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On the Mechanism of ATP Synthesis in Oxidative Phosphorylation: A REVIEW

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It is shown that the presently available evidence supports the existence of two entry points for water oxygen in the mitochondrial oxygen exchanges. This in turn provides support for the pseudorotation reaction mechanism of ATP synthesis which is the only mechanism of ATP synthesis proposed to date allowing for two entry points of water oxygen. It is also shown that the pseudorotation mechanism can resolve the apparent paradox of a $P_i \rightleftharpoons H_2O$ exchange separate from the reversal of phosphorylation yet dependent on the mechanism of P_i activation. In addition an interpretation consistent with the experimental observations concerning As_i-induced stimulation of respiration and effects associated with oligomycin and aurovertin is shown to follow from the analysis of the oxygen exchanges. Implications of the pseudorotation mechanism for the mechanism of energy coupling in oxidative phosphorylation are discussed.

INTRODUCTION

It has been recognized for many years that any mechanism of ATP synthesis in oxidative phosphorylation must involve a mechanism of P_i activation. However, there has not yet emerged any general agreement as to what the mechanism of P_i activation is. The most widely accepted paradigm has been that P_i activation occurs by the formation of some phosphorylated intermediate (*1*). Another proposal for P_i activation has been the formation of metaphosphate from P_i (*2*).

One might hope to define more precisely the mechanism of P_i activation by considering the various phenomena associated with ATP synthesis in oxidative phosphorylation. For example, the mitochondrial ATP synthetase catalyzes at least two and perhaps three oxygen exchanges: (1) $P_i \rightleftharpoons H_2O$ (*3*), (2) ATP $\rightleftharpoons H_2O$ (*4*) and (3) $As_i \rightleftharpoons H_2O$ (*5*), as measured by the transfer of ^{18}O label.[3] Although the $As_i \rightleftharpoons H_2O$ exchange is catalyzed both by mitochondria and submitochondrial particles (*6*), it has not been conclusively established that this exchange is catalyzed by the mitochondrial ATP synthetase itself. It seems likely that the pathway of oxygen in the $P_i \rightleftharpoons H_2O$, ATP $\rightleftharpoons H_2O$, and perhaps the $As_i \rightleftharpoons H_2O$ exchange as well, is the same as in ATP synthesis. In addition to the oxygen exchanges there are a number of phenomena

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[3] Abbreviation: As_i, inorganic arsenate.

associated with the antibiotics oligomycin and aurovertin that impose rather restrictive conditions on acceptable models of ATP synthesis (*7*).

The sum total of the various experimental observations related to ATP synthesis implicitly defines a restricted class of ATP synthesis mechanisms. It is therefore important to critically evaluate the conditions imposed by the various observations in order to make as explicit as possible the nature of these restrictive conditions. This is especially important in view of the widespread uncritical acceptance of paradigms which enjoy neither a solid experimental foundation nor a firm chemical basis.

In the present paper we will attempt to do four things: We will first show that the presently available oxygen exchange data are inconsistent with all of the ATP synthesis schemes proposed to date with the exception of the pseudorotation reaction mechanism proposed by Korman and McLick (*8–10*). Secondly, it will be shown that the pseudorotation mechanism can readily rationalize the previously unresolved problem of how a significant $P_i \rightleftharpoons H_2O$ exchange can occur by a mechanism separate from the reversal of phosphorylation yet still depend on the mechanism of P_i activation. Thirdly, it will be shown that the experimental results with the inhibitors oligomycin and aurovertin are inconsistent with all of the explanations proposed to date for their mode of action, but that the pseudorotation mechanism offers the possibility of explanations that at least meet the minimal requirement of consistency with the experimental data. Finally, some of the implications of the pseudorotation reaction mechanism for the mechanism of energy coupling in oxidative phosphorylation will be discussed.

OXYGEN EXCHANGES

A number of reaction schemes have been proposed for rationalizing the characteristics of the mitochondrial oxygen exchanges. Rather than considering each of the existing proposals individually, it is more instructive to consider a very general scheme (Fig. 1) proposed by Boyer (*2, 11*). In this scheme there are two conformations of the

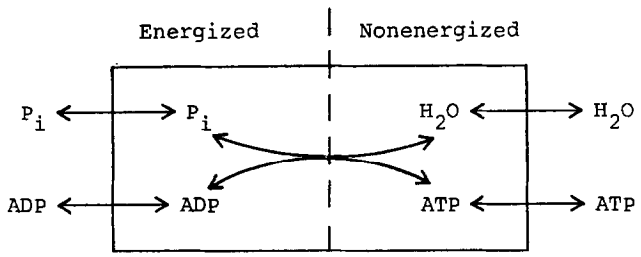

FIG. 1. Boyer's phenomenological scheme depicting transformation of reactants and products in ATP synthesis. This scheme involves only a single mode of entry of water oxygen.

ATP synthetase corresponding to the energized and nonenergized states of the mitochondrion. In the energized state bound ADP and P_i can exchange with free ADP and P_i, and in the nonenergized state bound ATP and H_2O can exchange with free ATP and H_2O. By dynamic reversal of certain steps in this scheme, including the phosphorylation step of oxidative phosphorylation, the ATP $\rightleftharpoons$ ADP, ATP $\rightleftharpoons$ P_i, ATP $\rightleftharpoons$

H_2O, and $P_i \rightleftharpoons H_2O$ exchanges can be rationalized, at least in a schematic, non-mechanistic manner. This scheme also provides a rationalization for the catalytic requirement of the $P_i \rightleftharpoons H_2O$ exchange for energy, which can be satisfied either by electron transfer or ATP hydrolysis (*12*, *13*), as well as the requirement for ADP in the $P_i \rightleftharpoons H_2O$ exchange (*14–16*).

