RECONSTITUTED MITOCHONDRIAL OLIGOMYCIN-SENSITIVE ATPase (F_0F_1) WITH INTERMEDIATE $P_i \rightleftharpoons$ HOH EXCHANGE BUT NO $P_i \rightleftharpoons$ ATP EXCHANGE ACTIVITY

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1. Introduction

Evidence accumulated in recent years suggests that the mitochondrial ATPase F_1 (and similar ATPases of chloroplasts and bacteria) can undergo energy-linked conformational changes that may be intrinsically involved in ATP synthesis coupled to electron and proton transport [1–14]. It has been proposed [12,15–17] that energy transduced through such conformational changes serves to release ATP from [7–17] and to promote productive binding of ADP and P_i to the enzyme [11–14, 17,18]. The actual conversion of bound ADP and P_i into ATP is regarded as proceeding without direct coupling to an energy supply. Important evidence suggesting this concept is based on the observation [10–15] that submitochondrial particles catalyze an oligomycin- and/or DCCD-sensitive, uncoupler-insensitive exchange of oxygen between P_i and H_2O accompanying the formation of P_i from ATP ('intermediate $P_i \rightleftharpoons$ HOH exchange'). Based on detailed studies of this exchange reaction [17,18] an alternating catalytic site mechanism was recently formulated [18], according to which an energy-requiring conformational change of F_1 promotes ADP and P_i binding in a manner capable of forming ATP at one catalytic site of the enzyme and simultaneously promotes the release of ATP from another catalytic site.

The insensitivity of the intermediate $P_i \rightleftharpoons$ HOH exchange to uncouplers suggests that this reaction is not dependent on a transmembrane proton gradient and, thus, that one might be able to observe it with ATPase preparations not capable of generating such a gradient. Earlier studies [19,20] have shown that preparations of soluble, oligomycin-insensitive F_1 ATPase exhibit little or no intermediate $P_i \rightleftharpoons$ exchange However, preparations of particulate, oligomycin-sensitive ATPase exhibit an appreciable intermediate $P_i \rightleftharpoons$ HOH exchange [21]. These preparations are also known to exhibit a $P_i \rightleftharpoons$ ATP exchange [22,23] and to be capable of generating an ATP-dependent proton gradient when incorporated into liposomes [23]. Further examination of relations of the capacity for $P_i \rightleftharpoons$ ATP exchange and generation of a proton gradient to the capacity for intermediate $P_i \rightleftharpoons$ HOH exchange thus seemed warranted.

In the experiments described below, an oligomycin-sensitive ATPase (OS-ATPase) was reconstituted [24] by combining purified, soluble F_1 with a 'membrane fraction' (F_0) derived from a preparation of oligomycin-sensitive ATPase by treatment with NaBr. This reconstituted system was found to exhibit no $P_i \rightleftharpoons$ ATP exchange and to generate no ATP-dependent proton gradient when incorporated into liposomes. On the other hand, the reconstituted particulate F_0F_1 complex exhibited an appreciable $P_i \rightleftharpoons$ HOH exchange that was sensitive to oligomycin and insensitive to uncouplers.

Abbreviations: ape, atom percent excess; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; OS-ATPase, oligomycin-sensitive ATPase; OSCP, oligomycin sensitivity conferring protein; PEP, phospho(enol)pyruvate; PK, pyruvate kinase

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2. Materials and methods

F_1 was purified from beef-heart mitochondria according to [25]. It had an ATPase activity of approx. 75 units/mg at 25°C. The enzyme was stored at 0°C in 50% saturated ammonium sulfate suspension containing 0.125 M sucrose, 5 mM Tris–SO_4, 1 mM EDTA, 3 mM ATP (pH 7.6). Before use, an aliquot of the suspension was centrifuged, and the sediment was dissolved in 0.25 M sucrose, 10 mM Tris–Ac, pH 7.8.

