ENERGY-DEPENDENT VARIATION OF THIOL GROUPS REACTIVITY OR ACCESSIBILITY IN RAT LIVER MITOCHONDRIA, REVEALED BY MEASUREMENTS OF LABELLED THIOL REAGENTS INCORPORATION

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SUMMARY : Permeant and impermeant labelled thiol reagents were incubated with rat liver mitochondria, and incorporation of reagent into mitochondria estimated. With permeant thiol reagents, incorporation depends on the energetic state of mitochondria ; when coupled electron-transfer takes place, incorporation is fairly increased ; the stimulation is abolished in the presence of an uncoupler, an electron-transfer inhibitor or inorganic phosphate. With an impermeant thiol reagent, the incorporation is unaffected by the energetic state of the mitochondria. These results favour the view of a participation of thiol groups in the energy-conserving mechanism but it cannot be ruled out that part of the unmasked thiol groups are implicated in the phosphate transport system. The observed stimulation may reflect either an increase in accessibility or in reactivity of some mitochondrial thiol groups.

Several authors (1-8) using thiol groups inhibitors or estimating mitochondrial SH groups, came to the conclusion that SH groups might be implicated in the mitochondrial energy transfer process.

In order to check this hypothesis, we measured the incorporation of labelled thiol-reagents into rat liver mitochondria incubated in different conditions ; we compared the incorporation of three thiol-reagents : ethacrynic acid* and NEM which are permeant thiol-reagents, and PCMB which is an impermeant thiol-reagent. The ability of thiol-reagents to penetrate (or not) into the mitochondrial matrix was elucidated previously (9) by measuring the inactivation of intramitochondrial reduced glutathione.

The results presented in this paper show unequivocally an energy-dependent stimulation of the permeant thiol-reagents incorporation, while the in-

* Ethacrynic acid : 2,3-dichloro-4-(2-methylenebutyryl) phenoxyacetic acid.

corporation of the impermeant thiol-reagent is unaffected by the mitochondrial energy level. This stimulation may be due to an energy-linked increase of the reactivity or the accessibility of some mitochondrial thiol groups.

MATERIAL AND METHODS : Rat liver mitochondria were prepared as previously described (10) ; proteins were estimated by the "quick biuret" method (11) ; mitochondria (4,5 mg - 6 mg protein) were incubated 10 minutes at 30°C in the following medium : 62 mM sucrose , 8 mM $MgCl_2$, 50 mM KCl, 20 mM glycylglycin, pH 7 ; 10 mM substrate (when present) ; thiol reagents 2.10^{-4}M. After incubation, 0.5 ml aliquots were centrifuged at room temperature (Eppendorf centrifuge 3200) over 0.5 ml 0.33 M sucrose for 2 minutes at 11.000 g. The pellets were resuspended in 1 ml 0.33 M sucrose and mitochondria were centrifuged again ; 0.5 ml 0.2 M TCA was added to the pellets and after shaking vigorously, a third centrifugation gave the pellets which were solubilized for 12 hours in 0.5 ml formic acid ; radioactivity was estimated in a liquid scintillation counter using PPO + dimethyl-POPOP as scintillant in 10 ml toluen + 4 ml ethanol. Oxygen uptake was measured with an Oxygraph (Gilson Medical Electronics) equipped with a Clark electrode.
Labelled thiol reagents were obtained from the Commissariat à l'Energie Atomique (Saclay, France) and diluted with cold thiol reagent to get the following solutions : 2.10^{-4}M, $[^{14}C]$-ethacrynic acid, specific activity : 100-200 d.p.m./nmole ; 2.10^{-4}M $[^{3}H]$ -N.E.M., specific activity 200 d.p.m./nmole and 2.10^{-4}M $[^{14}C]$ -PCMB, specific activity : 610 d.p.m./nmole. Ethacrynic acid and N.E.M. were ethanolic solutions, PCMB was diluted in 0.1 M NaOH.
All reagents were of analytical grade ; rotenone, oligomycin and antimycin A were obtained from Sigma Chemical Co., St-Louis, Mo., U.S.A..

RESULTS AND DISCUSSION

Influence of the energetic state of mitochondria on ^{14}C -ethacrynic acid incorporation

In Table 1, it appears that $\left[^{14}C\right]$ -ethacrynic acid incorporation into rat liver mitochondria was stimulated when mitochondria oxidized a substrate, the stimulation depending on the substrate oxidized, succinate being the most effective substrate.

The stimulation was cancelled either by an inhibitor of electron-transfer or by an uncoupler. The fact that addition of rotenone lowers significantly the incorporation of ethacrynic acid in the presence of succinate is not surprising, since it was found (10) that the addition of rotenone + ethacrynic acid to mitochondria oxidizing succinate uncouples the oxidation after a few minutes incubation.

Oligomycin slightly increased the incorporation while P_i abolished it completely ; these results will be discussed further down.

