MITOCHONDRIAL ATP-Pi EXCHANGE COMPLEX*

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Summary: An enzyme complex with high ATP-Pi exchange activity has been purified from beef heart mitochondria, using the general procedure which also yields electron transfer complexes I, II, III and IV from the same batch of mitochondria. The ATP-Pi exchange activity of the preparation, designated complex V, is inhibited by various uncouplers, rutamycin, venturicidin, dicyclohexylcarbodiimide, arsenate, azide, adenylyl imidodiphosphate, and valinomycin plus potassium. The ATP-Pi exchange activity of complex V is specific with respect to ATP; ITP, GTP and UTP are essentially ineffective. Complex V is deficient in cytochromes, but 2-3 times enriched as compared to mitochondria with respect to binding sites for the uncoupler 2-azido-4-nitrophenol. As in mitochondria, this binding is competitively inhibited by other uncouplers. Complexes I, III and IV, which in mitochondria contain the three energy coupling sites, do not bind the above uncoupler.

It has been shown in earlier studies that the mitochondrial electron transport system can be divided, in a reversible manner, into four enzyme complexes, which were designated complexes I, II, III and IV (1,2). This paper is a preliminary report on the isolation of another enzyme complex from mitochondria, which is capable of uncoupler- and oligomycin-sensitive ATP-Pi exchange. This enzyme complex, designated hereafter as complex V, is derived from the same batch of mitochondria from which the electron transfer complexes I, II, III and IV are isolated.

METHODS AND MATERIALS

Complex V was prepared as follows. Mitochondria isolated from beef-heart were treated with deoxycholate and KCl, centrifuged, and the red supernatant dialyzed as described before for the preparation of the electron transfer

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complexes (3,4). After dialysis, the red supernatant was centrifuged as before for 90 min at 78,500 xg, and the upper two-thirds of the supernatant removed from the loosely packed, red sediment and stored frozen at -70° until used. This supernatant material was thawed at room temperature, passed through a Sephadex G25 (coarse) column equilibrated and eluted at 5° with 10 mM Tris-acetate, pH 7.5, at 100 to 150 ml per each column of 10 x 22 cm. The effluent, which is pink, was collected, brought to 42% saturation with the addition of neutral saturated ammonium sulfate at 4°, and centrifuged for 15 min at 78,500 xg. The pellet was suspended in cold 0.25 M sucrose containing 10 mM Tris-acetate, pH 8.0, at a protein concentration of 15-20 mg/ml. Potassium cholate was added from a 20% (w/v) solution (pH 7.9) to a final concentration of 0.35 to 0.38 mg cholate/mg protein, and the mixture was fractionated at 0-4° with saturated ammonium sulfate. The fraction precipitating between 25% and 42% ammonium sulfate saturation was collected by centrifugation for 15 min at 105,000 xg, frozen in liquid nitrogen, and stored at -70° until used. This material constituted the preparation of complex V. The protein yield is about 10 mg of complex V per gram of mitochondria.

ATPase and ATP-Pi exchange, using ^{33}Pi, were assayed essentially according to published procedures (5,6). Protein was measured by the biuret method (7). Phospholipid microsuspensions in 20 mM Tris-acetate, pH 8.0, containing 1 mM ethylenediamine tetraacetate were prepared either from commercial soybean phospholipids by sonication and centrifugation of a 20 mg/ml suspension or from mitochondrial phospholipids by published procedures (8). The latter was kindly supplied by Dr. S. Fleischer. Both preparations were equally competent in the assays described below. Flavin was extracted and estimated as before (9). Cytochromes were estimated by the spectrophotometric method of Williams (10), and DPNH dehydrogenase (9), succinate dehydrogenase (11), cytochrome oxidase (12) and antimycin-sensitive DPNH-cytochrome *c* reductase (3) were assayed as already described. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate and mercaptoethanol was performed according to Weber and Osborn (13).

