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pH-JUMP-INDUCED ADP PHOSPHORYLATION IN MITOCHONDRIA

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Mitochondria, uncoupled by aging or by freeze-thaw treatment, are able to synthesize ATP from ADP and P_i after a fast increase (but not decrease) in the external pH. The maximal ATP yield (approx. 2.5 ATP molecules/electron-transport chain per pH jump) can be obtained under the following conditions: (1) the pH change during the jump must exceed 0.7 pH units; (2) in the course of this change, the pH of the mitochondrial suspension must cross the pH 8.1–8.3 value. This pH-jump-induced ATP synthesis is completely inhibited by oligomycin.

Introduction

It was shown in our previous work [1] that intact rat liver as well as beef heart mitochondria are able to perform stoichiometric synthesis of ATP (in addition to the normal oxidative phosphorylation) after a fast increase (but not decrease) in the external pH value by approx. 1.0 pH unit. Undamaged coupled mitochondria in which all three coupling sites are functioning were able to synthesize three additional ATP molecules/pH jump per electron-transport chain. The value and sign of the pH that could be formed across the inner mitochondrial membrane were in this case incompatible with the requirements of the chemiosmotic hypothesis [2], and the stoichiometric synthesis of ATP could not be explained by the formation of a transmembrane pH gradient. In Ref. 1 we have also published preliminary results according to which mitochondria uncoupled by aging or by freeze-thaw procedure are able to synthesize practically the same amount of ATP in response to a pH jump as intact mitochondria although their ability to perform oxidative phosphorylation was completely lacking. The use of uncoupled mitochondria permits measurement of ATP synthesis caused solely by the fast pH increase without interference due to normal oxidative phosphorylation. In the present work, we have carried out, therefore, a detailed study of the above-described phenomena using uncoupled mitochondria. It is possible to conclude from this study that in this case, ATP synthesis proceeds in the course of a conformational transition of the ATP-synthetase complex induced by a sufficiently fast ionization of certain acid groups of the electron-transport chain proteins and/or ATP-synthetase with pK values of 8.1–8.3. This can explain the results of authors [3] who claimed to perform ATP synthesis in mitochondria by decreasing the external pH. In the course of their experiments each pH decrease was preceded by a fast increase in pH.

Methods

Rat liver mitochondria were prepared according to Ref. 4 and were 'aged' before use by incubation

Abbreviation: TMPD, N,N,N',N-tetramethyl-p-phenylenediamine.

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at 4°C for 3–7 days or uncoupled by freeze-thawing [2]. Results obtained with both types of uncoupled mitochondria did not differ. Protein content was estimated according to Ref. 5. Electron-transport chain content was supposed to be equal to that of cytochrome a_3 which was measured spectrophotometrically according to Ref. 6. Phosphorylation activity was determined by two methods the results of which were practically identical.

ATP determination by radioisotopic method. ATP was determined according to Ref. 7 modified as described in Ref. 1. ([^{14}C]glucose 6-phosphate obtained by ATP reaction with [^{14}C]glucose in the presence of yeast hexokinase). The sensitivity was high enough to determine 10^{-11} mol ATP in a sample. The samples were fixed in three different manners: (1) by lipid extraction and ATP precipitation with the help of acidic methanol [7], (2) by protein precipitation in 20% trichloroacetic acid [8], and (3) by 3-min boiling of the sample diluted 1 : 10 with 0.1 M Tris-HCl buffer (pH 9.0) containing 2 mM EDTA [8]. The results obtained with any of these techniques were identical. Before every series of experiments with a new mitochondria preparation, a new ATP calibration curve was obtained: the known ATP quantities were added to the preliminarily boiled mitochondria suspensions. A typical experiment was carried out in the following way. To 0.1 ml of sucrose (0.26 M), EDTA (1 mM), $MgCl_2$ (2 mM), KH_2PO_4 (20 mM) and Tris-HCl buffer containing mitochondria suspension, 0.1 ml of aerated Tris-HCl buffer solution (having the pH value required for this particular experiment) containing sodium succinate (10 mM) or sodium ascorbate (10 mM) + TMPD (10 mM) and ADP (1 mM) were added. The initial distribution of reagents between mitochondrial suspension and added Tris-HCl buffer could be varied according to the requirements of each particular experiment. The mixing time of the added buffer solution and mitochondria suspension must be short enough (0.5 s).