F_0 was prepared from OS-ATPase by a method worked out [24]. The OS-ATPase used in these experiments consisted of a preparation of 'Complex V' isolated from beef-heart mitochondria according to [22] with minor modifications. To obtain F_0, Complex V was treated with 3.5 M NaBr as described [26]. F_0 was recovered as the membrane fraction appearing as a floating pellet after centrifugation of the NaBr-treated Complex V. It consisted of 5 major polypeptide components, including OSCP, F_6, and the DCCD-binding protein, and about 20% phospholipid [24].

Reconstitution of OS-ATPase was performed in the following manner: F_0 suspended in 0.33 M sucrose, 25 mM Tris–Cl, pH 7.5, and 0.5 mM histidine at a protein concentration of approx. 5 mg/ml was incubated with an aliquot of F_1, dissolved in 0.25 M sucrose and 10 mM Tris acetate, pH 7.8, at a protein concentration of 8 mg/ml at room temperature (~ 25°C). The extent of reconstitution of OS-ATPase was dependent on the time of incubation and the protein ratio F_0/F_1 [24]. In the present work the time of incubation was 4.5–6 h and the F_0/F_1 ratio 5; the extent of reconstitution, expressed as % oligomycin sensitivity, varied between 75 and 80.

ATPase activity was asayed routinely either by determining the amount of P_i liberated according to [27], or by coupling the reaction to the pyruvate kinase and lactate dehydrogenase systems and following the oxidation of NADH spectrophotometrically at 340 nm. In some experiments ATPase activity was assayed by using [γ-^{32}P]ATP as substrate and measuring the amount of $^{32}P_i$ formed by the isobutanol–benzene extraction method [28].

$P_i \rightleftharpoons$ HOH and $P_i \rightleftharpoons$ ATP exchange activities were measured by procedures routinely used in this laboratory [17]. Conditions employed in the individual experiments are specified in the table legend.

3. Results and discussion

The results are summarized in table 1.

Experiment 1 shows that no extra oxygen atoms were incorporated into P_i when the F_1 preparation alone was incubated in the presence of 10 mM ATP and an ATP-regenerating system. This confirms earlier data [19,20].

In exp. 2, the $P_i \rightleftharpoons$ HOH exchange was measured in the presence of the reconstituted F_0F_1, $H^{18}OH$, ATP, and in the absence of an ATP-regenerating system. Under these conditions submitochondrial particles show both intermediate and medium $P_i \rightleftharpoons$ HOH exchange. There was a significant $P_i \rightleftharpoons$ HOH exchange, which was completely abolished by oligomycin but was to a large extent uncoupler-insensitive. F_0 alone exhibited no $P_i \rightleftharpoons$ HOH exchange activity.

Experiment 3 shows that F_0F_1 exhibited a substantial intermediate $P_i \rightleftharpoons$ HOH exchange during hydrolysis of ATP in the presence of an ATP-regenerating system. This exchange was completely uncoupler-insensitive. Also, as shown in exp. 4, the exchange was insensitive to valinomycin + nigericin in the presence of K^+. The concentrations of valinomycin, nigericin and K^+ used in this experiment abolished the oligomycin-induced respiratory control of submitochondrial (EDTA) particles (data not shown).

From the data presented above it may be concluded that the reconstituted F_0F_1 system exhibits an intermediate $P_i \rightleftharpoons$ HOH exchange that is sensitive to oligomycin but insensitive to high concentrations of the uncoupler FCCP and to uncoupling concentrations of valinomycin and nigericin in the presence of K^+. The reconstituted F_0F_1 system exhibited no $P_i \rightleftharpoons$ ATP exchange. Furthermore, although the membrane fraction used here as F_0 does seem to contain an oligomycin- and DCCD-sensitive proton translocator [24], the reconstituted F_0F_1 system has so far not been found capable of generating an ATP-dependent proton gradient when incorporated into liposomes. It would thus appear that this system lacks a functional link needed for the coupling of ATPase activity to proton translocation and probably also for $P_i \rightleftharpoons$ ATP exchange It is conceivable that the $P_i \rightleftharpoons$ ATP exchange requires energy transfer between ATPase molecules, possibly via a transmembrane proton gradient.