The utility of this scheme is its great generality which derives from the fact that it involves only the reversible binding of reactants and products to the enzyme. This scheme makes no assumptions whatever about the mechanism by which reactants are transformed into products or how many steps are involved. Thus Boyer's scheme could accommodate mechanisms of P_i activation involving either the generation of a phosphorylated intermediate, or activation simply by the binding of P_i to the enzyme in the proper orientation and conformation. By placing the expulsion of H_2O before the reversible phosphroylation reaction, it could also accommodate a mechanism of P_i activation involving generation of metaphosphate. However, as noted by Boyer (*2*), this scheme involves only a single mode of entry of water oxygen. This appears to be its only restrictive assumption.

Mitchell, Hill and Boyer (*14*) measured the rates of the $P_i \rightleftharpoons H_2O$, ATP $\rightleftharpoons H_2O$, and ATP $\rightleftharpoons P_i$ exchanges in submitochondrial particles and found them to be in the ratio 50:13:1, respectively, under high ATP to ADP conditions. These authors point out that the high rates of both the $P_i \rightleftharpoons H_2O$ and the ATP $\rightleftharpoons H_2O$ exchanges relative to the ATP $\rightleftharpoons P_i$ exchange *cannot* be rationalized by Boyer's scheme. They concluded that either there is more than a single entry point of water oxygen or that much of the $P_i \rightleftharpoons H_2O$ exchange occurs by a reaction separate from the reversible phosphorylation reaction of oxidative phosphorylation. It should be noted, however, that these are *not* mutually exclusive alternatives. On the basis of difierences in the ATP $\rightleftharpoons H_2O$ and $P_i \rightleftharpoons H_2O$ exchanges other than the differences in relative rates, these authors suggested that most of the $P_i \rightleftharpoons H_2O$ exchange occurs by a reaction *separate* from the reversible phosphorylation reaction. However, they point out that the basic P_i activation of oxidative phosphorylation could nevertheless be involved. Although this proposal may appear rather paradoxical, it provides a phenomenological rationalization of their experimental results. However, it provides no basis for correlating the suggested involvement of the P_i activation mechanism and the separate character of the $P_i \rightleftharpoons H_2O$ exchange.

In addition to the evidence reported by Mitchell, Hill and Boyer (*14*) there is further evidence which supports the separate character of much of the $P_i \rightleftharpoons H_2O$ exchange. At low levels (0.2 μg/mg protein) oligomycin stimulates oxidative phosphorylation, reversed electron transfer, energy linked transhydrogenation, and ATP $\rightleftharpoons P_i$ exchange in certain submitochondrial particles (*17*). Although oligomycin at this low level enhances the ATP $\rightleftharpoons P_i$ exchange, it inhibits the $P_i \rightleftharpoons H_2O$ exchange (*15*). The simultaneous inhibition of the $P_i \rightleftharpoons H_2O$ exchange and enhancement of the reversible phosphorylation reaction lends strong support to the conclusion of Mitchell, Hill and Boyer (*14*) that most of the $P_i \rightleftharpoons H_2O$ exchange occurs by a reaction separate from the reversal of phosphorylation.

The stimulation of the $P_i \rightleftharpoons H_2O$ exchange by ADP (*14–16*) and the stimulation of the ATP $\rightleftharpoons H_2O$ exchange by ADP and P_i (*14*, *15*) might be interpreted as providing support for the scheme depicted in Fig. 1, in which both exchanges depend upon the

reversal of phosphorylation. However, a critical evaluation of the data shows this interpretation to be unlikely.

First of all, the apparent separateness of much of the $P_i \rightleftharpoons H_2O$ exchange is inconsistent with this exchange requiring the reversal of phosphorylation. Jones and Boyer (*16*) ignored the separateness of the $P_i \rightleftharpoons H_2O$ exchange and favored the involvement of ADP as a substrate in the reversible phosphorylation reaction as the basis for the apparent absolute requirement for ADP in the $P_i \rightleftharpoons H_2O$ exchange. However, these authors also recognized that ADP might act as an allosteric effector or that ADP might stabilize a pentacoordinate phosphorus intermediate in the $P_i \rightleftharpoons H_2O$ exchange. Both of these suggestions are consistent with a $P_i \rightleftharpoons H_2O$ exchange separate from the reversible phosphorylation reaction.

Concerning the former suggestion Huang and Mitchell (*18*) have recently reported that the inhibition by P_i and As_i of the ATP-dependent NAD^+ reduction is ADP-dependent. The inhibition by ADP is *noncompetitive* with ATP. Since both ATP and ADP must bind to the active site, this indicates the existence of a separate site to which ADP but not ATP can bind. This site is highly specific for ADP since IDP, UDP, CDP, GDP and the α,β-methylenephosphonate analog of ADP have no inhibitory effect at a concentration of 2 m*M* (*18*). The existence of a separate site for ADP on the mitochondrial ATPase more specific for ADP than the catalytic site has recently been directly confirmed by the binding studies of Hilborn and Hammes (*19*). This result is consistent with the suggestion of Jones and Boyer (*16*) that ADP might act as an allosteric effector. It is not apparent how a specific effector site for ADP would be consistent with the report of Mitchell, Hill and Boyer (*14*) that ATP can also meet the nucleotide requirement for the $P_i \rightleftharpoons H_2O$ exchange. It should be noted, however, that even if ATP could meet the nucleotide requirement for the $P_i \rightleftharpoons H_2O$ exchange, this would provide no support for the scheme depicted in Fig. 1.