10 s after the pH jump, the sample was fixed and the ATP content measured. The error of ATP determination in a sample of one and the same mitochondrial preparation did not exceed 30%. In parallel with each series, two types of control experiments were always carried out. In the first, P_i was not added, and in the second there was no pH jump (the pH value of the added solution was equal to that of the mitochondria suspension) the other components of the reaction mixture being the same as in the main experiment. The amount of ATP synthesized due to the pH jump was assumed to be equal to the difference between ATP contents in the experiment (where pH was changed and P_i present) and that in the control (where pH was not changed or P_i was absent).

Phosphorylation activity determined by P_i consumption. In some cases, the amount of ATP formed was calculated by the decrease in P_i content after the pH jump. The experimental procedure was the same as that described above with the following modifications. P_i and ADP concentrations were $2 \cdot 10^{-5}$ and $5 \cdot 10^{-4}$ M, respectively. Not later than 2 s after the pH jump, preparations were fixed by 0.2 ml of 10% trichloroacetic acid with 4 mM EDTA. After 5 min incubation at 0°C the samples were diluted with 1 ml of water centrifuged, and the P_i content in the supernatant was measured according to Ref. 9.

Mean values of the results of not less than five experiments with mitochondria of different preparations are listed in Table I. Some experiments were carried out with anaerobic solutions prepared by bubbling argon or carbon monoxide through the samples for 30 min or by sodium hydrosulfite (1 mg/ml solution). In order to perform several pH jumps with one and the same sample the gradually increasing volumes of buffer solutions (pH 9.0 and 6.0) were alternatively added in such a way that the sample underwent sequential pH transition from 7.4 to 8.5 and backwards. In the experiments with antimycin A (Sigma) the latter was added in quantities of 100 μg/g sample protein. Oligomycin (Boehringer) was used in concentrations of 10 μg/mg sample protein.

Results and Discussion

In all samples listed in Table I, we could not register any normal phosphorylating activity in the whole pH range covered (pH 5–10). It can be seen that the ATP yield is, as a rule, about 2.5 ATP molecules/pH jump per electron-transport chain. This yield depends neither on the final pH in the range 8.4–9.6, nor on the initial pH in the range 5.0–7.8. In order to obtain the maximal ATP

TABLE I

No.	Mitochondrial suspension	Buffer solution	pH		Number of jumps	ATP yield (molecules/ chain)
			Initial	Final		
1	ADP, P_i, succinate	O_2	7.4	8.7	1	2.5
2		O_2	7.4	7.4	1	0
3		O_2	8.7	8.7	1	0
4	ADP, succinate	O_2	7.4	8.7	1	0
5	P_i, succinate	O_2	7.4	8.7	1	0
6	ADP, P_i, succinate	CO	7.4	8.7	1	0.3
7		Ar	7.4	8.7	1	0.6–1.0
8	P_i, succinate	O_2, ADP	7.4	8.7	1	2.5
9		O_2, ADP added 5 s after jump	7.4	8.7	1	2.5
10		O_2, ADP added 15 s after jump	7.4	8.7	1	0
11	ADP, P_i, succinate	O_2	9.5	6.0	1	0
12		O_2	7.4	8.3	1	1.7
13		O_2	7.4	9.6	1	2.5
14		O_2	6.5	8.4	1	2.5
15		O_2	6.0	8.4	1	2.5
16		O_2	6.5	9.3	1	2.5
17		O_2	5.2	7.5	1	0
18		O_2	7.8	8.7	1	2.2
19		O_2	8.2	9.1	1	0
20		O_2	9.0	10.1	1	0
21		O_2	7.4	8.5	1	2.4
22		O_2	7.4	8.5	3	6.0
23		O_2	7.4	8.5	4	10.0
24		O_2	7.4	8.5	7	17.0
25	ADP, P_i, ascorbate, TMPD	O_2	7.4	8.7	1	1.2
26	ADP, P_i	O_2	7.4	8.7	1	0
27		hydrosulfite	7.4	8.7	1	0
28	ADP, P_i, succinate antimycin A	O_2	7.4	8.7	1	2.7
29	ADP, P_i, succinate, oligomycin	O_2	7.4	8.7	1	0

yield, two requirements must be fulfilled: (1) the pH change during the jump must exceed 0.7 units, and (2) in the course of this change, the pH of mitochondrial suspension must cross the pH 8.1–8.3 value. pH jumps of opposite sign (fast pH decrease) never led to ATP synthesis.

