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Influence of the Redox State of Pyridine Nucleotides on Mitochondrial Sulfhydryl Groups and Permeability Transition

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This work addresses a correlation between the redox state of pyridine nucleotides and that of sulfhydryl groups of the mitochondrial membranes. Several major observations emerge: (1) Conditions leading to an oxidation of the pyridine nucleotides such as incubation with *tert*-butyl hydroperoxide or acetoacetate determine a decrease of total mitochondrial sulfhydryl groups. Glutathione does not follow the same pattern since it decreases in the presence of *tert*-butyl hydroperoxide but not in the presence of acetoacetate. In addition, only in the presence of *tert*-butyl hydroperoxide is the decrease of sulfhydryl groups concomitant with a membrane protein polymerization, observed by polyacrylamide gel electrophoresis. (2) Under all conditions tested, the oxidation of sulfhydryl groups is further stimulated by the presence of calcium and phosphate ions. (3) Respiratory substrates, which prevent the swelling of mitochondria, also partially prevent the decrease of sulfhydryl groups.

Key Words: **liver mitochondria; permeability transition; pyridine nucleotides; respiratory substrates; sulfhydryl groups.**

Respiratory substrates, added to mitochondria incubation medium, prevent pyridine nucleotide loss (1), lipid peroxidation (2–4), swelling, and increase of the mitochondrial inner membrane permeability (5–8). The latter condition, also called "permeability transition," is considered to depend on the opening of an "unselective pore" (9–16; see 13–16 for extensive reviews) not yet clearly characterized. The critical involvement of membrane thiol status in mitochondrial membrane permeability is well known (17–28). In particular, the shift of the redox state of thiol groups toward a more oxidized condition has been considered to play a fundamental role in the general increase of the mitochondrial inner membrane permeability since the latter is enhanced by different thiol reagents such as *N*-ethylmaleimide, phenylarsine oxide, mersalyl, heavy metals, and diamide[2] (13). In mitochondria it is therefore possible to infer (29) a close redox communication among pyridine nucleotides, matrix thiols, and inner membrane thiols. Recently, Costantini *et al.* (30) reported that the mitochondrial membrane permeability transition is controlled at two sites; the first site is in an apparent redox equilibrium with the pyridine nucleotides, while the second site, being a redox sensitive dithiol, is in equilibrium with the glutathione pool.

Recent papers (31, 32) indicate that in isolated and permeabilized cells different metabolic conditions can alter the mitochondrial redox state.

In the present paper we report a direct measurement of mitochondrial thiols (protein thiols and glutathione) under different redox conditions; thiol modifications were correlated to the mitochondrial protein alterations examined with polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Rat liver mitochondria were isolated with differential centrifugation essentially as described by Myers and Slater (33) using a medium containing 220 mM mannitol, 70 mM sucrose, 2 mM Hepes (pH

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[2] Abbreviations used: diamide, diazenedicarboxylic acid bis(*N*,*N*-dimethylamide); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitrol; Hepes, *N*-(2-hydroxyethylpiperazine)-*N*-2-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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TABLE I

Effect of Ca^{2+}/Phosphate, *tert*-Butyl Hydroperoxide, and Acetoacetate on Total Sulfhydryl Group Content of Mitochondria Incubated in the Presence and Absence of Substrates

			Succinate		Succinate + isocitrate	
	nmol SH	%	nmol SH	%	nmol SH	%
None	95.8 ± 2.7	100	95.6 ± 2.7	100	96.4 ± 2.8	100
Ca^{2+}/P_i	92.1 ± 2.1*	96	92.7 ± 2.5	97	93.4 ± 2.8**	97
t-BHP	84.4 ± 2.7***	88	86.7 ± 1.7***	91	90.0 ± 3.0***	93
t-BHP/Ca^{2+}/P_i	82.7 ± 2.0***	86	84.3 ± 1.8***	88	85.2 ± 2.3***	88
AcAc	90.8 ± 2.3**	95	92.1 ± 2.4	97	94.0 ± 2.8	98
AcAc/Ca^{2+}/P_i	88.7 ± 2.0***	92	90.5 ± 1.9***	95	92.2 ± 2.4***	96