Table 1. [^{14}C]-*ethacrynic acid incorporation into rat liver mitochondria in different energetic states*

Mitochondria (4.5 mg - 6 mg of protein) were incubated in 1 ml of medium consisting of 62 mM sucrose, 8 mM $MgCl_2$, 50 mM KCl, 20 mM glycylglycin, pH 7 for 10 minutes at 30°C ; substrates (when present) : 10 mM, except pyruvate : 1 mM ; [^{14}C]-ethacrynic acid : specific activity 100-200 d.p.m./nmole, final concentration : 2.10^{-4}M ; after the incubation, mitochondria were spun down by quick centrifugation and treated as described under "Material and Methods" and radioactivity estimated (see Text).

Results are expressed in nmoles [^{14}C]-ethacrynic acid incorporated.mg.prot^{-1} and represent the mean ± S.E.M. ; numbers in parentheses denote the number of observations contributing to means.

Additions	no substrate	succinate	β-hydroxybutyrate	glutamate	malate + pyruvate
none	10.43 ± 0.22 (16)	15.70 ± 0.45 (17)	14.54 ± 0.29 (12)	14.06 ± 0.59 (6)	12.59 ± 0.75 (6)
rotenone 4 µg	7.99 ± 0.51 (3)	12.98 ± 1.28 (3)	-	7.29 ± 0.80 (2)	6.56 ± 0.28 (2)
antimycin 10 µg	8.51 ± 1.65 (3)	7.75 ± 0.31 (3)	7.68 ± 0.34 (2)	7.61 ± 0.31 (2)	7.02 ± 0.42 (2)
oligomycin 5 µg	12.88 ± 0.95 (4)	17.62 ± 1.01 (4)	15.42 ± 0.62 (4)	17.89 ± 0.52 (4)	-
CCCP 1.25 µM	7.56 ± 0.26 (3)	8.37 ± 0.50 (3)	8.06 ± 0.02 (2)	9.25 ± 0.08 (2)	-
phosphate 1 mM	8.70 ± 0.14 (5)	9.52 ± 0.14 (6)	11.10 ± 0.64 (4)	10.16 ± 0.26 (4)	-

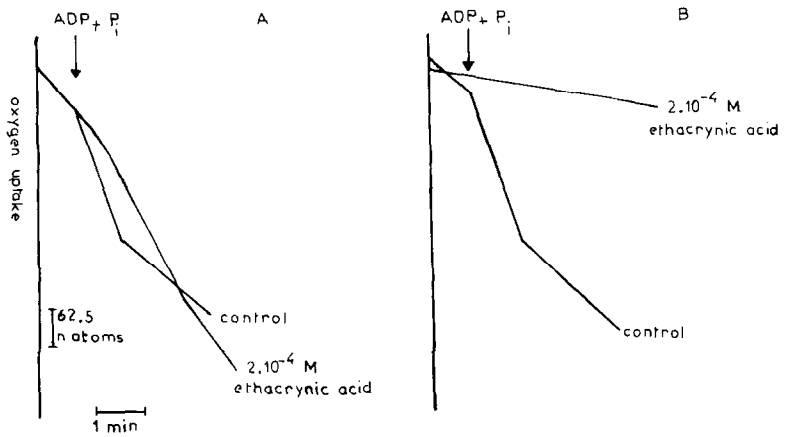

FIGURE 1

INFLUENCE OF ETHACRYNIC ACID ON SUCCINATE OXIDATION BY RAT LIVER MITOCHONDRIA

Mitochondria (5.44 mg of protein) were preincubated 10 minutes at 30°C in 1 ml of the following medium : 62 mM sucrose, 8 mM $MgCl_2$, 50 mM KCl, 20 mM glycylglycin, pH 7.
A : without substrate during preincubation ;
B : 10 mM succinate present during preincubation.
After 10 minutes, 0.5 ml aliquots were centrifuged (Eppendorf 3200) over 0.5 ml 0.33 M sucrose ; then the pellets were homogeneized and washed in 0.5 ml 0.33 M sucrose and mitochondria were centrifuged again ; they were resuspended in 0.5 ml of medium and transfered into the oxygraph cell containing 2 ml of medium + 8 mM succinate. 4 mM phosphate + 0.16 mM ADP were added (+).

Inhibition of succinate oxidation by ethacrynic acid : influence of substrate preincubation

The above results are quite in agreement with the findings (Fig. 1) that ethacrynic acid strongly inhibits succinate oxidation when preincubated with the substrate, while the inhibition is very low when ethacrynic acid is preincubated without substrate.