Let us now consider the $ATP \rightleftharpoons H_2O$ exchange and its stimulation by P_i (*15*). If one assumes stereospecific tight binding of substrates to the enzyme during the course of a reaction, then the scheme depicted in Fig. 1 can rationalize the $ATP \rightleftharpoons H_2O$ exchange only as the sum of a $P_i \rightleftharpoons H_2O$ exchange and an $ATP \rightleftharpoons P_i$ exchange. However, as already noted by Chan, Lehninger and Enns (*13*) the rapid $ATP \rightleftharpoons H_2O$ exchange *cannot* be rationalized as the sum of a $P_i \rightleftharpoons H_2O$ and an $ATP \rightleftharpoons P_i$ exchange. If one relaxes the constraint of stereospecific tight binding and allows for a rotation of partially bound P_i in the active site, then the scheme depicted in Fig. 1 would yield an $ATP \rightleftharpoons H_2O$ exchange without the need for P_i coming on and off the enzyme. It should be noted, however, that even if such a rotation were to occur, it would not allow one to rationalize the rapid rates of both the $P_i \rightleftharpoons H_2O$ and $ATP \rightleftharpoons H_2O$ exchanges relative to that of the $ATP \rightleftharpoons P_i$ exchange. Furthermore, the rotation of partially bound P_i in the active site would not be directly affected by the solution concentration of P_i. One must therefore conclude that the scheme depicted in Fig. 1 does not provide an adequate rationalization either for the relative rate of the $ATP \rightleftharpoons H_2O$ exchange or for its stimulation by P_i.

It might be noted here that Mitchell and Moyle (*20*) have reported that P_i stimulates the ATPase activity of sonic particles in a manner that is *noncompetitive* with ATP. This indicates the existence of a site other than the active site to which P_i but not ATP can bind and suggests that P_i may be an allosteric effector for the $ATP \rightleftharpoons H_2O$ exchange.

In view of the two binding sites for ADP (*19*) a similar interpretation seems plausible for the stimulation of the ATP $\rightleftharpoons$ H_2O exchange by ADP (*14*, *15*).

Since the only restrictive assumption in the scheme depicted in Fig. 1 appears to be the single entry point of water oxygen, the inadequacy of this scheme for rationalizing the characteristics of the ATP $\rightleftharpoons$ H_2O and P_i $\rightleftharpoons$ H_2O exchanges clearly suggests that there is more than one entry point of water oxygen. One is therefore led to ask whether allowing for more than one entry point of water oxygen could provide a basis for correlating the apparent separateness of much of the P_i $\rightleftharpoons$ H_2O exchange and the involvement of P_i activation. This would clarify the basis of the apparently paradoxical proposal of Mitchell, Hill and Boyer (*14*) concerning the P_i $\rightleftharpoons$ H_2O exchange.

Korman and McLick (*8–10*) have recently proposed a pseudorotation reaction mechanism for ATP synthesis in oxidative phosphorylation. Two essential features of this mechanism are: (1) The mechanism of P_i activation is the binding of P_i to the enzyme by way of hydrogen bonds and metal ion coordination all in the proper orientation and conformation, and (2) two oxoniums formed by protonation, when acting together as a *pair*, permit pseudorotation, thereby effecting the equatorial capture of ADP in ATP synthesis. The pair of oxoniums, which constitute the two entry points of water oxygen, provides a simple direct explanation of the ATP $\rightleftharpoons$ H_2O exchange. In this mechanism the path of oxygen in the ATP $\rightleftharpoons$ H_2O exchange is the same as in ATP synthesis. Korman and McLick suggested that the P_i $\rightleftharpoons$ H_2O exchange is analogous in this regard, but no analysis has yet been presented. In the next section it will be shown that a self-consistent development of the pseudorotation mechanism can correlate the separateness of the P_i $\rightleftharpoons$ H_2O exchange with its dependence on P_i activation.

Before proceeding, however, it is instructive to consider briefly the characteristics of the As_i $\rightleftharpoons$ H_2O exchange. Since As_i is a competitive inhibitor of the ATP $\rightleftharpoons$ P_i exchange (*6*), one might expect the As_i $\rightleftharpoons$ H_2O exchange to exhibit characteristics similar to those of the P_i $\rightleftharpoons$ H_2O exchange. However, De Master and Mitchell (*21*) have shown that, unlike the P_i $\rightleftharpoons$ H_2O exchange, the As_i $\rightleftharpoons$ H_2O exchange is oligomycin and dinitrophenol insensitive. Furthermore, Mitchell *et al.* (*6*) have also shown that the As_i $\rightleftharpoons$ H_2O exchange has no catalytic requirement for either energy or ADP. These facts have led Mitchell and coworkers to conclude that the As_i $\rightleftharpoons$ H_2O exchange is not related to oxidative phosphorylation. However, we will point out in the next section that an alternative explanation based simply on the difference in the P–O and As–O bond lengths also exists.

P_i $\rightleftharpoons$ H_2O EXCHANGE

Figure 2 viewed from top to bottom is a representation of ATP hydrolysis via the microscopic reversal of the pseudorotation mechanism for ATP synthesis. The ADP moiety has been represented as ADP–O in order to explicitly represent the bridge oxygen between the β and γ phosphate groups of ATP (*22*). The stabilization of the two oxonium moieties would require the existence of four proton transfer groups in the enzyme (labeled Tr), and the perspective of the drawing is purposely chosen to depict clearly the positions and protonation states of these hypothesized proton transfer groups.

The transition from state (a) or (a′) to state (b) or (b′) represents the binding of ATP and H_2O to the active site. Note that the enzyme active site is depicted in two possible isomeric forms depending upon which pair of proton transfer atoms is protonated. The terminal phosphate group of ATP is drawn in idealized tetrahedral geometry.

