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STUDIES OF THE OLIGOMYCIN-SENSITIVE ATPASE FROM YEAST MITOCHONDRIA. Reconstitution of ATP-$^{32}P_i$ Exchange in the Presence of Phospholipids

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A purified preparation of the oligomycin-sensitive ATPase from yeast mitochondria has been shown to elicit an oligomycin- and uncoupler-sensitive ATP-$^{32}P_i$ exchange in the presence of phospholipids. Reconstitution was normally achieved by dialysis of an ATPase-phospholipid-cholate mixture. Following this procedure, vesicles with diameters between 200 and 1,500 Å were seen by electron microscopy. As in mitochondria, ATPase activity in the reconstituted system was stimulated by a range of uncouplers which inhibited ATP-$^{32}P_i$ exchange. These and other findings suggest that the coupling mechanism may still be intact within the ATPase complex.

INTRODUCTION

The structure and function of the mitochondrial ATPase has received considerable attention since the discovery of its role as the catalytic site of ATP formation (1). The protein can be isolated in two forms, either as the 90-Å F_1* spheres or as the larger oligomycin-sensitive ATPase complex, which contains, in addition to F_1, the oligomycin-sensitivity-conferring protein (OSCP), the hydrophobic F_o sector required for conferral of oligomycin sensitivity, and tightly bound P-lipid. The latter complex contains nine or more nonidentical subunits compared with only five in F_1 (1–3). When prepared free of membranes, neither preparation seems capable of catalyzing phosphorylation or the associated ATP-P_i or ATP-ADP exchange reactions, which strengthens the belief that a vesicle structure contianing P-lipids is required for these energy-linked functions.

According to Mitchell (4) the electrochemical energy of the proton gradient is utilized to drive phosphorylation by an electrogenic movement of protons through the F_o-F_1 complex. The first important step towards demonstrating such a complex was achieved by Kagawa and Racker (5) who reconstituted vesicles with ATP-P_i exchange activity by using a hydrophobic membrane fraction (containing F_o), OSCP, F_1, and P-

*Abbreviations: F_1, coupling factor 1 ATPase; F_o, membrane sector required for conferral of oligomycin sensitivity; OSCP, oligomycin sensitivity-conferring protein, required for binding of F_1 to F_o; P-lipid, phospholipid; CCCP, carbonyl cyanide m-chlorophenylhydrazone; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

lipids. The same system was later shown to catalyze net phosphorylation by using either bacteriorhodopsin (6) or electron flow through cytochrome c-cytochrome oxidase (7, 8) to generate the proton gradient. These findings, therefore, are in line with the notion that the F_O-F_1 complex may itself contain the complete assembly of coupling proteins. It is the purpose of this paper to support this conclusion by showing that vesicles capable of ATP-P_i exchange can be reconstituted by using only a mixture of soybean P-lipids and the purified oligomycin-sensitive ATPase complex isolated from yeast mitochondria. Some of the findings presented here are currently in press elsewhere (9).

METHODS

Saccharomyces cerevisiae (diploid wild type) was grown at 28°C in a medium containing 1% galactase as carbon source (9). Preparation of the ATPase (9) was carried out by modification of the method of Tzagoloff and Meagher (10); stabilization against cold inactivation was important and could be achieved with methanol and ATP. Reconstitution of ATP-$^{32}P_i$exchange was carried out as described by Kagawa and Racker (5) by combining the ATPase (600 μg) with sonicated soybean P-lipids (5 mg), and sodium cholate (5 mg, added as a 20% [w/v] solution, pH 8.0) in a final volume of 0.5 ml, and dialyzing for 20 hr at 4°C against a medium containing 0.1 mM ATP, 0.2 mM EDTA, 1 mM dithiothreitol, 5% (w/v) methanol, and 10 mM tricine-NaOH, pH 8.0. The medium was replaced three times during dialysis.