Note. Rat liver mitochondria, at the final concentration of 4 mg/ml, were preincubated for 2 min in 213 mM mannitol, 71 mM sucrose, 20 mM Hepes/Tris (pH 7.4), 25 μM rotenone, and, when present, 20 mM substrate (succinate and/or isocitrate). Aliquots of 1.5 mL were transferred to test tubes containing, where indicated, 0.4 mM Ca^{2+}, 20 mM phosphate, 0.2 mM *tert*-butyl hydroperoxide (t-BHP), and 4 mM acetoacetate (AcAc) to a final volume of 1.650 ml; 4 mM EGTA was present in all the experiments except where Ca^{2+}/P_i were added. Reactions were carried out for 5 min. At the end of the incubation, aliquots of 0.2 ml of the mitochondrial suspension were transferred to cuvettes containing 2.3 ml of a mixture formed by 1.2 mM DTNB, 1% SDS, 10 mM EDTA, and 0.2 M Tris–HCl (pH 8.1). Readings were taken at 412 nm until the complete stabilization of the reaction was achieved. Values are nmol SH groups/mg mitochondrial protein. In each column values are compared versus the control (no additions).

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

7.0), and 0.5 mg/ml bovine serum albumin. Proteins were measured with the biuret test (34). Mitochondrial swelling was estimated spectrophotometrically by following the decrease of absorbance at 540 nm (35). The reduction of DTNB, consequent to the titration of sulfhydryl groups, was followed at 412 nm (36). Glutathione was measured with the method of Tietze (37) modified according to Anderson (38), for the determination of glutathione disulfide. Formation of mitochondrial protein aggregates was followed by SDS polyacrylamide gel electrophoresis. After incubation under the conditions indicated in Fig. 2, mitochondria were rapidly frozen with liquid nitrogen. Prior to electrophoresis, mitochondrial protein (1 mg/100 μl) were thawed and solubilized by the addition of 20 μl of 0.1 M EDTA followed by 200 μl of loading buffer [2% SDS, 50 mM Tris–HCl (pH 6.8), 0.1% bromophenol blue in 10% glycerol] with or without 0.16 M DTT. Samples were incubated for 5 min at 80°C, and subsequently aliquots of 15 μl were subjected to one-dimensional SDS–PAGE according to Laemmli (39). Running gel and stacking gel were 10 and 3.5% acrylamide, respectively. Electrophoresis was performed at 10 V/cm for 4 h. The gels were stained with Coomassie brilliant blue R-450.

Statistical analysis. All values are the mean ± SE of not less than five measurements. Statistical significance for the comparison of two groups was evaluated with the Student's *t* test. Multiple comparisons were made by one-way analysis of variance followed by the Tukey posttest (Graphpad Inc., Sorrento Valley, CA).

RESULTS

In rat liver mitochondria incubated with agents able to largely oxidize mitochondrial pyridine nucleotides (6, 40) such as *tert*-butyl hydroperoxide or acetoacetate and in the absence of substrate, a significant decrease of sulfhydryl groups, particularly with *tert*-butyl hydroperoxide, is observed (Table I). The presence of Ca^{2+} and phosphate ions further stimulates the total thiol groups disappearance; the effect elicited by Ca^{2+}/phosphate ions is observable even in the absence of pyridine nucleotide oxidizing agents (Table I). The addition of succinate or succinate + isocitrate to the incubation mixture does not substantially modify the total amount of sulfhydryl groups in the control while in the Ca^{2+}/phosphate experiment a slight increase in the total concentration of sulfhydryl groups is observed. Probably, the endogenous substrates are sufficient to mask the effect of the added substrates. On the contrary, when *tert*-butyl hydroperoxide or acetoacetate is present, the addition of the substrates significantly increases the concentration of the total sulfhydryl groups and isocitrate appears particularly effective. This effect is still evident in the presence of calcium and phosphate. The action of acetoacetate is especially interesting since this substrate does not directly oxidize sulfhydryl groups and therefore its effect depends only on the shift in the redox state of the pyridine nucleotides; when acetoacetate is present, the addition of succinate + isocitrate restores the sulfhydryl groups to values close to that of the control.

