Occurrence and Significance of Oxygen Exchange Reactions Catalyzed by Mitochondrial Adenosine Triphosphatase Preparations

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The capacity of various ATPase preparations from beef heart mitochondria to catalyze exchange of phosphate oxygens with water has been evaluated. Oligomycin-sensitive ATPase preparations retain a capacity for considerable intermediate \( \text{P_i} + \text{HOH} \) exchange per \( \text{P_i} \), formed during ATP hydrolysis at relatively high ATP concentration (5 mM). Submitochondrial particles prepared by an ammonia-Sephadex procedure with 5 mM ATP showed more rapid ATPase, less oligomycin sensitivity, and less capacity for intermediate exchange. With these particles, intermediate \( \text{P_i} + \text{HOH} \) exchange per \( \text{P_i} \) formed was increased as ATP concentration was decreased.

The purified, soluble ATPase from mitochondria catalyzed little or no intermediate \( \text{P_i} + \text{HOH} \) exchange at 5 mM ATP but showed pronounced increase in capacity for such exchange as ATP concentration was lowered. The ATPase also showed a weak catalysis of an ADP-stimulated medium \( \text{P_i} + \text{HOH} \) exchange. The results support the alternating catalytic site model for ATP synthesis or cleavage. They also demonstrate that a transmembrane protonotive force is not necessary for oxygen exchange reactions. At lower ATP concentrations, ADP and \( \text{P_i} \) formed at a catalytic site appear to remain bound and continue to allow exchange of \( \text{P_i} \) oxygens until ATP binds at another site on the enzyme.

The extent of incorporation of oxygen from water into \( \text{P_i} \) during ATP cleavage or into ATP during ATP synthesis by submitochondrial particles serves as a valuable probe of the reaction rates of bound intermediates. Modulation of the oxygen exchange reactions by the state of energization, presence of uncouplers and inhibitors, or change in substrate concentrations has contributed to development of an alternating catalytic site model for ATP synthesis (1, 2). Although the mechanism of the oxygen exchanges has not been conclusively established, our studies are interpreted on the premise that the exchanges reflect the reversible cleavage of bound ATP to bound ADP and \( \text{P_i} \), at a catalytic site. The premise gains support by observations of ADP stimulation of the exchange (3), lack of exchange of the oxygens of imido-ATP (4), and demonstration with chloroplasts that ATP cleavage accompanies oxygen exchange (5). This premise is also supported by studies with myosin where reversal of bound ATP cleavage has been shown to be sufficiently rapid to account for the oxygen exchange (6, 7). Because of torsional symmetry of the bound \( \text{P_i} \), an oxygen gained from water when ATP is cleaved is not necessarily lost during resynthesis of ATP from bound ADP and \( \text{P_i} \). The measurement of the oxygen incorporation into \( \text{P_i} \), formed during ATP cleavage thus allows estimation of the extent of reversal of bound ATP cleavage prior to release of the \( \text{P_i} \), formed from ATP to the medium.

Early studies with the purified ATPase from beef heart mitochondria showed no appreciable oxygen exchange accompanying ATP cleavage. In contrast, prominent exchange accompanies ATP cleavage by submitochondrial particles (1, 9-11). The present studies were initiated to study exchange reactions with various partially purified ATPase preparations, with the aim of delineating any protein or lipid factors that might be necessary for expression of the oxygen exchange reaction. Some relationships are reported in this paper, including the important finding that at low substrate concentration the ATPase from beef heart mitochondria catalyzes a prominent intermediate \( \text{P_i} + \text{HOH} \) exchange.

EXPERIMENTAL PROCEDURES

Enzyme Preparations and Materials—Soluble ATPase from beef heart mitochondria was isolated by the method of Knowles and Penefsky (12) or of Epstein and Blair (13). Glycerol-treated ATPase was prepared as described by Garrett and Penefsky (14). The ATPase was cold-inactivated (15) by incubating 2 mg in 1 ml of 50 mM Tris-Cl, 2 mM EDTA, and 0.2 M KNO\(_3\) at 4°C and pH 8.0.

Oligomycin-sensitive ATPase was prepared according to Serrano et al. (16) and complex V ATPase as described by Hattei et al. (17). Ammonia-Sephadex particles were prepared as described by Ferguson et al. (18).

Assays—Procedures for measurement of various catalytic activities followed closely those described in earlier publications from this laboratory (1, 2, 19). Neutralized K⁺ salts of P₃, phosphoenolpyruvate, ADP, and ATP were used.

RESULTS

Exchanges with an Oligomycin-sensitive ATPase Preparation—At the onset of these studies, it was deemed important to ascertain if the intermediate \( \text{P_i} + \text{HOH} \) exchange would

1 An unpublished measurement with relatively high ATP concentration was made over 10 years ago by one of the present authors (P. D. B.) using a preparation made available by Professor E. Racker of Cornell University. No oxygen exchange was noted. Penefsky has mentioned similar unpublished results (8).