Comparison of thiol-reagents incorporation into mitochondria in different energetic states

In order to explain these results, two hypothesis were considered. In the first hypothesis, the coupled energy-transfer would increase ethacrynic acid incorporation by favouring its penetration : the protons ejected during the electron transfer would increase the amount of the uncharged form

Table 2. *Influence of the energetic state of mitochondria on the incorporation of different thiol-reagents*
Conditions and results as in Table 1.
2.10^{-4}M [^{3}H] -NEM : specific activity 200 d.p.m./nmole ; 2.10^{-4}M [^{14}C]-PCMB : specific activity 610 d.p.m./nmole

	Rotenone 4 µg	Antimycin 10 µg	CCCP 1,25 µM	P_i 1 mM	[^{14}C]-ethacrynic acid nmoles.mg.prot^{-1}	[^{3}H]-NEM nmoles.mg.prot^{-1}	[^{14}C]-PCMB nmoles.mg.prot^{-1}
no substrate	0	0	0	0	10.43 ± 0.22 (16)	12.94 ± 0.90 (2)	22.52 ± 0.67 (2)
	+	0	0	0	7.99 ± 0.51 (3)	11.39 ± 0.15 (2)	-
	0	+	0	0	8.51 ± 1.65 (3)	11.56 ± 0.22 (2)	25.48 ± 0.16 (2)
	0	0	+	0	7.56 ± 0.26 (3)	9.85 ± 0.53 (2)	22.25 ± 0.45 (2)
	0	0	0	+	8.70 ± 0.14 (5)	10.63 ± 0.33 (2)	22.15 ± 0.08 (2)
succinate	0	0	0	0	15.70 ± 0.45 (17)	19.75 ± 1.00 (2)	21.19 ± 0.71 (2)
	+	0	0	0	12.98 ± 1.28 (3)	17.26 ± 0.38 (2)	-
	0	+	0	0	7.75 ± 0.31 (3)	11.70 ± 0.15 (2)	23.52 ± 0.69 (2)
	0	0	+	0	8.37 ± 0.50 (3)	13.25 ± 1.14 (2)	20.83 ± 0.93 (2)
	0	0	0	+	9.52 ± 0.14 (6)	15.92 ± 0.34 (2)	22.14 ± 0.07 (2)
β-hydroxybutyrate	0	0	0	0	14.54 ± 0.29 (12)	15.80 ± 0.39 (2)	21.50 ± 0.74 (2)
	+	0	0	0	-	10.15 ± 1.01 (2)	-
	0	+	0	0	7.68 ± 0.34 (2)	10.53 ± 0.46 (2)	23.60 ± 0.06 (2)
	0	0	+	0	8.06 ± 0.02 (2)	9.57 ± 0.83 (2)	22.65 ± 0.58 (2)
	0	0	0	+	11.10 ± 0.64 (4)	13.39 ± 1.05 (2)	20.76 ± 0.71 (2)

of ethacrynic acid, supposed to be the penetrant form. In the second hypothesis, the stimulated incorporation of ethacrynic acid is assumed to reflect an increase in the reactivity or accessibility of mitochondrial thiol groups, this increase being related to the high energy state of mitochondria induced by the electron-transfer.

The results shown in Table 2 make possible to eliminate the first hypothesis. As can be seen, similar results were obtained - that is, stimulated incorporation of thiol reagent by coupled electron transfer - when mitochondria were incubated with another permeant, but uncharged thiol reagent, i.e. N.E.M.. Since N.E.M. is an uncharged molecule, its penetration into mitochondria cannot be proton-dependent.

With PCMB, an impermeant thiol reagent, the incorporation is unchanged whatever the conditions are ; the fact that the amount of PCMB incorporated is much higher than the amount of ethacrynic acid or N.E.M. may be due to the higher affinity of PCMB towards thiol groups.

Consequently the stimulation of incorporation obtained only with a permeant thiol-reagent reflects an increase of reactivity or accessibility of thiol groups as proposed by Chude and Boyer (12) ; since this stimulation of incorporation takes place when mitochondria are in energized state, we assume that the transition from the non energized to the energized state induces an unmasking of thiol groups located either in the matrix or in the inner membrane.

Goldschmidt et al. (13) estimating the total mitochondrial thiol groups with Ellman's reagent, obtained similar results with mitochondria oxidizing succinate : decrease of ethacrynic acid accessible thiol-groups by an uncoupler, antimycin or P_i.

The inhibition of the stimulated incorporation of ethacrynic acid by P_i is in agreement with the fact that P_i, preincubated with mitochondria, prevents almost completely coupled succinate oxidation inhibition by ethacrynic acid, P_i and ethacrynic acid being competitive (14) ; these findings

agree with a possible interaction between P_i and ethacrynic acid in the oxidative phosphorylation process. On the other hand such an interaction can take place on the phosphate carrier known to be sensitive to thiol reagents (15,16) ; it has been found recently (17,18) that P_i protects the phosphate carrier against inhibition by thiol reagents. The increase of ethacrynic acid incorporation by oligomycin is in agreement with the fact that this inhibitor, by preventing the formation of a hypothetical phosphorylated high-energy intermediate (19) maintains a high energy level in mitochondria, which, as we just reported, is characterized by an increase of accessible thiol groups.

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