Table II shows total glutathione and glutathione disulfide content of mitochondria treated under conditions similar to those of Table I. In the absence of substrate, the addition of Ca^{2+} and phosphate does not significantly decrease the content of glutathione nor increase the disulfide form, while the presence of succinate or succinate + isocitrate slightly increases the measured glutathione and decreases the disulfide form. With *tert*-butyl hydroperoxide there is a net apparent decrease of total glutathione indicating that protein thiolation occurs, probably linked to the formation of

TABLE II

Effect of Ca^{2+}/Phosphate, *tert*-Butyl Hydroperoxide, and Acetoacetate on Glutathione Content of Mitochondria Incubated in the Presence and Absence of Substrates

			Succinate		Succinate + isocitrate	
	GSH + GSSG	GSSG	GSH + GSSG	GSSG	GSH + GSSG	GSSG
None	5.17 ± 0.15	0.20 ± 0.05	5.37 ± 0.14	0.26 ± 0.07	5.41 ± 0.19	0.13 ± 0.04
Ca^{2+}/P_i	4.96 ± 0.20	0.30 ± 0.06	5.12 ± 0.11	0.28 ± 0.04	5.33 ± 0.07	0.18 ± 0.04
t-BHP	3.34 ± 0.15*	2.18 ± 0.21*	3.77 ± 0.31*	1.08 ± 0.32	4.76 ± 0.08*	0.31 ± 0.07
t-BHP/Ca^{2+}/P_i	3.95 ± 0.08*	2.26 ± 0.30*	3.78 ± 0.11*	1.76 ± 0.29*	4.07 ± 0.18*	1.05 ± 0.17*
AcAc	5.08 ± 0.22	0.23 ± 0.07	5.14 ± 0.11	0.24 ± 0.04	5.22 ± 0.17	0.22 ± 0.04
AcAc/Ca^{2+}/P_i	4.95 ± 0.26	0.31 ± 0.06	5.12 ± 0.17	0.33 ± 0.09	5.19 ± 0.12	0.25 ± 0.04

Note. Rat liver mitochondria, at the final concentration of 4 mg/ml, were preincubated for 2 min in 213 mM mannitol, 71 mM sucrose, 20 mM Hepes/Tris (pH 7.4), 25 μM rotenone, and, when present, 20 mM substrate (succinate and/or isocitrate). Aliquots of 1.5 ml were transferred to test tubes containing, where indicated, 0.4 mM Ca^{2+}, 20 mM P_i, 0.2 mM *tert*-butyl hydroperoxide (t-BHP), and 4 mM acetoacetate (AcAc) to a final volume of 1.650 ml; 4 mM EGTA was present in all the experiments except where Ca^{2+}/P_i were added. Reactions were carried out for 5 min. At the end of the incubation, aliquots of 0.1 ml of the mitochondrial suspension were rapidly deproteinized with 6% metaphosphoric acid and centrifuged. After neutralization with 15% Na_3PO_4, total and oxidized glutathione were determined as described under Materials and Methods. Values are nmol/mg mitochondrial protein. In each column values are compared versus the control (no additions).