ATP-$^{32}P_i$ exchange assays contained, in 0.5 ml, 10 mM ATP, 20 mM $MgSO_4$, 10 mM KH_2PO_4, (containing 5×10^6 cpm of $^{32}P_i$), 1 mM dithiothreitol, 2 mg of defatted bovine serum albumin, 50 mM tricine-NaOH, pH 7.5, and 20 μg of ATPase protein. Reactions were terminated after 10 min at 37°C, and $^{32}P_i$ was separated before counting as described by Avron (11). ATPase activity was estimated for 5 min at 37°C in a reaction mixture which contained, in 1.0 ml, 5 mM ATP, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.5, and 2–5 μg of protein. The phosphate liberated (12), and protein (13), were determined as described previously.

RESULTS

Properties of the Oligomycin-Sensitive ATPase

When purified the protein was properly dispersed in the presence of detergent and appeared as a single major band on analytical ultracentrifugation and polyacrylamide gel electrophoresis (9). The specific ATPase activity varied between 30 and 60 μmoles P_i released/min/mg protein but was activated to more constant values of 75–85 μmoles P_i released/min/mg protein by heating the ATPase for 2 min at 50°C in a solution containing 20 mM ATP, 1 mM EDTA, 50 mM tricine-NaOH, pH 8.0, and 500 μg of protein/ml. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed nine major bands (14) with the following apparent molecular weights: I, 60,000; II, 54,000; III. 42,000; IV, 32,000; V, 28,000; VI, 22,000; VII, 20,000; VIII, 13,000; IX, 10,500.

Contamination of the ATPase by respiratory chain components was low (9); neither NADH- nor succinate oxidase activities were detectable, although NADH- and succinate dehydrogenase and cytochrome c oxidase activities were present at levels of 24, 9, and 9%, respectively, compared to mitchondria. Spectral analysis revealed some minor absorption in the cytochrome b–c region. No ATP-$^{32}P_i$ exchange activity could be detected in the purified protein.

Reconstitution of ATP-$^{32}P_i$ Exchange

Results in Table I show that ATP-$^{32}P_i$ exchange activity could be measured following dialysis of an ATPase-P-lipid-cholate mixture. No activity was observed where P-lipid was omitted. As in native membranes, [^{32}P]ATP formation was markedly inhibited by the energy transfer inhibitors oligomycin, rutamycin, and dicyclohexylcarbodiimide, by the protonophorous uncouplers CCCP, FCCP, and dinitrophenol, by the amine uncouplers NH_4Cl and atebrin, and by nigericin. Interestingly, both valinomycin and monactin stimulated the exchange activity in the presence of KCl (or K_2SO_4), indicating that the effect is due to an ionophore-induced K^+ transport. Both valinomycin and monactin in the presence of monovalent cations have similarly been found to stimulate acid-base and postillumination ATP formation in chloroplasts, apparently by establishing a diffusion potential across the membranes, positive inside (15, 16). Such conditions would favor an electrogenic efflux of protons through the ATPase complex, presumably with a resultant increase in phosphorylation.

The exchange activity was not significantly effected by the electron transport inhibitors rotenone, antimycin A, or KCN, or by the respiratory substrates NADH or succinate, moreover, no oxygen uptake was detected during ATP-$^{32}P_i$ exchange. These and other findings (9) clearly demonstrate that the exchange activity is independent of electron flow.

That the reconstituted system consists of vesicle structures was clearly shown by electron microscopy after negative staining with phosphotungstate (9). In general the vesicles ranged between 200 and 1,500 Å in diameter. Only amorphous aggregates of the ATPase were seen where P-lipids were omitted during reconstitution.

A further similarity between the reconstituted system and mitochondria is the stimulation of ATPase activity by uncouplers. As shown in Table II, uncouplers which inhibited ATP-$^{32}P_i$ exchange (Table I) significantly stimulated ATPase activity in the reconstituted system, but not in the purified ATPase. Such effects are normally ascribed to relief of the proton back pressure by an uncoupler-induced dissipation of the membrane potential.

Reconstitution of functional vesicles by dialysis in the presence of P-lipid is apparently a slow process requiring bile detergents (5). It is notable, therefore, that exchange activity could also be measured after direct addition of sonicated P-lipid to the ATPase (Table III). Though the rates of ATP-$^{32}P_i$ exchange reconstituted in this way were comparable to those achieved by the cholate-dialysis method, much higher levels of P-lipid were required. Moreover, while the exchange activity remained sensitive to oligomycin and CCCP, almost no inhibition was observed with nigericin or NH_4Cl. The reasons for the altered inhibitor effects, and for the high P-lipid requirement, are not at present understood.

