

A New Concept for Energy Coupling in Oxidative Phosphorylation Based on a Molecular Explanation of the Oxygen Exchange Reactions

(protein conformational change/uncouplers/mitochondria)

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ABSTRACT The $P_i \rightleftharpoons \text{HOH}$ exchange reaction of oxidative phosphorylation is considerably less sensitive to uncouplers than the $P_i \rightleftharpoons \text{ATP}$ and $\text{ATP} \rightleftharpoons \text{HOH}$ exchanges. The uncoupler-insensitive $P_i \rightleftharpoons \text{HOH}$ exchange is inhibited by oligomycin. These results and other considerations suggest that the relatively rapid and uncoupler-insensitive $P_i \rightleftharpoons \text{HOH}$ exchange results from a rapid, reversible hydrolysis of a tightly but noncovalently bound ATP at a catalytic site for oxidative phosphorylation, concomitant with interchange of medium and bound P_i . Such tightly bound ATP has been demonstrated in sub-mitochondrial particles in the presence of uncouplers, P_i , and ADP, by rapid labeling from $^{32}\text{P}_i$ under essentially steady-state phosphorylation conditions. These results lead to the working hypothesis that in oxidative phosphorylation energy from electron transport causes release of preformed ATP from the catalytic site. This release could logically involve energy-requiring protein conformational change.

A basic premise in most studies of oxidative phosphorylation has been that energy from oxidations serves to form a "high-energy" precursor to ATP or in some manner to cause formation of the terminal covalent anhydride bond in ATP. The purpose of this communication is to present experimental evidence for a distinctly different mode of energy input for net ATP synthesis, namely that energy is used to bring about release of a preformed, noncovalently bound ATP from the catalytic site.

The experimental evidence for this new concept and for a molecular explanation of the ^{18}O exchanges is of two principal types. One is the measurement of the effect of uncouplers of oxidative phosphorylation on the relative rates of ^{32}P and ^{18}O exchanges characteristic of oxidative phosphorylation. The observation of a $P_i \rightleftharpoons \text{HOH}$ exchange not blocked by uncouplers of oxidative phosphorylation but inhibited by oligomycin predicts that ATP might be formed at a catalytic site even though sufficient energy for net ATP synthesis is not available. The second type of evidence is the detection of such firmly bound ATP and the characteristics of its labeling by $^{32}\text{P}_i$.

The discovery of the ability of mitochondria to catalyze a rapid exchange of oxygens of inorganic orthophosphate (P_i) with water ($P_i \rightleftharpoons \text{HOH}$ exchange) (1) prompted experiments demonstrating the $P_i \rightleftharpoons \text{ATP}$ exchange (2, 3) and the $\text{ATP} \rightleftharpoons \text{HOH}$ exchange capacities of mitochondria (2, 4, 5). Every ATP molecule formed in net oxidative phosphorylation shows extensive incorporation of water oxygens into the γ -phosphoryl

group (4, 5). Any mechanism proposed for oxidative phosphorylation must account for this oxygen exchange. Suggestions have included a dynamic reversal of the overall process of oxidative phosphorylation (2, 6), the reversible formation by addition of water of pentavalent phosphorus intermediates (7, 8), or the exchange in an unspecified manner of a phosphorylated intermediate of oxidative phosphorylation (9).

Previous findings have shown that the $P_i \rightleftharpoons \text{HOH}$ exchange is less sensitive to 2,4-dinitrophenol than the $P_i \rightleftharpoons \text{ATP}$ exchange or the capacity for net oxidative phosphorylation (10-12). However, the significance or the source of this exchange has not been known. The possibility exists that it might reflect activities of enzymes such as alkaline phosphatase or pyrophosphatase known to catalyze a $P_i \rightleftharpoons \text{HOH}$ exchange (7), perhaps activated in some manner by 2,4-dinitrophenol. In addition, whether such behavior is limited to 2,4-dinitrophenol or might be shown by more potent uncouplers of oxidative phosphorylation has not been shown. Results with other uncouplers and with oligomycin inhibition reported here cover these points.