* $P < 0.001$.

mixed disulfides between protein thiols and glutathione disulfide produced by *tert*-butyl hydroperoxide. When mitochondria are incubated with *tert*-butyl hydroperoxide in the absence of Ca^{2+}/phosphate and substrate, glutathione is largely transformed to its disulfide form (more than 60%), while the addition of substrate to the incubation medium dramatically changes the redox condition, particularly in the presence of isocitrate, since glutathione appears completely reduced (more than 90%). The total glutathione content is also increased, indicating that substrates act in a dethiolation process by reducing mixed disulfides formed between glutathione and protein. Interestingly, in the presence of Ca^{2+}/phosphate the substrates are able to reduce glutathione only partially. This can be related to the Ca^{2+}/phosphate-dependent swelling of mitochondria and the consequent release of glutathione. As reported in Fig. 1, a large swelling occurs in the presence of Ca^{2+}/phosphate and further addition of *tert*-butyl hydroperoxide or acetoacetate does not substantially increase the amplitude of this swelling. Moreover, under our conditions, the latter two agents do not elicit swelling in the absence of Ca^{2+}/phosphate and in the presence of EGTA. Therefore, in this system, the glutathione reductase/peroxidase system is ineffective on the released glutathione and, in addition, is probably less efficient in the mitochondrial matrix because of the protein dilution. In the presence of acetoacetate or acetoacetate + Ca^{2+} there are no significant alterations in the total content of glutathione nor a significant shift toward the oxidized form; this is at variance with the total thiol group measurements that significantly decrease upon acetoacetate or acetoacetate + Ca^{2+} addition.

The decrease of sulfhydryl groups observed in the presence of Ca^{2+} and phosphate might be due to membrane alterations caused by swelling of mitochondria (Fig. 1) which might change the spatial arrangement

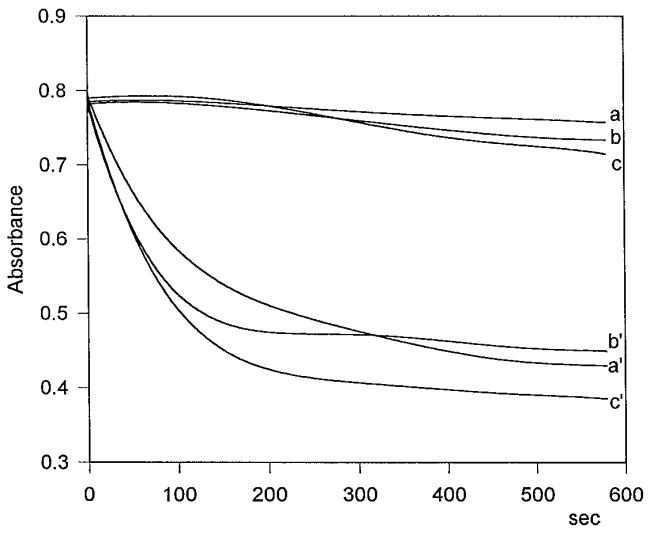

FIG. 1. Swelling induced by Ca^{2+}/phosphate and oxidizing agents in rat liver mitochondria. Rat liver mitochondria (0.25 mg/ml) were incubated at 25°C in 213 mM mannitol, 71 mM sucrose, 5 mM Hepes/Tris (pH 7.4), 0.5 mM *tert*-butyl hydroperoxide (c, c′) or 10 mM acetoacetate (b, b′) in a final volume of 1.5 ml. Other additions were 1 mM EGTA (a, b, c). In a′, b′, and c′ swelling was initiated by the addition of 1 mM phosphate followed, after 1 min, by 40 μM $CaCl_2$.