2 Definitions of and methods of measurement of oxygen exchanges have been given earlier (19). In hydrolysis catalyzed by an ATPase in presence of \( \text{H}_2\text{O} \), a minimum of 1 oxygen atom from water must appear in each \( \text{P_i} \) formed because of the reaction stoichiometry. Additional \(^{18}\text{O}\) may appear by exchange reactions, namely by prior incorporation of water oxygen into medium ATP by ATP = \( \text{HOH} \) exchange, by incorporation of more than 1 atom of water oxygen into...
be manifest in ATPase preparations separated from the bulk of the membrane components. Some results with an oligomycin-sensitive ATPase (16) are given in Table I. Results are given for a 15-min incubation period. Other data showed the reaction was close to linear with time. These and other experiments not reported here show that the preparation retains a definitive capacity for an intermediate $P_i = HOH$ exchange. Addition of phospholipid stimulated the ATPase considerably but did not change the relative amounts of oxygen exchange appreciably. The preparation also catalyzed a weak $P_i = ATP$ exchange (0.8 nmol/min/mg).

Tests were also made with the oligomycin-sensitive ATPase of Hatefi et al. designated as complex V (17). Results in Fig. 1 show the observed time course of the $P_i$ release, the total $P_i = HOH$ and ATP $= HOH$ exchanges, and the $P_i = ATP$ exchange. Some capacity for all the exchange reactions is retained in the complex V preparation, with relative activities much lower but roughly in the same relative proportions as with submitochondrial particles catalyzing ATP.

**Exchanges with Ammonia-Sephadex Particles**—These particles have most of the ATPase inhibitor protein removed, are not capable of oxidative phosphorylation, and have a relatively high ATPase activity (18). It was of interest to check such particles because changes in rate-limiting steps of the catalytic sequence accompanying the high ATPase activity might be reflected by reduced intermediate $P_i = HOH$ exchange. Results presented in Table II show that with 5 mM ATP present the particles catalyze some intermediate $P_i = HOH$ exchange but definitely less than particles competent for oxidative phosphorylation.

Also shown in Table II is another important result, namely that reduction in the ATP concentration results in a marked increase in the amount of intermediate $P_i = HOH$ exchange per $P_i$ formed. A similar effect with particles competent for oxidative phosphorylation was first observed by Russo et al. (11) and confirmed by us.

The ATPase activity of the ammonia-Sephadex particles was increased by about one-third in the presence of the uncoupler "S-13" plus valinomycin or decreased by about one-half in presence of rutamycin. The intermediate exchange per $P_i$ formed with 5 mM ATP was not modified under these conditions.

**Exchanges with Purified ATPase**—The observation mentioned above of increased intermediate exchange by particles as ATP concentration is reduced, and the observation by Hackney and Boyer (2) of markedly increased intermediate $P_i = HOH$ exchange per ATP formed during net oxidative phosphorylation as ADP or $P_i$ concentrations are reduced, prompted measurement of the effect of ATP concentration on intermediate $P_i = HOH$ exchange by the purified ATPase. Results are presented in Fig. 2 that show a definitive increase in the number of water oxygens incorporated per $P_i$ formed as the ATP concentration is decreased.

Tests were also made of the possible ability of the purified ATPase to catalyze a medium $P_i = HOH$ exchange. Results presented in Table III demonstrate that, upon prolonged incubation, some oxygen incorporation from water into $P_i$ is observed and that this incorporation is strongly stimulated by ADP. Other experiments showed the exchange was linear with time. Also, ATPase preparations made by either procedure used (12, 13) showed a similar exchange rate. The stimulation by ADP of water oxygen incorporation into $P_i$ did not result from hydrolysis of ATP formed by contaminating adenylate kinase. The oxygen exchange was not accompanied by an increase in medium $P_i$ concentration nor prevented by addition of hexokinase-glucose to trap any ATP that might be formed. A more marked ADP stimulation of oxygen exchange was observed with a glycerol-extracted ATPase (14); such extraction reduces the content of bound nucleotides and may

### Table I

**Oxygen exchange during ATP cleavage by an oligomycin-sensitive ATPase preparation**

<table>
<thead>
<tr>
<th>Additions</th>
<th>$P_i$ released</th>
<th>Observed $^{18}O$ in $P_i$</th>
<th>Water oxygens present in each $P_i$ formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.0</td>
<td>0.349</td>
<td>1.8</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>5.2</td>
<td>0.440</td>
<td>1.6</td>
</tr>
<tr>
<td>Rutamycin</td>
<td>&lt;0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipid + rutamycin</td>
<td>&lt;0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Partially purified soybean phospholipid (20).*

