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FREE –SH VARIATIONS DURING ATP SYNTHESIS BY OXIDATIVE PHOSPHORYLATION IN HEART MUSCLE MITOCHONDRIA*

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SUMMARY

To investigate the possible role of thiols in the coupling mechanism, free –SH variations have been estimated along with ATP synthesis during oxidative phosphorylation in heart mitochondria.

1. In well-coupled mitochondria, State 4 free –SH values amount to 43 nmoles/mg protein.

2. The addition of ADP + P_i, which initiates State 3 of oxidation and ATP synthesis, provokes an increase of free –SH (30 % of State 4 values). If ADP or P_i is added alone, no increase of free –SH occurs.

3. *m*-Chlorocarbonyl cyanide phenylhydrazone prevents any –SH release upon addition of ADP + P_i; moreover, free –SH levels decrease in mitochondria while the respiratory chain is stimulated by the uncoupling agent.

4. *N*-Ethyl maleimide added to well-coupled mitochondria blocks free –SH normally found in State 4 and strongly inhibits the respiratory level. Upon further addition of ADP, –SH release and stimulation of respiratory level as well as ATP synthesis are totally suppressed.

5. Oligomycin does not affect the free –SH level in State 4 of oxidation but suppresses totally the –SH increase due to ADP + P_i as well as ATP synthesis linked to respiratory chain oxidations.

6. If cholate is added after the incubations and just before the 5,5′-dithiobis-(2-nitrobenzoic acid) estimation to eliminate membrane phenomena, the number of –SH evaluated are not significantly different and the increase observed in State 3 remains the same.

These results are very much in favour of the participation of –SH with processes related to the coupling mechanism, since the –SH increase upon addition of ADP + P_i cannot be related to changes in the redox state of the respiratory chain or the phosphate entry.

Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); PCMB, *p*-chloromercuribenzoate; PHMB, *p*-hydroxymercuribenzoate; CCCP, *m*-chlorocarbonyl cyanide phenylhydrazone.

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INTRODUCTION

In a previous work, several results led us to propose a scheme implying the formation of an intermediary thiol ester in the coupling mechanism of oxidative phosphorylation[1,2]. This point of view is shared by other workers[3,4], but as early as 1960, FLUHARTY AND SANADI[5] suggested the possible involvement of a vicinal dithiol in oxidative phosphorylation. In a different approach, thiol reagents or agents have been used to inhibit processes related to oxidative phosphorylation. For example, *p*-chloromercuribenzoate (PCMB) and *p*-hydroxymercuribenzoate (PHMB) were used to uncouple and to inhibit specific exchanges in mitochondria[6]; in 1962 LEHNINGER[7] gave a general review concerning the implication of thiols in swelling and contractions. Although the possible implication of thiols in the coupling mechanism has been stressed in recent years, most workers attack the problem *via* indirect methods. We chose a direct approach. If a thiol ester or thiols are implicated in the coupling mechanism, one can expect to measure free –SH variations during ATP synthesis linked to oxidative phosphorylation.

In the present work, free –SH have been estimated in pig heart mitochondria along with ATP synthesis under several conditions, according to states of oxidation (States 3 and 4[8]) and in the presence of specific inhibitors. Since the addition of cholate just before 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) estimation of –SH (which breaks the membranes) does not modify significantly the –SH levels evaluated in States 3 and 4 and thus the increase of –SH linked to the State 4–State 3 transition, the results presented are very much in favour of the participation of some –SH to processes related to the coupling mechanism. Preliminary results have been reported at the Federation of European Biochem. Socs. Meeting[9] and at the Warwick Meeting[10].

MATERIALS AND METHODS

Isolation procedures

Pig heart mitochondria were isolated at 15000 × *g* in a 0.25 M sucrose, 0.01 M potassium phosphate medium, pH 7.6, 0°. according to the method of CRANE *et al.*[11]. The pellets were washed in 0.25 M sucrose, pH 7.4, 0°, and recentrifuged at 15000 × *g*.

Rat liver mitochondria were sedimented at 8500 × *g* as described by WEINBACH[12], washed twice in the presence of 0.25 M sucrose, pH 7.4, 0° and recentrifuged.