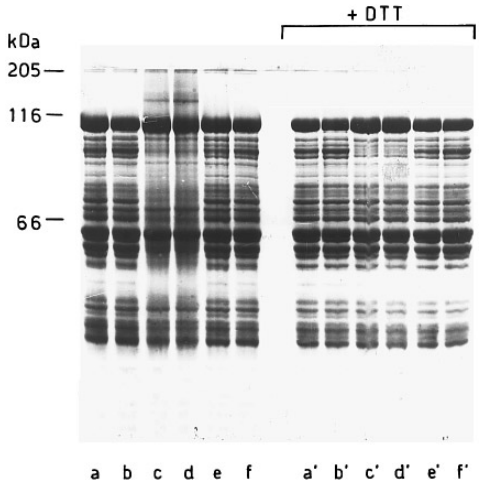

FIG. 2. SDS–polyacrylamide gel electrophoresis of proteins from rat liver mitochondria. Mitochondrial proteins were applied to the running gel after 6 min of incubation at room temperature in 213 mM mannitol, 71 mM sucrose, 5 mM Hepes/Tris (pH 7.4), and in the presence of 0.1 mM EGTA (lanes a, c, e, a′, c′, e′) or 1 mM Ca^{2+} (100 nmol/mg protein) and 50 mM phosphate (5 μmol/mg protein; lanes b, d, f, b′, d′, f′). Other additions were 0.5 mM *tert*-butyl hydroperoxide (lanes c, d, c′, d′) or 10 mM acetoacetate (lanes e, f, e′, f′). Lanes a′ to f′ represent the gels treated with DTT before application. Other conditions are reported under Materials and Methods.

of cysteinyl residues of mitochondrial proteins bringing to conditions of oxidizability. Therefore, experiments similar to those reported in Tables I and II were performed in the presence of cyclosporin, the specific inhibitor of the mitochondrial permeability transition (41), and no substantial differences in total mitochondrial thiol groups and glutathione were found (not reported).

Mitochondrial protein can be separated by SDS gel electrophoresis, and the cross-linkage of the protein, when occurring, can be detected (25–27). As can be seen in Fig. 2, in the presence of *tert*-butyl hydroperoxide there is an extensive polymerization above 116 kDa (lane c) that is increased by the presence of Ca^{2+}/phosphate (lane d). Ca^{2+}/phosphate alone does not detectably increase the formation of polymers (lane b), although a noticeable increase is apparent when submitochondrial particles, instead of whole mitochondria, are tested (not shown). In the presence of acetoacetate, which causes a consistent decrease of mitochondrial thiol groups (Table I) no cross-linkage formation is observed (lane e) except a very slight increase due to the presence of Ca^{2+}/phosphate (lane f). In Fig. 3, the extent of polymerization of the mitochondrial protein stained in the SDS gel was measured on the basis of the intensity of the areas between 116 and 200 kDa. The incubation conditions of mitochondria are the same as in Fig. 2 and, by comparison, the action of diamide, a well-known oxidizer, is reported (Fig. 3, lanes g and h); it gives rise to a very extensive formation of high-molecular-weight polymers in line with the results of Fagian *et al.* (25). Under reducing conditions, i.e., in the presence of dithiothreitol, the protein aggregates almost completely disappear (Fig. 2), indicating a predominant contribution of the intermolecular disulfide bridges to the formation of the observed aggregates.

The redox state of total pyridine nucleotides under conditions comparable to those of Tables I and II is reported in Fig. 4. In the presence of EGTA and in the absence of oxidizing agents, pyridine nucleotides are almost completely reduced (Fig. 4A); when *tert*-butyl hydroperoxide or acetoacetate is added (Figs. 4B and 4C, respectively) pyridine nucleotides are largely oxidized in the absence of substrates, while, in the presence of succinate + isocitrate, they are in a more reduced state. Comparing these results with those of Table I, a correspondence between sulfhydryl group decrease and pyridine nucleotide oxidation is apparent.

In the presence of Ca^{2+}/phosphate there is a large oxidation of total pyridine nucleotides in line with previous observations (40, 42); substrates are scarcely effective in preventing the oxidation of the pyridine nucleotides and, in general, this behavior corresponds to the large oxidation of SH groups (Table I). It should be noted that, in the absence of substrate, the oxidation of pyridine nucleotides proceeds at a slower rate due to the lack of Ca^{2+} uptake; on the contrary, in the presence of succinate, the oxidation of the pyridine nucleotides is accelerated, because of a rapid calcium uptake.

