{15}N , respectively; h is Planck's constant, r_{HN} is the ^{15}N - ^1H internuclear distance, $\Delta\delta_N$ is the difference of the two principal components of the axially symmetric ^{15}N chemical shift tensor, and $J(\omega)$ represents the spectral density function at the frequency ω :

$$J(\omega)=0.4\tau_c/[1+(\tau_c\omega)^2]$$

When the slow motion limit ($\omega\tau_c \gg 1$) is considered, $J(\omega)$ can be neglected and only $J(0)$ is used for data elaboration.

APPENDIX II

Abbreviations

A17R	Alanine 17 to Arginine mutation
Y85A	Tyrosine 85 to alanine mutation
APS	Ammonium persulfate
ATP	Adenosine -5'-Triphosphate
a.u	Arbitrary units
BH ₄	Tetrahydrobiopterin (6R)-2-Amino-6-[(1R,2S)-1,2-dihydroxypropyl]-5,6,7,8-tetrahydro-4(1H)-pteridinone
BSA	Bovine Serum Albumin
cAMP	Adenosine -3'-5'-cyclo phosphoric acid
CD	Circular dichroism
CD	Chromo-domain
Cip	Calf intestinal Phosphatase
CSA	Chemical shift anisotropy
CSD	Chromo shadow domain
DLB	Dementia with Lewy bodies
DNA	Desoxyribonucleic acid
D _{NH}	N-H Dipolar coupling
L-DOPA	3,4-dihydroxy-l-phenylalanine
dNTPs	Deoxy nucleotide triphosphates
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EPR	Electron paramagnetic resonance
FP	Fluorescence Polarization
^N H	Amide proton
HMQC	Heteronuclear double quantum coherence
Hp1 β	Associated heterochromatin protein 1, β isoform
HSQC	Heteronuclear single quantum coherence
Hz	hertz
INEPT	Insensitive nuclei enhanced by polarization transfer
I _{rel}	Relative intensity
coIP	Co-immunoprecipitation
IPAP	In-Phase-Anti-Phase
IPTG	Isopropyl- β -D-thiogalactopyranoside
J _{NH}	N-H scalar coupling

LB	Luria-Bertani medium
LBs	Lewy bodies
kb	Kilo bases
kDa	Kilo Daltons
K_M	Michaelis-Menten constant
ms	Milliseconds
MM	Molecular mass
MW	Molecular weight
MWCO	Molecular weight cut off
μ s	Microseconds
NAC	Non-A β Component of Alzheimer's disease amyloid plaques
NMR	Nuclear magnetic resonance
NOE	Nuclear overhauser effect
ns	Nanoseconds
nm	Nanometers
OD	Optical density
PBS	Phosphate buffered saline
PEG	Polyethylen glycol
PCR	Polymerase chain reaction
PD	Parkinson's disease
PKA/	Protein Kinase A/C
PMSF	Phenylmethysulfonyl fluoride
PPA2	Protein phosphatase A2
ppm	Parts per million
ps	Picoseconds
R_α	Relaxation rate constant of the spin α state
R_β	Relaxation rate constant of the spin β state
RDC	Residual dipolar coupling
R_g	Radius of gyration
R_h	Hydrodynamic radius
RNA	Ribonucleic acid
SDS	Sodium Dodecyl Sulphate
PAGE	Polyacrylamide gel electrophoresis
SNpc	Substantia nigra pars compacta
TEMED	N,N,N',N'-Tetramethylethylenediamine
TH	Tyrosine hydroxylase
Tyr	L-Tyrosine
T_m	melting temeperature
TRACT	<u>T</u> ROSY for <u>r</u> otational <u>c</u> orrelation <u>t</u> imes
Tri-mek9	Tri-methylated at lysine 9 (peptide)
TROSY	Transverse relaxation optimized spectroscopy
UV	Ultra-violet
τ_c	rotational correlation time

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