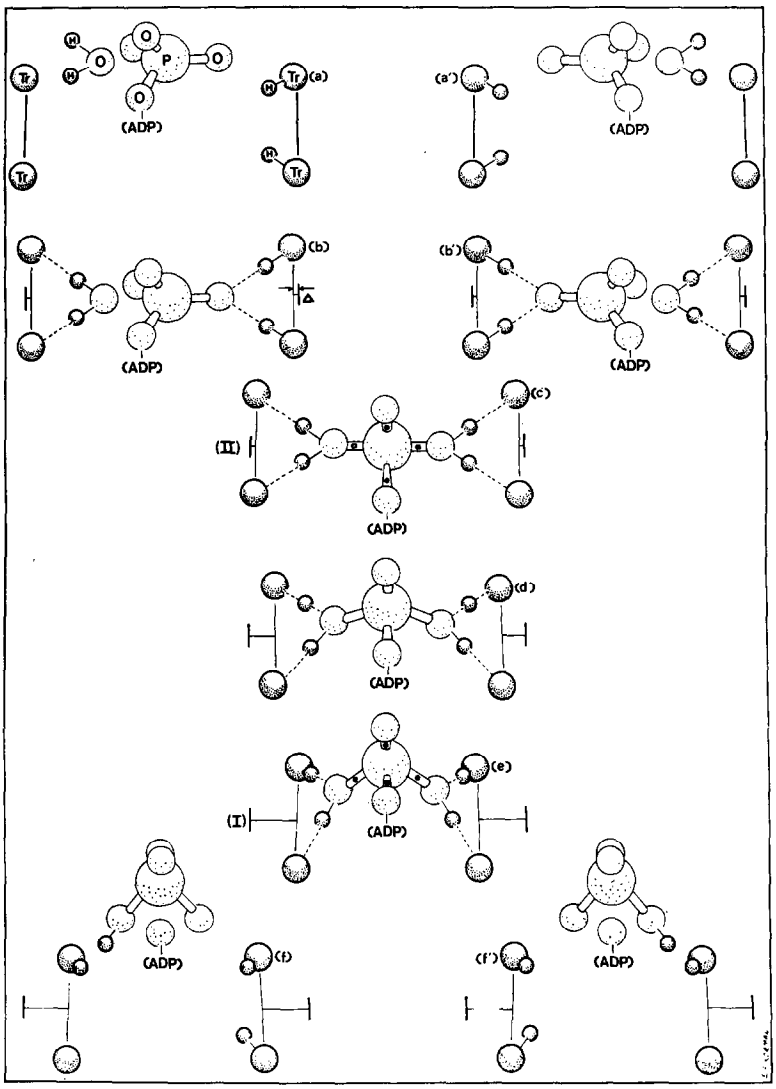

FIG. 2. Representation of the pseudorotation reaction mechanism of ATP hydrolysis. Bond lengths are depicted on a scale twice that of the atomic radii.

Since the binding of ATP and H_2O would probably perturb that tetrahedral configuration, the structures depicted in states (b) and (b′) are admittedly idealized. States (a′) and (b′) are drawn as mirror images of states (a) and (b), respectively.

State (c) depicts the trigonal bipyramidal reaction intermediate having two apical oxonium groups. This structure is designated reaction intermediate (II) in keeping with

its previous designation (*8*). Note that the proton transfer atoms are held fixed in states (b), (b′) and (c).

The reversible reaction sequence, (a) $\rightleftharpoons$ (b) $\rightleftharpoons$ (c) $\rightleftharpoons$ (b′) $\rightleftharpoons$ (a′) is sufficient to account for the rapid ATP $\rightleftharpoons$ H_2O exchange. With this in mind, let us consider a proposed displacement of pairs of proton transfer atoms by a distance Δ (Fig. 2b) in going from state (a) or (a′) to state (b) or (b′). Note carefully that there is no apparent requirement for this conformational transition in the active site to rationalize the ATP $\rightleftharpoons$ H_2O exchange. Indeed, when we compare states (b), (c) and (b′), we see that the terminal phosphate group of ATP undergoes oxygen exchange with H_2O within an active site whose proton transfer atoms are held fixed, since the phosphorus center can exist in either a tetrahedral or a trigonal bipyramidal configuration in such an active site. Also, ATP and H_2O could presumably come on and off the enzyme without a conformational transition in the active site being an absolute requirement.

However, if ATP and H_2O could come on and off the enzyme without a displacement of the proton transfer atoms from their positions in states (b) and (b′), then the enzyme would *spontaneously* catalyze a $P_i \rightleftharpoons H_2O$ exchange. The dimensions and configuration of P_i are essentially the same as those of the terminal phosphate group of ATP so that the pairs of proton transfer atoms held fixed in their positions in states (b), (c), and (b′) could stabilize a trigonal bipyramidal reaction intermediate formed from P_i and H_2O just as well as reaction intermediate (II) formed from ATP and H_2O. The spontaneous catalysis of a $P_i \rightleftharpoons H_2O$ exchange would contradict the experimental observation that the $P_i \rightleftharpoons H_2O$ exchange occurs only in the presence of either electron transfer or ATP hydrolysis.

Consequently, we have proposed that the active site undergoes a "closing in" conformational transition during the binding of ATP and H_2O in which the two pairs of proton transfer atoms are displaced by a distance Δ as represented in Fig. 2. Self-consistency requires that P_i and H_2O be unable to induce this "closing in" conformational transition. When ATP and H_2O come off the enzyme with a $\Delta \gtrsim 0.1$ Å, the consequent "spread" in the active site would amount to at least 0.2 Å. This spread would be sufficient to preclude the stabilization of a trigonal bipyramidal reaction intermediate about a phosphorus center.

The occurrence of a conformational transition in the active site when ATP and H_2O come on and off the enzyme is also suggested by the oligomycin sensitivity of the ATP $\rightleftharpoons$ H_2O exchange. Using submitochondrial A-particles, Hinkel, Penefsky and Racker (*15*) reported that rutamycin (oligomycin D) at a level of 1 μg/mg A-particle inhibits the ATP $\rightleftharpoons$ H_2O exchange. Kagawa and Racker (*23*) (1966) have shown that rutamycin does not bind to the mitochondrial ATPase (F_1) but does bind to an insoluble fraction (F_0) that has the capacity to restore oligomycin sensitivity to F_1. The fact that oligomycin binds to a subunit other than F_1 seems to imply that it inhibits ATPase activity by preventing some conformational transition required either for the binding or the hydrolysis of ATP. Since in the present model the ATP $\rightleftharpoons$ H_2O exchange does not require cleavage of ATP, self-consistency requires us to postulate that oligomycin prevents a conformational transition involved in the binding of ATP. Thus, oligomycin would prevent the formation of reaction intermediate (II) in state (c) of Fig. 2. This conclusion is consistent with the recent demonstration of oligomycin sensitive (*24*) binding of ATP apparently at the active site of the mitochondrial ATP synthetase (*25*).