The final suspensions of both types of mitochondria contained 30–40 mg protein per ml in 0.25 M sucrose, pH 7.4.

Incubations

Mitochondria (3 mg protein per 2.35 ml total volume) were incubated at 30° with substrate in 0.015 M Tris–HCl medium, pH 7.4. After 3 min ADP + P_i (400 nmoles/assay) were added to initiate State 3 of oxidation, unless otherwise stated. Glutamate is the substrate unless otherwise stated.

Estimation procedures

Protein contents were determined by the "quick" Biuret method[13].

ATP was estimated by the firefly luminescence method[14]. For determination of

the kinetics of ATP synthesis, the reaction was stopped by sudden addition of trichloroacetic acid (10 % final concentration) 15, 45 or 60 sec after ADP + P_i introduction. Trichloroacetic acid was eliminated by four extractions with ethyl ether at 0° before ATP estimations.

Thiol groups were estimated directly in the incubation mixture either by the method of BOYER[15] with PCMB or by the procedure of ELLMAN[16] using DTNB in a 0.1 M Tris–acetic buffer, pH 7.6, to avoid mitochondria swelling or volumes changes. It is important to precise that the addition of DTNB necessary for the colorimetric estimation blocks completely ATP synthesis within a few seconds[17]; oxygen consumption levels (State 3 and State 4) and respiratory control ratios were determined by oxypolarography with a Gilson's oxygraph.

RESULTS

Compared free –SH values in pig heart and rat liver mitochondria in State 4 of oxidation as described by CHANCE AND WILLIAMS[8].

Since the first data concerning –SH contents in mitochondria were obtained for rat liver particles[18] by an amperometric technique, we chose to compare the results obtained with our methods in rat liver mitochondria as well as in pig heart mitochondria.

Table I shows that the level of free –SH measured in State 4 of oxidation depended on the type of particles and on the procedure of estimation. More –SH was determined by the DTNB technique. This was very striking with liver mitochondria whose –SH content seemed particularly affected by the physiological state and estimation procedure; indeed, KLOUWEN[19] using diphenylpicryl phenyl hydrazine found a third type of values for rat liver mitochondria: 37 ± 1 nmoles/mg protein, but values given by the amperometric method (85–100 nmoles/mg protein)[18] are comparable to those obtained here by the DTNB technique. So we used the DTNB technique for most of our further experiments since it was more sensitive than the amperometric technique and since the adenylic nucleotides absorption did not interfere with readings at 412 nm as it did in the PCMB procedures.

In the same way, BRIERLEY and co-workers[20,21] reported that the reactivity of

TABLE I

FREE –SH IN MITOCHONDRIA (STATE 4 OF OXIDATION)

Pig heart mitochondria and rat liver mitochondria were incubated (3 mg protein per 2.35 ml) in a 0.015 M Tris–HCl medium, at pH 7.4, 30°, in the presence of 10^{-2} M glutamate. After 3 min, the –SH was estimated directly on aliquots of the incubation mixture, either by the method of BOYER using PCMB or by the procedure of ELLMAN using DTNB (*cf.* MATERIALS AND METHODS) at room temperatures. Figures in parentheses indicate the number of experiments. Results are the same when the incubation was carried our fot 10 min and when either P_i or ADP alone was added to the medium.

Method of estimation	*–SH (nmoles/mg protein)*	
	Pig heart	*Rat liver*
PCMB	30.5 ± 1.0 (42)	18.5 ± 3.0 (6)
DTNB	39.8 ± 3.0 (31)	92 ± 5.0 (16)

various agents on mitochondria membrane –SH groups may vary with the physiological state and the ionic environment of mitochondria. However, the –SH values for pig heart mitochondria remain quite close when the particles are well coupled.

Free –SH release during the State 4–State 3 transition in pig heart mitochondria

In Table II we see that the addition of ADP + P_i, which initiated the State 4–State 3 transition and ATP synthesis, provoked a significant increase of the free –SH level which remained constant during ATP synthesis; 1 mM EDTA did not affect this increase of –SH level. Simultaneously the ATP synthesis and respiratory control ratios were measured to evaluate if mitochondria were well coupled or not.