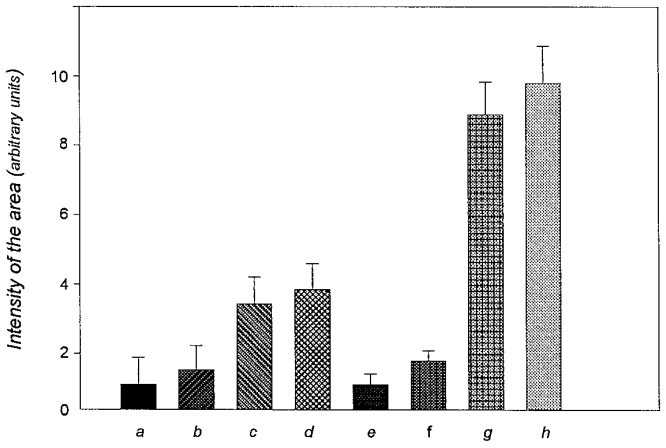

FIG. 3. Extent of polymerization of mitochondrial proteins under different experimental conditions. The images of the Coomassie-stained gels were imported with a gel scanner in a personal computer and the intensity of the areas between 116 and 200 kDa was analyzed. The values obtained were then transferred to a graphic software. The intensity values of four gels were mediated and utilized for the reported data. Samples were prepared as in Fig. 2. Other additions were 0.1 mM EGTA (a, c, e), 1 mM Ca^{2+} (100 nmol/mg protein), and 50 mM phosphate (5 μmol/mg protein; lanes b, d, f), 0.5 mM *tert*-butyl hydroperoxide (c, d), 10 mM acetoacetate (e, f), 1 mM diamide (g, h). Other conditions are reported under Materials and Methods.