TABLE I

ATP-Pi Exchange Activity of Complex V

Conditions	Activity nmoles/min x mg
Complex V + PL + BSA	100-120
Complex V + PL	80-100
Complex V + BSA	10-25
Complex V	10-25

ATPase: 3-4 μmoles/min x mg at 30°

Conditions: Complex V was dissolved in 0.66 M sucrose, containing 50 mM Tris-HCl, pH 8.0, and 1 mM histidine, and mixed with the phospholipid microsuspension to a final concentration of 1.0 mg protein/ml and 350 μg phospholipid phosphorus/ml. Of this mixture, 0.2 ml was added to 0.8 ml of the reaction mixture at 30°. The latter contained 25 μmoles Tris-acetate, pH 7.5, 15 μmoles $MgSO_4$, 15 μmoles ATP, 3 mmoles sucrose, 10 μmoles KH_2PO_4, 3 mg BSA and ^{33}Pi (1-3 x 10^6 cpm). The reaction was stopped after 10 min with the addition of 0.1 ml of 35% $HClO_4$. $AT^{33}P$ was determined as described (17). Where indicated BSA and phospholipid (PL) were omitted.

The sources of the materials used were as follows. Cholic acid, deoxycholic acid, and ammonium sulfate from Mann, nucleotides from PL Biochemicals, crystalline BSA* from Miles Research Laboratories, DCCD from K and K Laboratories, AMP-PNP from ICN, and ^{33}P from New England Nuclear. The sources of uncouplers and valinomycin were the same as before (14). Nigericin was kindly donated by Professor H.A. Lardy, and venturicidin and triethyl tin were the generous gifts of Dr. D.E. Griffiths.

RESULTS

Ammonium sulfate-precipitated preparations of complex V are stable at -70°, and dissolve readily in buffer to form a clear, straw-colored solution. The

* Abbreviations: BSA, bovine serum albumin; F_1, ATPase of mitochondria; OSCP, oligomycin sensitivity-conferring protein; PL, phospholipid microsuspension; S-13, 5-chloro-3-*t*-butyl-2'-chloro-4'-nitrosalicylanilide; Cl-CCP, carbonyl-cyanide *m*-chlorophenylhydrazone; 1799, bis-(hexafluoroacetonyl)acetone; PCP, pentachlorophenol; DCCD, dicyclohexylcarbodiimide; and NEM, N-ethylmaleimide.

TABLE II

Nucleotide Specificity of the
ATP-Pi Exchange Activity of Complex V

Nucleotide	% Activity
ATP	100
ITP	7
GTP	3
UTP	0.0

Conditions were the same as in Table I. Where indicated 15 μmoles ITP, GTP or UTP was used instead of ATP.

solution remains clear upon dilution to concentrations as low as 1 mg/ml, and upon standing, even at room temperature. Table I shows the ATP-Pi exchange activity of complex V in the absence and presence of phospholipids and BSA.** By itself, the preparation has an ATP-Pi exchange activity of 10 to 25 nmoles/min x mg protein at 30°. This activity is enhanced several-fold in the presence of phospholipids, and about 20% upon further addition of BSA. Unlike the reconstituted systems of Racker and coworkers, which are composed of cholate-ammonium sulfate extracted mitochondrial membrane fragments plus F_1, OSCP and phospholipids (15,16), the ATP-Pi exchange activity of complex V does not require the formation of enzyme-phospholipid vesicles by prolonged dialysis or special sonication procedures. In our experiments, ATP-Pi exchange assay is performed simply by mixing complex V with the phospholipid suspension, and adding them together to the assay mixture. It is also possible to add the phospholipid suspension to the assay mixture, then start the reaction by the addition of

** By differential centrifugation of lysolecithin-treated submitochondrial particles Sadler *et al.* have isolated membrane fragments which also have high ATP-Pi exchange activities (private communication from Dr. D.E. Green).

TABLE III

Effect of Uncouplers on the
ATP-Pi Exchange Activity of Complex V

Uncoupler	Conc. (μM)	% Inhibition
S-13	10	98
Cl-CCP	10	94
1799	50	98
NPA	30	90
PCP	30	80
Picrate	150	95
Triethyl Tin	10	97

Conditions were the same as in Table I. S-13, Cl-CCP, 1799, PCP and triethyl tin were added in 0.25 to 1% ethanol. One percent ethanol by itself caused about 15% inhibition. Triethyl tin was used as the sulfate salt.

complex V. That the presence of phospholipids in the assay is not an absolute requirement is shown in Table I.