In Fig. 2 the pseudorotation event is depicted as the transition from state (c) to state (e) [reaction intermediate (I)] with state (d) being the tetragonal pyramidal transition state. Since the perspective optimal for representing the positions and protonation states of the four proton transfer atoms is less suitable for clearly representing the pseudorotation event, the apical bonds have been labeled *a* and the equatorial bonds *e*. States (f) and (f′) depict two possible isomeric forms of the active site when ADP and P_i come off the enzyme. Note the difference in protonation state of these two isomeric forms relative to states (a) and (a′). No conformational transition is required in this model for the desorption of ADP and P_i, although such a transition cannot be ruled out.

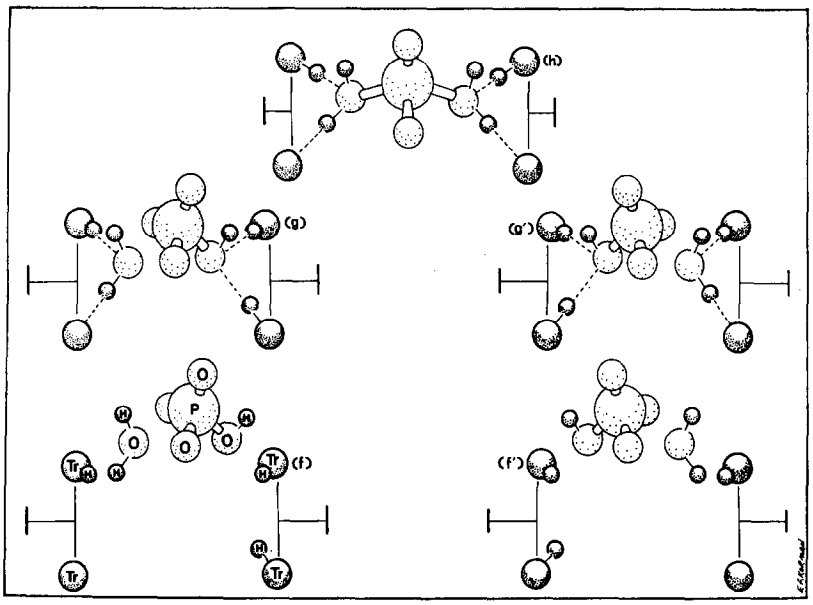

FIG. 3. Representation of the reaction mechanism of $P_i \rightleftharpoons H_2O$ exchange. Bond lengths are depicted on a scale twice that of the atomic radii.

We can now consider the capacity of the active site in states (f) and (f′) to catalyze a $P_i \rightleftharpoons H_2O$ exchange that is separate from the reversal of phosphorylation. The binding of P_i and H_2O to the active site, the exchange of oxygens at the phosphorus center, and the release of P_i and H_2O are shown in Fig. 3. It is apparent that the "closing in" of the active site induced by the binding and hydrolysis of ATP makes the enzyme competent to catalyze a $P_i \rightleftharpoons H_2O$ exchange separate from the reversal of phosphorylation. Since the coupling of ATP hydrolysis with the conformational change of the enzyme depicted in Fig. 2 is a reversible process, the energy requirement supplied by respiration for ATP synthesis in oxidative phosphorylation must be to change the conformation and protonation state of the active site. For the $P_i \rightleftharpoons H_2O$ exchange this energy requirement is only a catalytic requirement since the restructured active site can catalyze multiple $P_i \rightleftharpoons H_2O$ exchanges (*13*).

In Fig. 3 note that it is necessary to "spread" the active site from its conformation in states (f) and (f′) to its conformation in state (h) at the tetragonal pyramidal transition

state. This allows a partial apical entry of the oxygen atom of H_2O at the phosphorus center to form the pentacoordinate structure. There are at least three ways to rationalize this conformational transition: (1) The binding of P_i and H_2O to state (f) or (f′) as depicted in Fig. 3 may itself be able to stabilize a "spread" conformation, (2) The desorption of ADP and P_i may lead to a "spreading" of the active site not depicted in Fig. 2. (3) The trigonal bipyramidal structure of reaction intermediate (I) depicted in state (e) of Fig. 2 may not be the structure of the first reaction intermediate formed upon the binding of ADP and P_i. A pure apical "attack" is only a limiting case, and it is conceivable that reaction intermediate (I) has a structure between the structures depicted in states (e) and (d) of Fig. 2. There is no apparent basis at present for choosing between these three alternatives.

Finally, it should be noted that the model proposed here for the ATP $\rightleftharpoons$ H_2O and $P_i \rightleftharpoons H_2O$ exchanges suggests an explanation for the $As_i \rightleftharpoons H_2O$ exchange. A tetrahedral As–O bond length is about 1.67 Å (*26*), whereas a tetrahedral P–O bond length is only about 1.54 Å (*26, 27*), a difference of 0.13 Å. A comparable difference would be anticipated for apical As–O and P–O bond lengths. Thus if $\Delta \approx 0.13$ Å, the enzyme in states (a) or (a′) would be competent to catalyze an $As_i \rightleftharpoons H_2O$ exchange but incompetent to catalyze a $P_i \rightleftharpoons H_2O$ exchange. Thus the fact that an As–O bond is longer than a P–O bond could provide a simple explanation for the energy independence of the $As_i \rightleftharpoons H_2O$ exchange as well as its insensitivity to oligomycin and dinitrophenol. Thus the mere *potential* for P_i activation in this model would be sufficient for rationalizing the characteristics of the $As_i \rightleftharpoons H_2O$ exchange. Obviously, no mechanism of P_i activation involving either the formation or cleavage of a covalent bond prior to the formation of the pertinent P–O bond in ATP could be related to an $As_i \rightleftharpoons H_2O$ exchange that is both energy independent and insensitive to oligomycin and dinitrophenol.