TABLE II

–SH VARIATIONS IN PIG HEART MITOCHONDRIA AFTER ADDITION OF ADP + P_i (STATE 3 OF OXIDATION)

After 3 min incubation (see conditions in Table I), 400 nmoles ADP and 400 nmoles P_i were added to mitochondria. Then –SH was estimated by the DTNB technique on aliquots of the mixture, 15, 45 and 60 sec after the addition of ADP + P_i to mitochondria. The figures below give the increase in free–SH in mitochondria expressed in % of the values estimated before addition of ADP + P_i (State 4). Figures in parentheses indicate the number of experiments.

Time (sec) following addition of ADP + P_i	*Δ–SH (% of the corresponding State 4–SH values)*
15	+ 32 ± 5 (21)
45	+ 31 ± 8 (4)
60	+ 36.5 ± 6.5 (6)

This –SH increase depended on the coupling state of mitochondria. In well-coupled mitochondria, State 4 –SH levels were low and the release of –SH upon addition of ADP + P_i amounted to about 30 % of State 4 values. In badly coupled mitochondria, State 4 –SH levels approached State 3 values, and there was no significant release of free –SH after the addition of ADP + P_i.

Besides, where either ADP or P_i was added alone, there was no increase of free –SH.

It is important to precise that if 0.15 % cholate is added to mitochondria just after the incubation and just before the DTNB estimation, to eliminate membrane phenomena, the –SH levels estimated are not significantly different and the increase of –SH level observed after ADP + P_i addition remains the same.

Influence of specific inhibitors of oxidative phosphorylation on the –SH levels and on the release of –SH initiated by ADP + P_i addition

The influences of *m*-chlorocarbonyl cyanide phenylhydrazone (CCCP) and oligomycin are presented in Table III.

Uncoupling agents, such as CCCP, prevented significant release of –SH provoked by the addition of ADP + P_i; moreover free –SH levels decreased by 30 % as compared to State 4 values while the respiratory chain was stimulated (as measured by polarography) and ATP synthesis was inhibited. The small amount of ATP still synthetized could be related to substrate-level phosphorylation.

Oligomycin did not affect the free –SH level in State 4 of oxidation which is in agreement with the fact that the State 4 respiratory level was not modified upon addition of this inhibitor. However, the inhibitor did totally suppress the –SH release upon addition of ADP + P_i as well as it suppressed the respiratory chain oxidations. We verified that the small quantity of ATP synthetized could be related to the functioning of adenylate kinase (or myokinase, EC 2.7.4.3.) upon addition of ADP.

TABLE III

INFLUENCE OF OLIGOMYCIN AND CCCP ON FREE –SH CONTENTS AND –SH VARIATIONS DURING THE STATE 4–STATE 3 TRANSITION OF OXIDATION IN PIG HEART MITOCHONDRIA

Incubation conducted as in Table I. After 2 min incubation, oligomycin (2 μg/mg protein) was added to mitochondria and allowed to react for 1 min with the mitochondria in the incubation medium; then, ADP + P_i (400 nmoles of each) were added. –SH was estimated by the DTNB method before (State 4) and 15 sec after the ADP + P_i addition (which provoked the State 4–State 3 transition of oxidation). In other assays, CCCP (10^{-6} M) was introduced together with the mitochondria in the incubation medium; as above, the –SH was estimated by the DTNB method after 3 min incubation (State 4) just before the addition of 400 nmoles of ADP + P_i and 15 sec after the addition (State 3). In all assays, ATP is estimated by the firefly luminescence method at the same time as the –SH. Figures in parentheses give the number of experiments.