Table II shows the nucleotide specificity of complex V for ATP-Pi exchange, and Tables III and IV show, respectively, the effect of uncouplers and various inhibitors. The ATP-Pi exchange and the ATPase activities of complex V are not affected by rotenone and antimycin A, and the ATPase activity is only slightly enhanced by uncouplers (DNP, Cl-CCP; none by S-13). The latter is similar to the effect of uncouplers on the ATPase activity of submitochondrial particles or purified preparations of F_1 (ATPase)(17,18).

By equilibrium-binding experiments with the use of our new, radioactive uncoupler, 2-azido-4-nitrophenol (NPA), it has been shown elsewhere (14) that the mitochondrial inner membrane contains a specific uncoupler-binding site at a concentration which is comparable to that of the individual electron carriers

TABLE IV

Effect of Inhibitors and Ionophores on the ATP-Pi Exchange Activity of Complex V

Additions	Conc.	% Inhibition
Rutamycin	7 µg/mg prot.	> 99
DCCD	5 µg/mg prot.	> 99
Venturicidin	2 µM	99
Arsenate	20 mM	65
NaN_3	100 µM	72
AMP-PNP	10 mM	80
Valinomycin ($+K^+$)	1 µg/mg prot.	92
Nigericin ($+K^+$)	5 µg/mg prot.	53
Mercurials, NEM ± ATP		15-30

Conditions were the same as in Table I.

TABLE V

NPA-Binding Capacity of Complexes I, III, IV and V

Complex	nmoles NPA/mg protein
I	$\leqslant$ 0.05
III	< 0.01
IV	< 0.01
V	0.81

Conditions: The NPA binding experiments were carried out at pH 8.0 and 4° according to ref. 14.

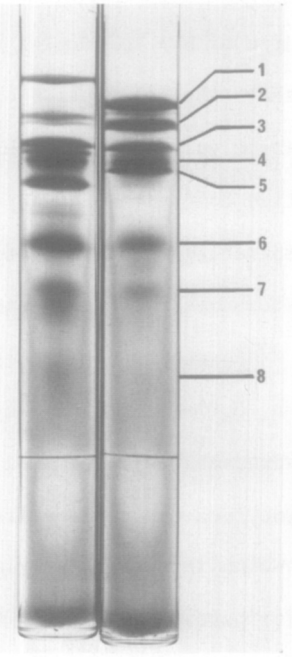

Fig. 1. Polypeptide composition of complex V (right gel, 31 µg protein) and oligomycin-sensitive ATPase (left gel, 36 µg protein) as depicted on SDS-10% polyacrylamide gels stained with Coomassie Blue.

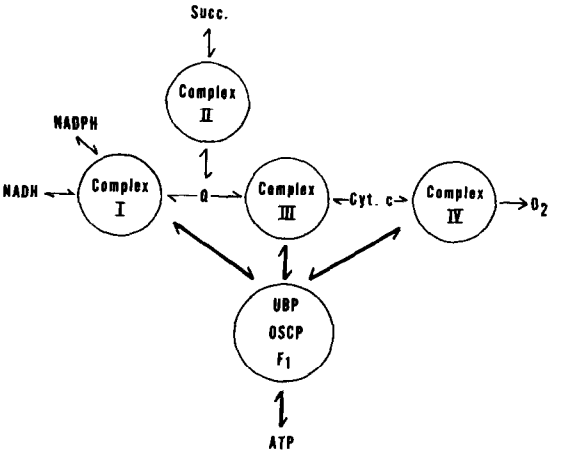

Fig. 2. Schematic representation of the functional relationship of the five enzyme complexes of the mitochondrial electron transport-oxidative phosphorylation system. UBP, uncoupler-binding proteins.