Both phosphorylation substrates (ADP and P_i) are absolutely obligatory in order to have any increase in the sample ATP content in response to a pH jump. ADP and P_i must be either present in the initial mitochondrial suspension, or added in the course of the pH jump, or even after the pH jump (but not later than 10 s after the jump).

The procedure of ATP synthesis due to the fast increase in the suspension pH can be repeated several times with the same sample. Each sequential pH jump is accompanied by ATP formation.

It could be supposed that aging or freeze-thaw-

ing can lead to the disruption of mitochondria and to formation of submitochondrial particles for which the pH induced by a pH jump would have the 'proper sign' required by the chemiosmotic hypothesis (although obviously too low a value). The contribution of submitochondrial particles to the observed ATP synthesis can, however, be ruled out due to the fact that, as stressed above, the intact mitochondrial before aging or freeze-thawing possess the same ability to synthesize an equal amount of ATP after a pH jump-like pH increase.

Table I shows also that the necessary condition of pH-induced ATP synthesis is the presence of electron donors in the incubation medium. With succinate the ATP yield/electron-transport chain per pH jump was approx. 2.5 molecules, and with ascorbate + TMPD, a lower yield (approx. 1 ATP molecule) was recorded.

Besides electron donors another necessary component of the reaction mixture is an electron acceptor – oxygen (the buffer solution with which the pH jump is performed must be aerated). The ATP yield decreases considerably if we remove oxygen by means of hydrosulfite or by argon or CO bubbling.

The addition of the electron-transfer inhibitor antimycin A does not influence the pH-jump-induced ATP formation. The addition of oligomycin, an inhibitor of ATP-synthetase, leads, on the other hand, to its complete retardation.

Our results exclude the possibility of explaining ATP formation after a jump-like pH increase by the release of previously formed tightly bound ATP or by adenylate kinase activity. The sum total of our data leads us almost inevitably to the conclusion that the primary effect of a pH jump is the fast ionization of certain acid groups with pK values of 8.1–8.3. It is well known that such ionization can lead to the formation of rather long-lived conformationally nonequilibrium states of proteins [10].

One can suggest two alternative interpretations of these results. According to the first, a pH jump subsequent conformational relaxation of the membrane-bound proteins (ATP-synthetase included) lead to the temporary 'switching on' of the coupling between respiration and phosporylation. The primary energy source is, in this case, electron transfer as in the coupled mitochondria. The obligatory presence of electron donors and O_2 can be regarded as evidence in favor of this interpretation.

The pH-jump-induced ATP synthesis in the presence of the electron-transfer inhibitor, antimycin A, is, however, at variance with this explanation. The latter is also at variance with the fact that the additional synthesis of ATP induced by a pH increase in intact coupled mitochondria is quantitatively the same as that in the case of uncoupled mitochondria.

We prefer, therefore, the second interpretation according to which the presence of electron donors and O_2 is necessary to ensure a proper distribution of redox states of electron carriers and of oxidizable groups in ATP-synthetase [10,11]. Under this condition, the conformational relaxation of the ATP-synthetase complex leads to the compulsory formation of ATP. The energy source is, in this case, the titration of acid groups with pK values between 8.1 and 8.3. A detailed description of the relaxation concept of membrane phosphorylation can be found in Refs. [12 and 13].

The classical experiments of Jagendorf and Uribe [14] have been considered by many scientists as one of the most important and direct proofs of the chemiosmotic hypothesis. It was shown in these experiments that chloroplasts are able to synthesize ATP in the dark if the pH gradient of the required sign (increase in the external pH) had been artificially induced. We have shown here, however, that ATP synthesis due to an increase in the external pH can also lead to ATP synthesis in mitochondria. It is clear, therefore, that the coincidence of the sign of the pH gradient induced in chloroplasts in Ref. 14 with the requirements of the chemiosmotic hypothesis cannot be considered as a proof of the latter.

Interpretation of our results in terms of the relaxation concept cannot, of course, be regarded as proven. Their explanation in the realm of the chemiosmotic hypothesis is, however, completely impossible.

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