The effects of increasing concentrations of the potent uncoupler 5-chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide (S-13) (13) on the exchanges catalyzed by mitochondria are shown in Fig. 1. In the absence of uncoupler, the relative rates of the reactions $P_i \rightleftharpoons \text{HOH}$, $\text{ATP} \rightleftharpoons \text{HOH}$, and $P_i \rightleftharpoons \text{ATP}$ are about 12:6:1, respectively, under the conditions used. At low concentrations of uncoupler, the $P_i \rightleftharpoons \text{ATP}$ and the $\text{ATP} \rightleftharpoons \text{HOH}$ exchanges are much more sensitive to the uncoupler than the $P_i \rightleftharpoons \text{HOH}$ exchange. At a concentration of S-13 sufficient to inhibit the $P_i \rightleftharpoons \text{ATP}$ and $\text{ATP} \rightleftharpoons \text{HOH}$ exchange by about 50%, the $P_i \rightleftharpoons \text{HOH}$ exchange is inhibited by less than 5%. At a concentration S-13 that gives a near zero value for the $P_i \rightleftharpoons \text{ATP}$ and $\text{ATP} \rightleftharpoons \text{HOH}$ exchanges and a maximum value for the uncoupler-stimulated ATPase activity, the $P_i \rightleftharpoons \text{HOH}$ exchange is still rapid and inhibited by only 35%. Responses similar to those reported in Fig. 1 for S-13 with mitochondria are also observed with 2,4-dinitrophenol and *m*-chlorocarbonylcyanide phenylhydrazine.

Important for the present considerations are the demonstrations, not given in detail here, of the effects of oligomycin. This antibiotic is a potent inhibitor of oxidative phosphorylation and inhibits the $P_i \rightleftharpoons \text{HOH}$ exchange (14). In the absence of uncouplers and under conditions like those described with Fig. 1, the presence of 0.1 μg of oligomycin per mg of protein nearly completely inhibits all exchanges, with smaller concentrations showing a greater proportional inhibition of the $P_i \rightleftharpoons \text{HOH}$ exchange. The uncoupler-insensitive $P_i \rightleftharpoons \text{HOH}$ exchange is similarly blocked by oligomycin.

Abbreviation: S-13, 5-chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide.

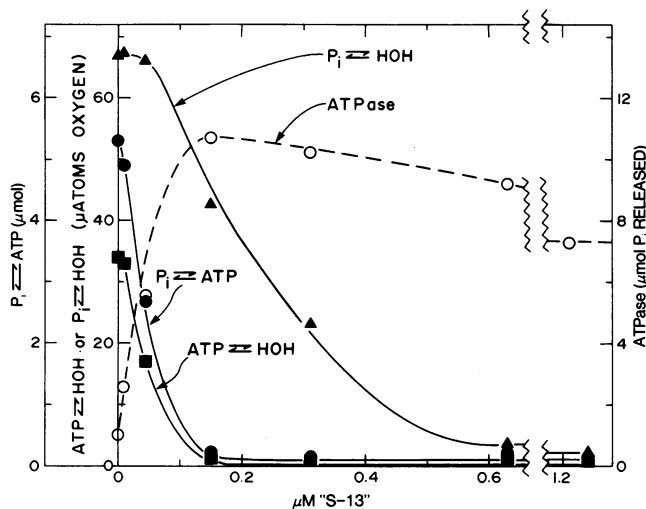
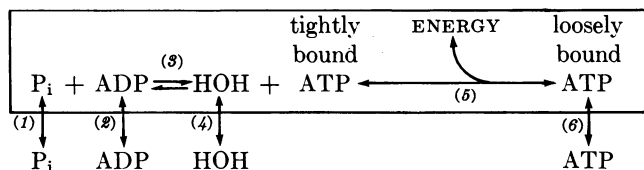


FIG. 1. Rat-liver mitochondria were incubated for 5 min in 2.0 ml total volume at pH 7.4 and 37° with 5 mM $^{32}\text{P}_i$ (equivalent to 10^6 cpm), 5 mM ATP, 5 mM MgCl_2 , 40 mM KCl, and 40 mM Tris-chloride, with 0.98 atom % of excess ^{18}O in the water of the medium and concentrations of S-13 as indicated. Protein concentration was 4 mg/ml, as determined by the Lowry procedure with bovine-serum albumin as a standard. Reactions were stopped by addition of perchloric acid and analyses were made as described (10).