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or of F_1 molecules. As shown in Table V, the NPA-binding sites of mitochondria are absent from the electron transfer complexes I, III and IV, but present in complex V. The dissociation constant of NPA binding to complex V (K_D = 18-28 µM) is the same as that found for submitochondrial particles. Similar to mito-

chondria, the equilibrium binding of NPA to complex V is competitively inhibited by other uncouplers. Fig. 1 shows the polypeptide composition of complex V as depicted on SDS-10% polyacrylamide gels stained with Coomassie Blue. The gel on the left shows the polypeptide composition of a preparation of oligomycin-sensitive ATPase (19) at a comparable amount of protein. It is seen that complex V does not contain a greater number of Coomassie-stainable bands than oligomycin-sensitive ATPase. Preparations of complex V contain per mg protein 0.15-0.2 nmole of cytochrome *b*, 0.07-0.1 nmole of cytochrome c_1 (+*c*), and about 1 nmole of flavin (acid-extractable). Complex V is devoid of cytochromes aa_3, and does not oxidize ferrocytochrome *c*. The flavin appears to be associated with DPNH dehydrogenase, which in the SDS-acrylamide gel of complex V is probably responsible for a major portion of band number 2. Bands 3, 4 and 8 are probably the subunits of F_1, and bands 7 and 6 appear to be, respectively, due to OSCP and an uncoupler-binding protein discovered in this laboratory (14,20). It is rather remarkable, therefore, that an enzyme complex, which is capable of energy conservation and of inhibition by the reagents shown in Tables III and IV, should have such an apparently simple polypeptide composition.

In conclusion, we wish to emphasize (a) the important property of complex V as an energy conserving system, (b) the finding that the mitochondrial uncoupler binding sites appear to be located exclusively in this complex, and (c) the fact that complex V is isolated by a fractionation procedure which from the same batch of mitochondria also leads to the isolation of complexes I, II, III, and IV, all in very high yields. The findings reported above are consistent with a scheme of the mitochondrial energy generation-conservation system as depicted in Fig. 2.

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REFERENCES

1. Hatefi, Y., Haavik, A.G. & Griffiths, D.E. (1961) *Biochem. Biophys. Res. Commun. 4,* 441-446 and 447-453.
2. Hatefi, Y., Haavik, A.G., Fowler, L.R. & Griffiths, D.E. (1962) *J. Biol. Chem. 237,* 2661-2669.
3. Hatefi, Y., Haavik, A.G. & Jurtshuk, P. (1961) *Biochim. Biophys. Acta 52,* 106-118.
4. Hatefi, Y. (1966) *Comprehensive Biochem. 14,* 199-231.
5. MacLennan, D.H., Smoly, J.M. & Tzagoloff, A. (1968) *J. Biol. Chem. 243,* 1589-1597.
6. Pullman, M.E. (1967) *Methods Enzymol. 10,* 57-60.
7. Gornall, A.G., Bardawill, C.J. & David, M.M. (1949) *J. Biol. Chem. 177,* 751-766.
8. Fleischer, S. & Fleischer, B. (1967) *Methods Enzymol. 10,* 406-433.
9. Hatefi, Y. & Stempel, K.E. (1969) *J. Biol. Chem. 244,* 2350-2357.
10. Williams, J.N., Jr. (1964) *Arch. Biochem. Biophys. 107,* 537-543.
11. Baginsky, M.L. & Hatefi, Y. (1969) *J. Biol. Chem. 244,* 5313-5319.
12. Smith, L. (1955) *Methods of Biochem. Anal. 2,* 427-434.
13. Weber, K. & Osborn, M. (1969) *J. Biol. Chem. 244,* 4406-4412.
14. Hanstein, W.G. & Hatefi, Y. (1974) *J. Biol. Chem. 249,* 1356-1362.
15. Kagawa, Y. & Racker, E. (1971) *J. Biol. Chem. 246,* 5477-5487.
16. Racker, E. (1973) *Biochem. Biophys. Res. Commun. 55,* 224-230.
17. Pullman, M.E., Penefsky, H.S., Datta, A. & Racker, E. (1960) *J. Biol. Chem. 235,* 3322-3329.
18. Penefsky, H.S., Pullman, M.E., Datta, A. & Racker, E. (1960) *J. Biol. Chem. 235,* 3330-3336.
19. Tzagoloff, A., Byington, K.H. & MacLennan, D.H. (1968) *J. Biol. Chem. 243,* 2405-2412.
20. Hanstein, W.G. (1974) *Fed. Proc. 33,* 1517.