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**Fig. 1.** ATPase and exchange activities of complex V. The final 0.5 ml reaction mixture at pH 7.5 and 30°C contained 0.3 M sucrose, 50 mM Tris-Cl, 20 mM MgSO$_4$, 15 mM K$^+$/ATP, 15 mM K$^+$/Pi, 3 mg of bovine serum albumin, 125 μg of complex V, and 36 μg of phospholipid in HOH containing 8.11 atom % excess $^{18}O$. For ATPase measurement, $^{32}P$ where $ATP$ (10$^7$ cpm) was present, and for $P_i = ATP$ exchange measurement $^{32}P_i$ (10$^7$ cpm) was present. The ordinate gives the millimolar concentration of the indicated reactions. For the $P_i = HOH$ and ATP $= HOH$ exchanges the values are for the millimolarity of water exchanged and represent the total of intermediate and medium exchanges (see Footnote 2), corrected for approach to isotopic equilibrium.
have other effects on enzyme structure.

As a test of whether the oxygen incorporation might be due to a contaminant or represents a catalytic activity of the ATPase, measurement was made on oxygen exchange capacity after the ATPase was cold-inactivated (15). It seemed unlikely that any contaminant responsible for the exchange would show similar cold inactivation. Preparations of cold-inactivated ATPase showed little or no oxygen exchange capacity when measured under conditions as used for Table III. The possibility that the oxygen incorporation might result from a P1= HOH exchange catalyzed by an enzyme such as propionyl-CoA carboxylase (see Refs. 22 and 23) accompanied by an exchange of water oxygens with CO2 was rendered unlikely by lack of inhibition by avidin. The results thus make it probable that the observed oxygen incorporation represents a weak capacity of the purified ATPase to catalyze an ADP-stimulated medium P1= HOH exchange.

DISCUSSION

The most interesting feature of the results is the finding that the ATPase purified from beef heart mitochondria acquires a capacity for catalysis of intermediate P1= HOH exchange as the ATP concentration is decreased. Such a capacity has important implications for the role of the ATPase as a coupling factor, as noted below. The demonstration of the intermediate exchange also answers one of the questions that served as part of the initial motivation for this series of experiments, namely what minimal structural features of sub-mitochondrial particles are necessary for expression of oxygen exchange capacity? The ability of the ATPase to catalyze exchange obviously shows that other proteins, phospholipids or membrane vesicles, are not required for oxygen exchange to occur. The ATPase itself suffices.

This behavior of the ATPase can be accounted for by participation of alternating catalytic sites on the ATPase (1, 27). In a model for oxidative phosphorylation and photophosphorylation based on participation of alternating catalytic sites (1, 2, 28), an energy-linked conformational change promotes ADP and P1 binding at one site in a mode competent for bound ATP formation and favors release of a tightly bound ATP from another site. A scheme for one-half of a complete reaction cycle, adapted from Hackney and Boyer (2), is given in Fig. 3; reaction proceeds from top left for ATP formation and from bottom left for ATP hydrolysis, with branched pathways of substrate release or reaction on each side of the key conformational change. The forms ATP>E and E<ATP have identical structural and kinetic properties, but in conversion of one form to the other the tightly bound ATP is found on a different catalytic site. Completion of a reaction cycle, one-half of which is depicted in Fig. 3, would regenerate the original enzyme form. Structural studies have indicated that F1 might contain three catalytic sites. Alternation between two or among three catalytic sites is plausible (1).

At low ATP concentration, the initial cleavage of ATP would take place with a tightly bound ATP molecule in Step 6. With low ATP concentration in the medium, reversal of Step 5 would lead to exchange of oxygens of the bound P1, giving rise to intermediate P1= HOH exchange in the continued net P1 formation. At high medium ATP concentrations, reversal of hydrolysis of bound ATP in Step 5 would be prevented because of a more rapid release of P1 that shows little or no oxygen exchange.

The scheme of Fig. 3 also readily accounts for another observation of interest, namely the difference shown by various ATPase preparations in capacity for intermediate P1= HOH exchange when excess ATP is present. For discussion,
suggestion of Mitchell (30) that oxygen removal is a direct how proton motive force may be coupled to ATP formation.