The fact that this F_0F_1 system exhibits an oligomycin-sensitive, uncoupler-insensitive intermediate

Table 1
$P_i \rightleftharpoons HOH$ exchange activity of reconstituted oligomycin-sensitive ATPase (F_oF_1)

Exp. no.	Conditions	P_i formed (μmol/min/mg F_1)	Atoms water oxygen incorporated/molecule of P_i formed
1	F_1	75	1.01
2	F_o	0	–
	F_oF_1	7.7	1.14
	F_oF_1	5.3	1.28
	F_oF_1 + 5 μg oligo	0.9	1.00
	F_oF_1 + 0.5 μM FCCP	5.1	1.22
	F_oF_1 + 5 μM FCCP	5.0	1.24
3	F_oF_1	8.1	1.38
	F_oF_1 + 2.5 μM FCCP	7.9	1.37
4	F_oF_1	4.2	1.31
	F_oF_1 + val + nig	4.1	1.37

F_1, F_o and reconstituted F_oF_1 were prepared as described in Materials and methods. The incubation mixtures consisted of the following. Exp. 1: 10 mM ATP, 10 mM $MgAc_2$, 15 mM PEP, 60 mM KAc, 25 mM Tris Ac, pH 7.8, 50 units PK, and 32 μg F_1. Exp. 2: 10 mM ATP, 3 mM ADP, 13 mM $MgAc_2$, 5 mM P_i, 25 mM Tris Ac, pH 7.8, and 120 μg F_oF_1. Exp. 3: 3 mM ATP and 3 mM $MgAc_2$, 15 mM PEP, 60 mM KAc, 25 mM Tris Ac, pH 7.8, 50 units PK, and 234 μg F_oF_1. Exp. 4: 10 mM ATP, 3 mM ADP, 13 mM $MgAc_2$, 5 mM P_i, 100 mM KAc, 25 mM Tris Ac, pH 7.8, and 100 μg F_oF_1. When indicated, FCCP, oligomycin (oligo.), valinomycin (val.) and nigericin (nig.) were added. Final vol. 1 ml. Temp. 30°C. Time of incubation as indicated. The reaction was terminated by the addition of 0.25 ml 25% Trichloroacetic acid and the samples were processed for ^{18}O analysis and calculations as described [17]. ATPase activity was determined in exps 1 and 3 by measuring P_i formed according to [25], and in exps 2 and 4 by using [γ-^{32}P]ATP and measuring the amount of $^{32}P_i$ [28]. All samples were run in duplicates

$P_i \rightleftharpoons HOH$ exchange further substantiates the concept that this exchange reflects a capacity of the ATPase system that is independent of a transmembrane proton gradient. The intermediate $P_i \rightleftharpoons HOH$ exchange may be regarded as resulting from reversal of the hydrolysis of bound ATP to bound ADP and P_i prior to the release of P_i to the medium. The capacity for reversible synthesis of ATP at the catalytic site has been suggested to depend upon a conformational state of the enzyme complex attainable by energy input from oxidation or ATP cleavage [17,18]. The lack of a $P_i \rightleftharpoons ATP$ exchange accompanying the intermediate oxygen exchange is readily explainable by the alternating site mechanism presented [18]. Release of P_i to the medium is accompanied by loss of the conformation that promotes ATP formation. The energization from ATP cleavage is thus lost. Even if bound ATP became labeled from $^{32}P_i$, such [^{32}P]-ATP would be tightly bound and would be released only if ADP and P_i could bind at the alternate catalytic site and be converted to ATP by an energy input. This occurs under conditions allowing $P_i \rightleftharpoons ATP$ exchange or during net synthesis.

The present data seem to provide conclusive evidence for the involvement of F_1 in the oligomycin-sensitive intermediate $P_i \rightleftharpoons HOH$ exchange and may open the way to its further reconstitution by means of isolated components. After the completion of this paper evidence was obtained [29] for an oligomycin-insensitive intermediate $P_i \rightleftharpoons HOH$ exchange catalyzed by soluble F_1 in the absence of F_o. Demonstration of this exchange requires the use of low (≤ 0.5 mM) concentrations of ATP. Possible interpretations and implications of these results are discussed elsewhere [29,30].

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