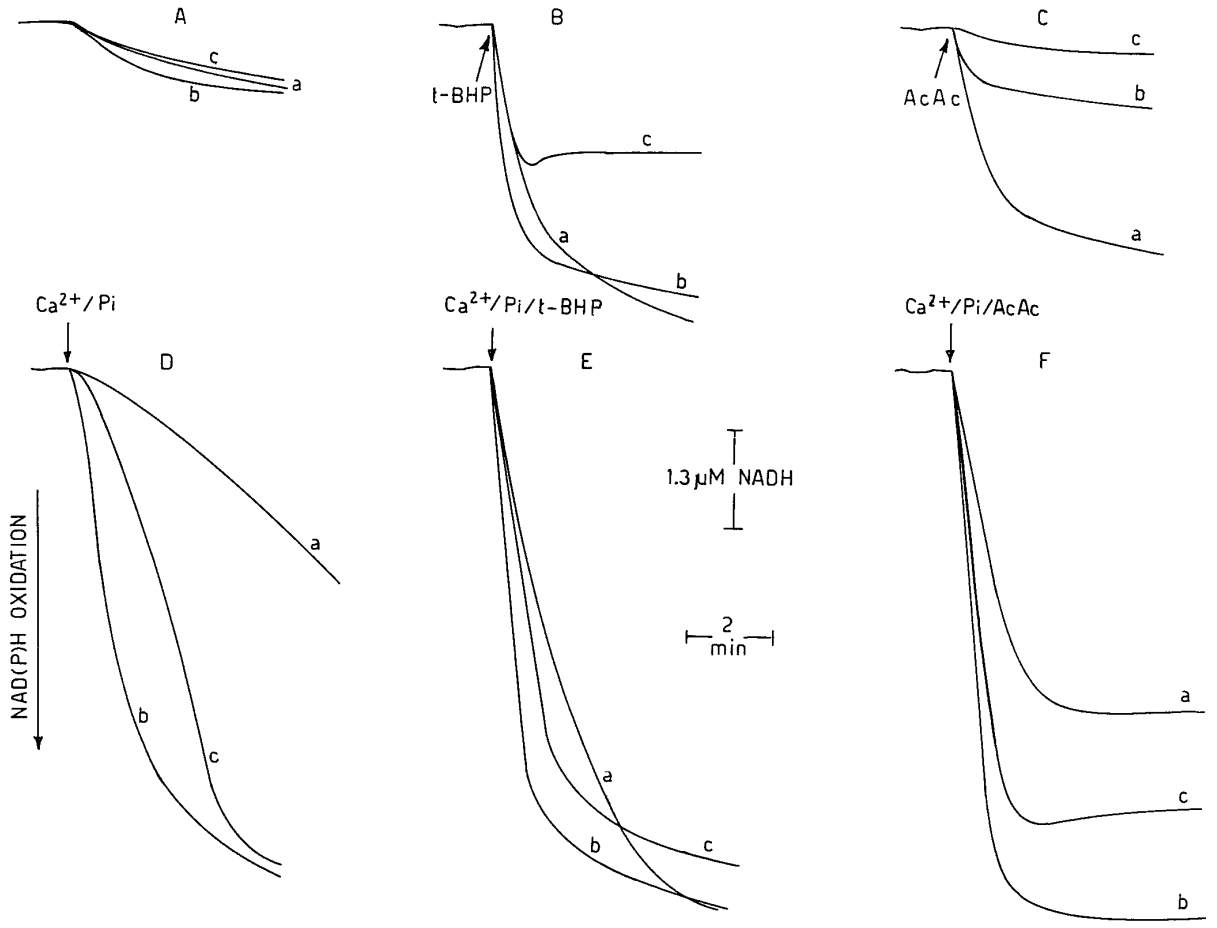

FIG. 4. Extent of reduction of mitochondrial pyridine nucleotides. Rat liver mitochondria (0.6 mg/ml) were incubated at 25°C in 213 mM mannitol/71 mM sucrose buffered with 5 mM Hepes/Tris (pH 7.4). After addition of mitochondria, 20 μM rotenone was also added, and, when indicated, 1 mM EGTA (A, B, C), 66 μM Ca^{2+}, 3 mM phosphate (D, E, F), 26 μM *tert*-butyl hydroperoxide (B and E), and 0.85 mM acetoacetate (C and F). The following substrates were added: 5 mM succinate (b), 5 mM succinate + 5 mM isocitrate (c), or no addition of substrates (a). The pyridine nucleotide redox state was followed fluorimetrically (E_x, 366 nm; E_m, 450 nm). Abbreviations: Pi, inorganic phosphate; t-BHP, *tert*-butyl hydroperoxide; AcAc, acetoacetate.

Nevertheless, if isocitrate is added together with succinate the rate of oxidation of pyridine nucleotides is slowed down. On the whole, the data on the total pyridine nucleotides fluorescence appear to clearly indicate a correlation between thiol group disappearance and pyridine nucleotide oxidation.

DISCUSSION

Total mitochondrial sulfhydryl groups measured with DTNB in the presence of SDS as detergent are about 95 nmol of sulfhydryl groups/mg protein. This figure is consistent with previous literature reports (43, 44). Lower values indicate an incomplete solubilization of mitochondria by the detergent or an incomplete titration with the reagent. In fact, the determination of the sulfhydryl groups of whole mitochondria requires treatment with a detergent able to expose all sulfhydryl groups to the titrating reagent (DTNB). In order to avoid nonspecific oxidation of sulfhydryl groups after unfolding with SDS, DTNB was included in the reaction mixture to ensure an immediate titration; EDTA was also present to prevent reoxidation of 2-nitro-5-thiobenzoate (the reduced form of DTNB) and autoxidation of protein sulfhydryl groups. Under these conditions differences in sulfhydryl groups of mitochondria incubated under different experimental conditions can be observed.

A relationship between the redox state of the pyridine nucleotides and the inner membrane permeability of rat liver mitochondria was established early by Lê Quôc and Lê Quôc (7) since conditions inducing the oxidation of NAD(P)H cause a slight but significant decrease of *N*-ethylmaleimide-reactive thiols of the adenine nucleotide translocase, depending on the orientation of the adenine nucleotide binding site across the membrane. Interestingly, the oxidation of sulfhydryl groups was not accompanied by an increase of glutathi-

one disulfide. The oxidation of these crucial thiols can be counteracted by the energization of the *trans*-hydrogenase (45) with the consequent reduction of $NADP^+$ by NADH.