INHIBITORS

The analysis of the oxygen exchanges leads naturally to a consideration of the effects associated with oligomycin, aurovertin and As_i. Oligomycin at high levels ($\geqslant 1$ μg/mg protein) and aurovertin inhibit oxidative phosphorylation and the ATP $\rightleftharpoons$ P_i and $P_i \rightleftharpoons H_2O$ exchanges (*28*). However, whereas oligomycin at high levels blocks all ATP-driven processes, aurovertin allows ATP-driven reversed electron transfer, transhydrogenation and ion translocation (*17, 29, 30*). As already noted, oligomycin at low levels (~0.2 μg/mg protein) stimulates oxidative phosphorylation, reversed electron transfer, energy linked transhydrogenation, and ATP $\rightleftharpoons$ P_i exchange in certain submitochondrial particles (*17*), but inhibits the $P_i \rightleftharpoons H_2O$ exchange (*15*). Oligomycin at low levels completely inhibits the As_i-induced stimulation of respiration (*31, 32*). Aurovertin at low levels (~0.2 μg/mg protein) also inhibits the As_i-induced stimulation of respiration, but at higher levels ($\geqslant 4$ μg/mg protein) it has no inhibitory effect at all (*31, 32*).

Since most workers have assumed that the mechanism of P_i activation in oxidative phosphorylation would be via the formation of a covalent bond, the interpretations offered to date for these various effects have all been based on the hypothesized inter-

mediates of oxidative phosphorylation, X ~ I and X ~ P. In order to see the problems posed by these effects, it is helpful to briefly review some of these interpretations.

Lee and Ernster (*17*) as well as Cross and Wang (*31*) have proposed that the oligomycin block occurs between X ~ I and X ~ P, whereas the aurovertin block occurs between X ~ P and ATP. However, it is not apparent how an aurovertin block between ATP and X ~ P could allow ATP-driven reversed electron transfer, transhydrogenation, and ion translocation. Furthermore, an oligomycin block between X ~ P and X ~ I would not explain the inhibition of the ATP $\rightleftharpoons$ ADP exchange by oligomycin (*33*).

Lardy, Connelly and Johnson (*28*) have proposed that the oligomycin block is between ATP and X ~ P and the aurovertin block between X ~ P and X ~ I. They have further proposed the existence of another intermediate W involved only in ion translocation and volume changes in order to bypass the aurovertin block. However, as Lee and Ernster (*17*) have pointed out, the existence of an intermediate W would not rationalize how aurovertin allows ATP-driven reversed electron transfer. Presumably, the same set of intermediates involved in oxidative phosphorylation would be involved in its reversal. Since aurovertin inhibits the ATPase activity of F_1 (*7*), Lardy (personal communication) has subsequently concluded that aurovertin does not act between X ~ P and X ~ I.

The fact that oligomycin has two very different activities in two different concentration ranges seems to imply that there is a heterogeneous population of oligomycin binding sites. Hinkle, Penefsky and Racker (*15*) have proposed that at high levels the oligomycin block is between ATP and X ~ P whereas at low levels oligomycin blocks the hydrolysis of X ~ I. The proposed action of oligomycin at low levels is to account both for its enhancement of energy coupling and its inhibition of the $P_i \rightleftharpoons H_2O$ exchange. However, if the hydrolysis of X ~ I were irreversible, as implicitly assumed by their proposal, then it could not account for the rapid $P_i \rightleftharpoons H_2O$ exchange.

Ter Welle and Slater (*32, 34*) as well as Cross and Wang (*31*) have interpreted the As_i-induced stimulation of respiration as arising from the arsenylation of ADP followed by the spontaneous hydrolysis of the arsenylated compound. It is interesting to note that these authors interpreted their results with the As_i-induced stimulation of respiration as providing strong evidence for the existence of a phosphorylated intermediate. However, if ADP were to undergo arsenylation, it is not apparent why high levels of aurovertin should not affect the aesenylation of ADP but completely inhibit its phosphorylation. Furthermore, it is not apparent why low levels of oligomycin should both inhibit the arsenylation of ADP in mitochondria and stimulate the phosphorylation of ADP in certain submitochondrial particles. These facts suggest that the As_i-induced stimulation of respiration does not involve the arsenylation of ADP. It might also be noted that should one attempt to explain the $P_i \rightleftharpoons H_2O$ exchange in terms of an intermediate such as X ~ I or X ~ P that can exchange oxygen with water, then the existence of an $As_i \rightleftharpoons H_2O$ exchange which is both energy independent and insensitive to dinitrophenol and oligomycin would be inconsistent with an arsenylated ADP.

It is apparent from this brief review that an interpretation of the effects of oligomycin, aurovertin, and As_i consistent with the available experimental data has not yet been offered. We will attempt to show that a consistent interpretation of these effects follows naturally from the analysis of the oxygen exchanges. It is, of course, beyond the

scope of the present stereochemical analysis to deal with the mechanism of noncompetitive enzyme inhibitors except in so far as they pertain directly to events about the phosphorus or arsenic reaction center. Nonetheless, it is instructive to consider the types of explanations suggested by the pseudorotation mechanism.

As previously discussed, we would propose that oligomycin at high levels prevents the conformational transition required for the stabilization of reaction intermediate (II). This would inhibit all ATP-driven processes as well as oxidative phosphorylation.

Since at low levels oligomycin inhibits the $P_i \rightleftharpoons H_2O$ exchange, we would propose that at this low level it prevents the stabilization of the intermediates represented in Fig. 3. Since the $P_i \rightleftharpoons H_2O$ exchange depicted in Fig. 3 is separate from the reversal of phosphorylation, the inhibition of this exchange is consistent with the enhancement of energy coupling by oligomycin at low levels. The proposal that oligomycin at low levels prevents the stabilization of the intermediates represented in Fig. 3 obviously says nothing about the mechanism by which energy coupling is enhanced by oligomycin.