Other studies in this laboratory have shown that comparative rates of isotope exchange can serve as powerful tools for probing the event occurring at catalytic sites (15, 16). Such studies, together with the requirement of ADP for the $\text{P}_i \rightleftharpoons \text{HOH}$ exchange (17), suggest that the exchange in the presence of uncouplers could be explained by a continued dynamic reversal of ATP formation limited to the catalytic site. Lack of the $\text{P}_i \rightleftharpoons \text{ATP}$ and $\text{ATP} \rightleftharpoons \text{HOH}$ exchanges in the presence of uncouplers would be explained if there exists an energy requirement for the release of the bound ATP. This concept may be diagrammed simply as follows:



Steps occurring at the catalytic site are depicted within the rectangle. Rapid interchange of water with the site in step 4 seems logical and has some support from the demonstration that the ^{18}O from labeled P_i readily appears in water of the medium (18). Dynamic reversal of steps 1 and 3 in the presence of uncouplers would thus suffice for the $\text{P}_i \rightleftharpoons \text{HOH}$ exchange. Inhibition by uncouplers of an energy-driven release of ATP by way of steps 5 and 6 would not inhibit the $\text{P}_i \rightleftharpoons \text{HOH}$ exchange but would block the $\text{P}_i \rightleftharpoons \text{ATP}$ and $\text{ATP} \rightleftharpoons \text{HOH}$ exchanges.

For recognition of the simple explanation outlined above we had to overcome a limitation in our own thinking, namely the supposition that in net oxidative phosphorylation, as in substrate-level phosphorylation, use of energy was limited to events occurring before or during but not after the formation of each ATP molecule.

An important prediction of the concept outlined above is that submitochondrial particles under oxidative phosphoryla-

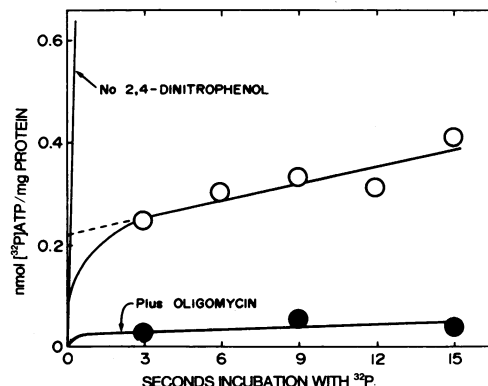


FIG. 2. Aliquots of 0.25 ml each containing 0.25 M sucrose, 10 mM Tris-chloride (pH 7.5), and submitochondrial particles equivalent to 5.0 mg of protein were brought to 30°. Additions made in small volumes gave concentrations of 12 mM succinate at 0 sec; 10.2 mM P_i , 2.0 mM ADP, 0.5 mM ATP, 3.3 mM MgCl_2 , and 102 μM 2,4-dinitrophenol at 30 sec; and 9.0×10^7 cpm of $^{32}\text{P}_i$ at 60 sec. After incubation with $^{32}\text{P}_i$ for the times indicated, reaction was stopped by addition of 1 ml of cold 0.375 M HClO_4 containing 12.5 mM P_i , 12.5 mM PP_i , and 12.5 mM ATP. Precipitated protein was removed by centrifugation at 0°. Variations in this sequence included addition of 3 μg of oligomycin per mg of protein 15 sec before succinate or omission of dinitrophenol from the 30-sec addition. In the blank reaction, $^{32}\text{P}_i$ and the acid quench were added together at 60 sec. Phosphate was extracted from an aliquot of the supernatant as its acid molybdate complex by five washes with two volumes of isobutanol-benzene (1:1, v/v) at 0°. An aliquot of the aqueous phase was counted as a measure of ^{32}P ATP, with correction for the blank (23% of the 6-sec value).

tion conditions but in the presence of uncouplers should have a small amount of bound ATP present that rapidly equilibrates with P_i but not with ATP of the medium. An indication that such bound ATP might be present comes from the important but previously unexplained observations of Eisenhardt and Rosenthal (19). They found that even in the presence of uncoupling concentrations of 2,4-dinitrophenol, a small "jump" in total amount of ^{32}P ATP present occurred upon addition of ADP to mitochondria in the presence of $^{32}\text{P}_i$, substrate, and O_2 . This "jump" could have resulted from a shift of the equilibrium of step 3 upon ADP addition.

Experiments that demonstrate detection of a rapidly labeled, bound ATP are shown in Fig. 2. In these experiments, submitochondrial particles are in a near steady-state condition with ADP, substrate, and P_i present, and with sufficient 2,4-dinitrophenol present to stop net oxidative phosphorylation. Upon addition of a trace of $^{32}\text{P}_i$, a rapid appearance of about 0.2 nmol of ^{32}P ATP per mg of protein occurs, followed by a much slower continued ^{32}P incorporation into ATP. A similar result has been observed in 36 other experiments, in about half of which no upward slope (Fig. 2), likely due to very low $\text{P}_i \rightleftharpoons \text{ATP}$ exchange activity, was observed.

