IMPLICATION OF SH-GROUPS IN THE MITOCHONDRIAL ENERGY-COUPLING SYSTEM REVEALED BY MEASUREMENTS OF ^{14}C-ETHACRYNATE* INCORPORATION INTO RAT LIVER MITOCHONDRIA

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Received 3 November 1970
Revised version received 18 January 1971

1. Introduction

In previous work [1, 2] it was shown that the thiol reagent [3, 4], ethacrynate, inhibits or uncouples oxidative phosphorylation, depending on the substrate used and the experimental conditions. Among these previous findings, it was found that succinate oxidation is inhibited by ethacrynate but that inorganic phosphate (Pi) prevents this inhibition, ethacrynate and phosphate being competitive. By using ^{14}C-ethacrynate [2], we have shown that with increasing amounts of Pi there is a proportional decrease of ethacrynate incorporation into mitochondria.

The present work was undertaken to study in different experimental conditions the incorporation of ethacrynate into mitochondria, and consequently, assuming that this incorporation is a measurement of the amount of free SH-groups [3, 4], to follow the level of mitochondrial free SH-groups; it is shown that this level is increased by ADP but not by ATP, that the ADP-increase is oligomycin-sensitive and that antimycin and uncouplers decrease the thiol level and abolish the Pi sensitivity. We propose and discuss the existence of at least two different populations of thiol groups, one in close relation to the energy-coupling system, the other in connection with the phosphate–anion exchange system.

* We used the sodium salt of ethacrynic acid: 2,3-dichloro-4-(2-methylene-butyryl) phenoxyacetic acid.

2. Materials and methods

^{14}C-Ethacrynic acid was supplied by Merck, Sharp and Dohme, Rahway (N.J.); ADP was from P.L. Biochemicals, ATP (free of Pi) from Calbiochem, antimycin and oligomycin from Sigma St. Louis. Rat liver mitochondria were prepared as already described [2] and incubated (4.5 mg protein in a final volume of 2 ml) in a thermoregulated water bath shaker at 30°, using the respiratory medium described by Ernster [10]; gas phase was air. After incubation, aliquots were centrifuged over cold 0.33 M sucrose for 3 min at 22,000 *g*; the supernatant was quickly sucked up and the pellets dissolved in formic acid for counting in a Nuclear Chicago gas flow detector.

3. Results and discussion

From table 1, it can be seen that addition of ADP in the absence of added Pi increases thiol groups (+ 1.8 nmoles/mg prot.) and that this increase is maintained in the presence of Pi (+ 1.5 nmoles/mg prot.). In the presence of Pi, however, there is a net decrease of thiol groups (– 2.5 nmoles/mg prot. with ADP, – 2.2 nmoles/mg prot. without ADP), results which confirm our previous findings [2]. In the presence of ATP or oligomycin, there is no increase in thiol groups but oligomycin completely inhibits the

Table 1
Effects of adenine nucleotides and oligomycin on ^{14}C-ethacrynate incorporation and SH-group levels in rat liver mitochondria, in the presence or absence of Pi.

Additions	Pi	cpm/mg prot.	SH-groups equivalents (nmoles/mg prot.)
Controls	0	1100	5.15
	+	630	2.95
ADP	0	1490	6.95
	+	950	4.45
ATP	0	940	4.40
	+	665	3.15
Oligomycin	0	1150	5.38
	+	645	3.02
ADP + oligomycin	0	870	4.08
	+	620	2.90

Incubation medium (cf. material and methods) with succinate 5 mM; Pi, ADP or ATP 2.5 mM were added 3 min after mitochondria; oligomycin (5 μg) was added immediately after mitochondria; ^{14}C-ethacrynate 1×10^{-4} M (214 cpm/nmole) was added 5 min after mitochondria and the experiment was stopped 10 min later by centrifugation.

Table 2
Effect of 2,4-dinitrophenol (2,4-DNP), antimycin and rotenone on ^{14}C-ethacrynate incorporation and SH-group levels in rat liver mitochondria in the presence or absence of Pi.

Additions	Pi	cpm/mg prot.	SH-group equivalents (nmoles/mg prot.)
Controls	0	1050	4.92
	+	570	2.67
2,4-DNP	0	665	3.15
	+	700	3.28
Antimycin	0	560	2.62
	+	580	2.72
Rotenone	0	1100	5.15
	+	560	2.62

Conditions similar to table 1; 2,4-DNP 1×10^{-4} M, antimycin (1 μg) or rotenone (1.5 μg) were added immediately after mitochondria, just before succinate.

increase of thiol groups induced by ADP; addition of Pi in these three conditions significantly decreases the level of thiol groups.

With an uncoupling agent (2,4-dinitrophenol) or an inhibitor of succinate oxidation (antimycin) the amount of thiol groups is decreased compared to the control, but unaffected by the presence of Pi (table 2). With rotenone, as could be expected, the level of thiol groups is almost identical to the control and phosphate sensitivity is maintained.

To explain these results, we postulate the existence of at least two different thiol group populations, one ADP-sensitive, the other Pi sensitive. By ADP-sensitive thiol groups, we mean the thiol groups which become accessible to the thiol reagent when mitochondria are in the presence of excess ADP, even if Pi is present; by Pi-sensitive thiol groups, we mean the thiol groups which are no longer accessible to the thiol reagent when Pi is added to the mitochondria.

In our view, the ADP-sensitive thiol groups would be involved in the coupling mechanism; this hypothesis is strengthened by the effect of oligomycin observed. We assume that addition of ADP to mitochondria in the presence of succinate induced conformational changes of proteins involved in the coupling mechanism, allowing increased accessibility or reactivity of these thiol groups.

Gautheron et al. [3] have described similar results with pig heart mitochondria, using Elmann's reagent to estimate thiol groups; however, these authors need to add ADP + Pi in order to obtain the increase in thiol groups, while we need to add only ADP.

Pi-sensitive thiol groups can be involved in phosphate transport across the mitochondrial inner membrane dependent on a phosphate carrier [6], since this postulated carrier is inhibited by thiol reagents [6–8]. The fact that oligomycin does not prevent the phosphate effect favors this hypothesis.

The results with 2,4-dinitrophenol can be explained by the uncoupling effect of this agent, and the fact that Pi is released from ATP through the 2,4-DNP-stimulated ATPase.

Finally, antimycin, by inhibiting succinate oxidation, blocks almost completely anion exchange across the inner membrane.

Acknowledgement

We wish to thank Dr. Gautheron for her helpful and interesting discussions.

Additional note

While this paper was in preparation, Dr. Zimmer [9] published findings dealing with estimation of thiol groups by labelling mitochondrial proteins with ^{14}C-NEM. Some of Dr. Zimmer's results are in agreement with ours, but the different experimental conditions (mitochondria in the presence of ATP + KCN) make it difficult to compare our results and conclusions with Dr. Zimmer's.

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