The fact that aurovertin inhibits the rapid $P_i \rightleftharpoons H_2O$ exchange to the same extent as the slower $ATP \rightleftharpoons P_i$ exchange suggests that the path of oxygen is affected. The generation of the two oxonium groups is essential in the pseudorotation mechanism of ATP synthesis in order to effect the equatorial capture of the poor nucleophile ADP. This requires that the four proton transfer atoms be able to *reversibly* transfer their protons to the appropriate phosphate oxygens. If aurovertin were to stabilize the covalent bonding of one or more protons to their respective proton transfer atoms, reaction intermediate (I) would be greatly stabilized relative to reaction intermediate (II), and the equatorial capture of ADP via a pseudorotation would become improbable. ATP could still be hydrolyzed, but it would be an essentially irreversible process, at least for large negative free energies of reaction. Despite such a perturbation of the reaction mechanism the stereochemical pathway of ATP hydrolysis would remain invariant so that ATP hydrolysis would still be coupled to a conformational transition of the enzyme. Thus some of the free energy of ATP hydrolysis could still be utilized to drive reversed electron transfer, transhydrogenation, or ion translocation. The $P_i \rightleftharpoons H_2O$ exchange would be inhibited by this mechanism of aurovertin action since the $P_i \rightleftharpoons H_2O$ exchange requires the *reversible* transfer of two protons from their proton transfer atoms to the neighboring phosphate oxygens.

The sensitivity of the As_i-induced stimulation of respiration to low levels of oligomycin is similar to that of the $P_i \rightleftharpoons H_2O$ exchange. This suggests that in order to stimulate respiration As_i must bind to the enzyme as depicted for P_i in Fig. 3. In order to consider the implications of such an As_i–enzyme complex we must briefly consider the mechanism of respiratory control in this model (*35*). The present model requires that conformations (f) and (f′) be long-lived or metastable in the absence of substrates in order to conserve the free energy of ATP hydrolysis. However, since this enzyme must also act as catalyst for the reversible phosphorylation of ADP, the binding of ADP and P_i and the subsequent formation of the reaction intermediates depicted in Fig. 2 must facilitate the relaxation of the enzyme. This implies that the enzyme–substrate interactions lower the activation barrier for the transformation of the enzyme as well as that of the substrates.

If As_i were to bind as depicted for P_i in Fig. 3, then it would be potentially capable of forming a reaction intermediate analogous to (II). Due to the longer As–O bond

length the binding of As_i to conformations (f) or (f′) would involve the distortion of bond angles in As_i. Thus a spreading of the proton transfer atoms, as in going from (f) or (f′) to (c), would tend to stabilize the As_i–enzyme complex. These considerations suggest that As_i might stimulate respiration by stabilizing the enzyme conformations either on the pathway or only slightly perturbed from the pathway of ATP synthesis. This would, of course, be an energy dissipating process. This proposal is consistent with the fact that high levels of aurovertin do not inhibit the As_i-induced stimulation of respiration. Even if one or more protons remained covalently bound to their transfer atoms, a pentacoordinate arsenic intermediate could stabilize a conformation in which the proton transfer groups are as depicted in Fig. 2c. Thus the As_i-facilitated relaxation of the enzyme need not involve the generation of two full oxonium moieties as proposed for oxidative phosphorylation.

It should also be noted that the strain induced in As_i upon binding to conformations (f) or (f′) could explain the very effective inhibition by P_i of the As_i-induced stimulation of respiration (*6*, *32*). Supporting this proposal is the observation that the As_i-induced stimulation of respiration in the presence of high levels of aurovertin is still inhibited by P_i (*32*). Thus P_i can effectively compete with As_i even in the absence of phosphorylation.

The enhancement by ADP of the As_i-induced stimulation of respiration (*32*) can be rationalized in a manner analogous to that proposed for the ADP requirement in the $P_i \rightleftharpoons H_2O$ exchange. Our proposals involving As_i bound to the active site obviously do not exclude other effects due to As_i bound at another site.

These proposals concerning oligomycin, aurovertin and As_i are admittedly very speculative. As already noted, it is beyond the scope of a stereochemical analysis to deal with the mechanism of noncompetitive enzyme inhibitors except in so far as they pertain directly to events about the reaction center. Our sole purpose in considering these effects here is to show that unlike the mechanisms involving $X \sim I$ and $X \sim P$, the pseudorotation mechanism offers the possibility of explanations that are at least consistent with the experimental data.

DISCUSSION

There is a close correspondence between Fig. 2 and the reaction scheme proposed by Boyer, Fig. 1. In Fig. 2 the isomeric conformations (a) and (a′) and the isomeric conformations (f) and (f′) might be viewed as corresponding to the nonenergized and energized states of the mitochondrion, respectively. In each conformation the appropriate bound substrates can exchange with free substrates. Thus Fig. 2 incorporates all of the features in Boyer's scheme. However, unlike in the Boyer scheme, the existence of the *two* isomeric conformations (a) and (a′) allows one to rationalize the $ATP \rightleftharpoons H_2O$ exchange while retaining stereospecific tight binding of substrates to enzyme throughout the exchange reaction.