Inhibitor	*Free –SH (nmoles/mg protein)*			*Inhibition of ATP synthesis* (%)
	State 4	*State 3 (ADP + P_i)*	*Δ–SH during transition*	
None	35.8 ± 3 (10)	48.7 ± 2.5 (10)	+ 12.9 (10)	
Oligomycin	38 (2)	38 (2)	0	–84
CCCP	25 ± 2.5 (10)	29.6 ± 2.5 (10)	+ 4.6	–80

DISCUSSION AND CONCLUSIONS

The –SH release observed in pig heart mitochondria after addition of ADP + P_i could be related to several types of phenomena: either to the redox state of respiratory chain carriers and dehydrogenases, to the mechanism of entry of phosphate and adenylic nucleotides, to the coupling mechanism of oxidative phosphorylation or to membrane conformational changes. The results presented here seem very much to indicate that this –SH increase was somewhat related to the coupling mechanism.

The –SH release depended on the coupling state of mitochondria. In tightly coupled mitochondria (high respiratory control ratio), State 4 –SH levels were low and the release upon addition of ADP + P_i amounted to about 30 %. In badly coupled mitochondria (low respiratory control ratio), State 4 –SH levels approached State 3 values and there was no significant release of free –SH after addition of ADP + P_i.

Since 1 mM EDTA did not affect this increase of –SH, it could not be due to adenylate kinase. In the same way, if ADP or inorganic phosphate was added alone, there was no increase of –SH. Both should be added together, and we know that adenylate kinase worked when ADP was added alone[22].

Similarly, the –SH increase could have been related to the phosphate entry. We know by the works of FONYO AND BESSMAN[23], FONYO[24] and TYLER[25,26] that some –SH are implicated in the transport of phosphate in mitochondria; it has been shown also that the position of the –SH groups of the phosphate carrier can vary depending on several parameters[27]. Here we chose to work in the presence of a limiting concentra-

tion of P_i to obtain a slow synthesis of ATP and not to displace totally the equilibrium of coupling reactions towards ATP synthesis. However, if the P_i concentration was increased in the preincubation medium (5 to 20 mM), no increase of –SH occurred unless ADP was added simultaneously; thus the State 4 –SH levels in heart mitochondria did not depend on the extramitochondrial phosphate concentration, and therefore, the –SH increase did not seem to be due to the phosphate transport system unless, of course, the phosphate transport system was part of the coupling mechanisms.

Finally, we also controlled, that the P_i concentration did not affect ADP translocation.

The observed –SH release cannot be related to the change in the redox state of respiratory chain. Upon addition of ADP + P_i, the redox state of the respiratory chain shifted to the more oxidized side; so one could expect a lowering of free –SH group content and not an increase. But we had actually a decrease of –SH level upon addition of uncoupling agents, such as CCCP and dinitrophenol, which stimulate the oxidation (and shift the redox state of the respiratory chain to the more oxidized state) but inhibit ATP synthesis.

However, the inhibition of the –SH release under the influence of CCCP could not be due to an effect on the phosphate transport. Indeed, even if this uncoupling agent at least partially acted by competition[28] towards phosphate entrance (and here owing to CCCP low concentration, the competition should be very weak) or by stimulating phosphate efflux[29], we have shown that the –SH content was independent of phosphate concentration.

Oligomycin, which did not affect the State 4 respiratory level, or the State 4 –SH level, did, however, suppress any –SH release upon addition of ADP + P_i, as well as the respiratory level stimulation and the ATP synthesis linked to respiratory chain oxidations. This is what should be expected from an inhibitor of phosphorylating oxidation.

Finally one could suggest that the titratable –SH could be a function of the conformation of the mitochondrial membranes and that the –SH increase would then reflect a change in membrane conformation under the influence of ADP + P_i[30]. However, since cholate did not significantly affect the amount of –SH titrated or the increase of –SH produced by the addition of ADP + P_i, we can say that our results are very much in favour of the participation of some –SH in processes related to the coupling mechanism of oxidative phosphorylation. Many hypotheses and results have suggested such participation[3,4,31,32]. Our results agree with FALCONE's[4] hypothetical scheme, but they do not seem to support directly LAMBETH AND LARDY's[33] recent model. PAINTER AND HUNTER's[34–38] model system is very interesting to consider in view of our results.

Our results should be compared to those obtained by LAM *et al.*[39] and LAM[40] on Factor B, a soluble –SH containing Energy Transfer Factor of oxidative phosphorylation in beef heart mitochondria.

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