The initial Pi formed by ATP cleavage remains tightly bound wisely, during either synthesis or cleavage, energy would be lost. The conformational transitions of Step 3 (Fig. 3) do not occur unless both catalytic sites are occupied. Other-

phosphorylation. One is that for coupling to occur, it is im-

consequences for suggested coupling mechanisms in oxidative

Pi + HOH exchange at low ATP concentrations has important

Steps 4 and 5 or 5' and result in continued intermediate Pi blocked by oligomycin. In particles competent for ATP synth-

plex to proton translocation (29), a sequence that can be summarized in Table IV. At these relatively high ATP con-

centrations, the purified ATPase shows very little or no ex-

change capacity, the ATPase on the ammonia-Sepha-

dex particles slightly more, and the oligomycin-sensitive ATPases and the submitochondrial particles show considerably more exchange activity. Conformational changes in Step 3 are regarded as being linked through proteins of the ATPase complex to proton translocation (29), a sequence that can be blocked by oligomycin. In particles competent for ATP syn-

thesis and in oligomycin-sensitive ATPase preparations, rate limitation in Step 3 would allow back up of reactions through Steps 4 and 5 or 5' and result in continued intermediate Pi = HOH exchange. With the purified ATPase such rate limit-

ation is absent, ATP hydrolysis is much more rapid, and little or no intermediate exchange occurs.

The capacity of the purified ATPase to give intermediate Pi = HOH exchange at low ATP concentrations has important consequences for suggested coupling mechanisms in oxidative phosphorylation. One is that for coupling to occur, it is im-

perative that the conformational transitions of Step 3 (Fig. 3) do not occur unless both catalytic sites are occupied. Other-

wise, during either synthesis or cleavage, energy would be lost. The initial Pi formed by ATP cleavage remains tightly bound until another ATP molecule binds. Release of this tightly bound Pi, may be regarded as being driven by the confor-

mational changes resulting when the loosely bound ATP forms a tightly bound ATP.

A second consequence of the capacity of the purified ATP-

ase to catalyze intermediate Pi = HOH exchange concerns how proton motive force may be coupled to ATP formation. Such exchange capacity gives additional evidence against the suggestion of Mitchell (30) that oxygen removal is a direct conse-

quence of protonation of the Pi and favors the confor-

mational coupling suggestion of Boyer (29).

It must be emphasized that the preceding explanations, although attractive, are not proven. As noted in the introduc-
tion to this paper, considerable evidence favors the view that the oxygen exchanges result from dynamic reversal of cleavage of bound ATP to bound ADP and P0, but alternative mecha-

isms of exchange remain possible. It does appear that, even if alternative mechanisms exist, their operation reflects reten-
tion of the competency to form bound ATP from bound ADP and P0. In addition, mechanisms other than alternating cata-
lactic sites could explain the increases in the oxygen exchange accompanying ATP cleavage and ATP synthesis at low sub-

strate concentration. As considered in an earlier publication, in which markedly increased intermediate ATP = HOH exchange per ATP formed was observed at low ADP or P0 concentration (2), explanations based on enzyme hysteresis, enzyme heterogeneity, or on control sites for ATP, ADP, and P0 seem less likely. Such possibilities are currently being investigated. It will be necessary for any mechanism proposed for oxidative phosphorylation to account for substrate modula-
tion of oxygen exchanges during ATP hydrolysis or cleavage. The findings in this paper are in harmony with other recent data from this laboratory demonstrating that catalysis of oxygen exchange occurred in a reconstituted ATPase prepara-
tion with oligomycin sensitivity but no capacity for Pi = ATP exchange (31). Exchange between medium P0 and medi-

um ATP is most readily explained if energy transfer between ATPase complexes can occur. Retention of energy gained

a With ATP regenerating system present.

b Data for 15-min sample (Table I).

c Average values for four experiments.
from ATP cleavage within one ATPase complex might allow reversal of the segment of the phosphorylation sequence depicted in Fig. 3. Such reversal, however, would only label tightly bound ATP with medium Pi. For exchange with medium ATP, reversal of an additional segment of the sequence would be necessary and, in the absence of energy from oxidations, this would require energy input from cleavage of ATP by another ATPase complex. Possibilities are energy transfer between ATPases, as suggested by evidence of energy domains of ATPase complexes (32), or energy transfer by creation of protonmotive force (33) in vesicles.

The finding of an apparent, weak capacity of the purified ATPase to catalyze an ADP-stimulated medium Pi + HOH exchange, in terms of the scheme of Fig. 3, would require transient formation of some tightly bound ATP. This would be analogous to myosin, which has been shown to catalyze a medium Pi + HOH exchange accompanied by formation of tightly bound ATP from 32Pi (34). However, it must be emphasized that the capacity of the purified mitochondrial ATPase to catalyze medium Pi = HOH exchange is far less than that of competent submitochondrial particles; we estimate the rate as roughly 1/1000 of the rate on particles. Preliminary experiments did not show presence of detectable [32P]ATP formation from 32Pi with the purified ATPase; any ATP present was considerably less than the relative amount observed with myosin (34). The lack of detection of bound ATP formation on the F1-ATPase may reflect the slow rate of oxygen exchange.

REFERENCES
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