DISCUSSION

The mechanism of the energy-linked ATP-P_i exchange reaction might reasonably be viewed within the framework of current chemiosmotic concepts as a composite of two contributing reactions, an initial proton translocation through the ATPase complex coupled with ATP hydrolysis (17), followed by a small rate of rephosphorylation driven by

TABLE I. Effect of Inhibitors on ATP-$^{32}P_i$ Exchange Activity

Experiment	Addition to assay	ATP-$^{32}P_i$ exchange	
		Specific activity[1]	% activity
1	None	247	100
	10 μg/ml oligomycin	44	18
	10 μg/ml rutamycin	60	24
	50 μM CCCP	5	2
2	None	945	100
	50 μM dicyclo-hexylcarbodiimide	17	2
	50 mM KCl	975	103
	50 mM KCl + 1 μg/ml valinomycin	1,442	153
	50 mM KCl + 10 μg/ml monactin	1,400	148
3	None	205	100
	10 M FCCP	28	14
	0.5 mM dinitrophenol	31	15
	10 mM NH_4Cl	32	16
	10 μM atebrin	23	11
	0.2 μg/ml nigericin	29	14

ATP-$^{32}P_i$ exchange activity was reconstituted by dialysis of the yeast oligomycin-sensitive ATPase in the presence of P-lipids and cholate. Details of the method and of the measurement of ATP-$^{32}P_i$ exchange activity are given in the text.

[1] nmoles [^{32}P]ATP formed/mg protein/10 min.

TABLE II. Effect of Uncouplers on the ATPase Activity

Additions	ATPase activity[1]	
	Purified ATPase	Reconstituted ATPase
None	31.0	11.1
50 μM CCCP	33.8	18.5
5 μM FCCP	31.9	16.9
0.2 μg/ml nigericin	31.6	15.4
10 mM NH_4Cl	31.7	14.8
0.5 mM dinitrophenol	31.1	13.4

[1] μmoles P_i released/mg protein/min.

the proton gradient thus generated. Such a mechanism is in line with the apparent requirement for vesicles and for the observed inhibition by protonophorous uncouplers. Whatever the precise mechanism, the discovery of ATP-P_i exchange following reconstitution with P-lipids indicates that the yeast ATPase complex may contain the complete assembly of polypeptides required for transducing the proton gradient energy into the anhydro-bond energy of ATP.

TABLE III. Reconstitution of ATP-$^{32}P_i$ Exchange Activity by Direct Addition of P-lipid[1]

P-lipid addition	Addition to assay	ATP-$^{32}P_i$ exchange[2]
mg P-lipid/mg protein		
0	–	0
29.8	–	147
59.5	–	296
119.0	–	427
119.0	5 μg/ml oligomycin	174
119.0	50 μM CCCP	15
119.0	0.2 μg/ml nigericin	398
119.0	10 mM NH_4Cl	433
178.6	–	485

[1] ATPase protein (84 μg) was combined with variable amounts of sonicated P-lipid and preincubated at 37°C in 0.4 ml of a solution containing 12.5 mM $MgSO_4$, 2 mg of defatted bovine serum albumin, and 62.5 mM tricine-NaOH (pH 7.5). After 5 min, a 0.1-ml aliquot containing 50 mM ATP, 5 mM dithiothreitol, and 50 mM $^{32}P_i$ was added to initiate the exchange reaction. Inhibitors were also added at this stage where indicated. No activity was obtained where the ATPase was omitted.
[2] nmoles [^{32}P] ATP formed/mg protein/10 min.

Preparations of the oligomycin-sensitive ATPase which have intrinsic ATP-P_i exchange activity have recently been obtained from bovine heart mitochondria. That of Sadler et al. (18) consists of small membrane particles with projecting F_1 spheres. Whether the preparation of Hatefi et al. (19) also contains vesicle fragments has not been clearly established, although it is noteworthy in this regard that the exchange activity was markedly stimulated by addition of P-lipid vesicles. Like the yeast ATPase, however, such complexes contain far fewer polypeptide species than the parent mitochondrial membranes, and thus seem likely to be of considerable advantage for future studies of the phosphorylation mechanism.

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