Both acetoacetate and hydroperoxides are able to oxidize mitochondrial pyridine nucleotides, though with different mechanisms. Acetoacetate is enzymatically reduced to β-hydroxybutyrate by NADH, while hydroperoxides oxidize mitochondrial glutathione and NADPH in an enzymatic process involving glutathione peroxidase and glutathione reductase. Moreover, in the latter case, in addition to the formation of glutathione disulfide, which does not occur in the former case, the production of free radicals is also elicited. In fact, in mitochondria, it was shown that *tert*-butyl hydroperoxide is able to generate methyl, *tert*-butoxyl, and *tert*-butyl peroxyl free radicals, after interaction with a tightly liganded metal such as heme iron (46). Therefore, *tert*-butyl hydroperoxide, in addition to the oxidation of NADPH, also gives rise to glutathione disulfide that can form mixed disulfides with protein thiols; in addition, alkoxyl and peroxyl radicals are able to oxidize protein thiols leading to the formation of protein cross-linkages involving inter and intra bonds, finally resulting in polymer formation. The different reaction mechanism between acetoacetate and *tert*-butyl hydroperoxide is apparent from the SDS–PAGE in which only *tert*-butyl hydroperoxide gives rise to cross-linkages able to produce high-molecular-weight polymers (Fig. 1).

The approach of observing the oxidation of membrane protein and the consequent cross-linking formation by SDS–PAGE was utilized by Vercesi and coworkers in a series of papers (25–27) in which they showed that diamide and *tert*-butyl hydroperoxide, particularly in the presence of Ca^{2+}, elicit the formation of protein aggregates able to increase the permeability characteristics of mitochondria. Interestingly, they also found that Ca^{2+} accumulation by mitoplasts caused protein polymerization even in the absence of an added prooxidant (25). The permeabilizing effect of Ca^{2+} alone or in the presence of *tert*-butyl hydroperoxide appears mediated by reactive oxygen species generated in the respiratory chain (26–28), and polymerization of inner-membrane protein could create an assembly with a central cavity large enough to explain the properties of the nonspecific transition pore. On the other hand, according to Zoratti and Szabò (15) the reproducible and specific characteristics of the permeability transition pore do not support the idea that it might arise from the random cross-linking of proteins. The SDS–PAGE pattern of the acetoacetate treatment shows essentially no difference from the control experiment while, on the contrary, acetoacetate under the same experimental conditions causes a significant decrease of sulfhydryl groups titrated with DTNB (compare Table I and Fig. 2). These results would indicate that the oxidation of NAD(P)H induced by acetoacetate probably determines an oxidation of sulfhydryl groups that does not give rise to a gross formation of protein polymers. This might depend on an intramolecular disulfide formation or to a limited and specific cross-linkage that does not appear in the gel electrophoresis pattern.

Further studies are required in order to determine the specific localization of the thiol groups involved in the mitochondrial membrane permeability increase; their identification might also disclose the molecular identity of the components taking part in the formation of the "unselective pore" possibly involved in the mitochondrial Ca^{2+}-dependent permeability transition (14, 15). Finally, it should be considered that the mitochondrial calcium-transport systems play an important role in maintaining cytosolic and mitochondrial Ca^{2+} homeostasis. Mitochondrial calcium release can occur nonspecifically when the inner membrane becomes leaky and membrane potential collapses or specifically with the preservation of the mitochondrial membrane potential (47). Therefore, pyridine nucleotide oxidation and the thiol redox state appear to regulate the specific release of calcium from mitochondria in a way not related to the opening of the "pore" (47). NAD hydrolysis and calcium release are stimulated when some specific vicinal thiols are cross-linked in the presence of phenylarsenoxide or gliotoxin; on the contrary, when the formation of the disulfide is prevented by derivatization of a single sulfhydryl group, Ca^{2+} release is also prevented (47). Therefore, the specific Ca^{2+} release depends on pyridine nucleotide oxidation and thiol oxidation probably linked by the thioredoxin system (47).

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