Conditions of the experiment as reported in Fig. 2 require that the rapid incorporation of $^{32}\text{P}_i$ into ATP occurs by exchange and not by net synthesis, that is, as a result of rapid reversible formation of a small steady-state level of ^{32}P ATP. As noted in Fig. 2, presence of a low concentration of oligomycin blocks the labeling, a clear indication that the reaction responsible is associated with oxidative phosphorylation.

That the small amount of rapidly labeled ATP is protein-bound is clearly indicated by the limited appearance of [^{32}P]-ATP after $^{32}\text{P}_i$ addition under near steady-state conditions.* The facile release of [^{32}P]-ATP upon addition of cold perchloric acid (see legend of Fig. 2) indicates noncovalent binding. That the released ^{32}P -labeled compound was ATP was shown by its identity with authentic ATP in acid hydrolysis, cochromatography on anion exchange resin, and susceptibility to hexokinase.

Previous experiments have shown that the $\text{P}_i \rightleftharpoons \text{HOH}$ exchange rate increases considerably with increase in P_i concentrations above 10 mM, the concentration used for experiments of Fig. 2. If the bound ATP is an intermediate in the $\text{P}_i \rightleftharpoons \text{HOH}$ exchange, increase in P_i concentration above 10 mM would be expected to give further increase in the bound ATP. This prediction has been verified; with 50 mM $^{32}\text{P}_i$ present, the apparent bound ATP was increased over 4-fold above that observed with 10 mM P_i .

Our findings indicate that a prominent function of energy in oxidative phosphorylation is to cause release of preformed ATP. This could be the only step for energy input. Also, the possibility of energy input at more than one step merits consideration. Mitochondria can apparently carry out processes with energy requirements less than equivalent to the hydrolysis of ATP. For example, energy-linked reduction of TPN by DPNH can still be driven by oxidation with concentrations of 2,4-dinitrophenol present that stop net oxidative phosphorylation (21), and reversal of energy-linked reduction can drive ion transport (22). Also, in preliminary experiments we have noted that the level of bound ATP in and the rate of the $\text{P}_i \rightleftharpoons \text{HOH}$ exchange catalyzed by submitochondrial particles in the presence of 2,4-dinitrophenol is decreased by lack of substrate and ATP in the medium. These shifts could reflect energy needs for maintaining integrity of the catalytic capacity, i.e., a "housekeeping" function, or energy input favoring formation of the bound ATP.

An explanation is desirable for how ATP may be formed by reversal of hydrolysis at the catalytic site with limited or perhaps even no energy input. Factors that may contribute include the existence or the formation of a tight-binding capacity for ATP, a higher effective concentration of ADP and P_i , or a reduced water activity at the catalytic site.

The simplest, and on the basis of present information the most likely, way to bring about release of a preformed ATP from a tight, noncovalent binding site is by protein conformational change. Additional impetus for consideration of conformational coupling in ATP synthesis comes from recent findings with actomyosin ATPase. These show rapid formation of appreciable amounts of a tightly bound [^{32}P]-ATP from ADP and $^{32}\text{P}_i$ †, and offer an explanation for the "intermediate" and "medium" exchanges of water oxygens with phosphate oxygens. These results, together with other data (23, 24), suggest that the principal energy-yielding step in

muscle contraction accompanies the binding of ATP, with hydrolysis serving for release of ADP and P_i . Nature thus appears to use a similar mechanism in contractility and in oxidative phosphorylation, with energy release or input accompanying binding or release of ATP.

A more detailed consideration of conformational coupling in oxidative phosphorylation and in other energy-linked transducing processes is given elsewhere (25). A conformationally interlinked matrix of proteins within the membrane provides an attractive hypothesis for capture, transmission, and use of energy in oxidative and photosynthetic phosphorylation, active transport, and energy-linked reduction. An interesting possibility is that in conformational coupling, energy use and donation to a membrane matrix may be in amounts considerably less than that equivalent to hydrolysis of one ATP molecule.

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* It is not known whether any relationship exists between the bound ATP present under steady-state phosphorylation conditions and the occurrence of a tight binding site for ATP in mitochondrial proteins precipitated by perchloric acid (20).

† Wolcott, R. W. & Boyer, P. D., unpublished observations.