Similarly, the existence of the isomeric conformations (f) and (f′) allows one to rationalize a $P_i \rightleftharpoons H_2O$ exchange both separate from the reversal of phosphorylation and stereochemically analogous to the $ATP \rightleftharpoons H_2O$ exchange. It should be noted that in order for the $P_i \rightleftharpoons H_2O$ exchange to be stereochemically analogous to the $ATP \rightleftharpoons H_2O$ exchange, P_i must bind to the enzyme as depicted in Fig. 3 rather than as depicted for ATP synthesis in Fig. 2. The $P_i \rightleftharpoons H_2O$ exchange is therefore *completely* off the

pathway of oxidative phosphorylation. Nonetheless, this $P_i \rightleftharpoons H_2O$ exchange still depends on the mechanism of P_i activation. Thus, allowing for two entry points of water oxygen provides a basis for correlating the apparent separateness of much of the $P_i \rightleftharpoons H_2O$ exchange and the involvement of P_i activation. The apparently paradoxical proposal of Mitchell, Hill and Boyer (*14*) concerning the $P_i \rightleftharpoons H_2O$ exchange therefore follows naturally from a self-consistent development of the pseudorotation mechanism.

Since the $ATP \rightleftharpoons H_2O$ exchange does not require the dynamic reversal of phosphorylation and since the $P_i \rightleftharpoons H_2O$ exchange is completely off the pathway of oxidative phosphorylation, the present model can readily rationalize the rapid rates of these two exchange reactions relative to the $ATP \rightleftharpoons P_i$ exchange.

It should also be emphasized that the pathway of oxygen in the $ATP \rightleftharpoons H_2O$, $P_i \rightleftharpoons H_2O$, and $As_i \rightleftharpoons H_2O$ exchange reactions proposed here is the same as in ATP synthesis. In this regard it might also be noted that whereas the three oxygen exchanges follow an inversion stereochemistry path, the ATP synthesis reaction follows a retention path.

We might note here that the mechanism of P_i activation by the stereospecific tight binding of P_i to the enzyme in the proper conformation implies the direct loss of an oxygen atom from P_i to H_2O in ATP synthesis. This "direct dehydration" is consistent with the results of Chaney and Boyer (*36*) for photophosphorylation.

We recall that Boyer's phenomenological scheme, Fig. 1, is a very general one insofar as it encompasses a very large variety of possible ATP synthesis mechanisms. The inadequacy of this scheme for rationalizing the characteristics of the $ATP \rightleftharpoons H_2O$ and $P_i \rightleftharpoons H_2O$ exchanges coupled with the simplicity and directness of the explanations provided by the pseudorotation mechanism constitute a strong argument in support of the latter mechanism. In particular, it is quite striking that the mechanistic heart of the pseudorotation mechanism of ATP synthesis, namely, the two oxoniums acting together as a pair to effect the equatorial capture of ADP, provides a straightforward rationalization of the apparent two entry points of water oxygen in the $ATP \rightleftharpoons H_2O$ and $P_i \rightleftharpoons H_2O$ exchanges. Thus the pseudorotation mechanism not only consistently rationalizes a far wider range of experimental data than has been achieved heretofore, but it does so in terms of fundamental phosphorus stereochemistry and mechanism.

There are some important implications of the pseudorotation reaction mechanism for the mechanism of energy coupling in oxidative phosphorylation. The direct-union of ADP and P_i implies that the phosphorylation-dehydration reaction is *mechanistically independent* of the oxidation–reduction reactions in oxidative phosphorylation. Mechanistic independence is supported by the following observations: (1) The inhibition of respiration in well coupled mitochondira by oligomycin can be completely relieved by dinitrophenol (*37*, *38*). (2) The dose–response curves for the inhibition of the $ATP \rightleftharpoons P_i$ and $P_i \rightleftharpoons H_2O$ exchanges by oligomycin and aurovertin are hyperbolic, whereas they are markedly signoidal for the inhibition of respiration (*28*). (3) Respiratory inhibitors have no or only partial inhibitory effect on the $ATP \rightleftharpoons P_i$, $ATP \rightleftharpoons H_2O$ and $P_i \rightleftharpoons H_2O$ exchanges (*39*). (4) Respiratory inhibitors have no effect on ATP-driven ion translocation and transhydrogenation, and, similarly, oligomycin has no effect on respiration-driven ion translocation and transhydrogenation (*40*, *41*). (5) Promitochondria catalyze an oligomycin and dinitrophenol sensitive $ATP \rightleftharpoons P_i$ and $P_i \rightleftharpoons H_2O$ exchange in the almost total absence of respiratory carriers (*42*). (6) A

soluble ATP synthetase catalyzes an oligomycin and uncoupler sensitive ATP $\rightleftharpoons$ P_i and ATP $\rightleftharpoons$ ADP exchange even in the absence of a membrane (*43*, *44*). These observations provide compelling support for the implication of the pseudorotation mechanism that electron transfer and ATP synthesis are mechanistically independent.

Despite the well-known nature of these observations, mechanisms for ATP synthesis continue to be proposed which link the phosphorylation and redox chemistries (*45*, *46*). In this regard it is also interesting to note that in commenting on the pseudorotation mechanism Hutchison (*47*) observes that "no mention is made of the role of oxidation in this [ATP synthesis] reaction." It is ironic that the omission of oxidation should be viewed as a deficiency of the pseudorotation mechanism rather than as fulfilling the condition imposed by the above-mentioned observations.

The direct-union of ADP and P_i via the pseudorotation mechanism implies that the immediate source of free energy for ATP synthesis is the ATP synthetase itself. That is, ATP synthesis must be coupled to a relaxation of the ATP synthetase from a conformation of higher to a conformation of lower free energy. This implies that once conformations (f) or (f′) are generated, by whatever means, the formation of reaction intermediates (I) and (II) and the desorption of ATP and H_2O are *spontaneous* processes. The enzyme–substrate interactions due to stereospecific tight binding are fully adequate to account both for P_i activation and for the stabilization of reaction intermediate (II) relative to reaction intermediate (I). Thus in the present model energy transduction emerges simply as an aspect of enzyme catalysis.

SUMMARY

The present paper demonstrates that the pseudorotation mechanism, besides meeting the fundamental requirement of P_i activation, also meets the following three conditions: (1) It rationalizes the various characteristics of all three oxygen exchanges in a manner mechanistically related to ATP synthesis. (2) It is consistent with the effects of oligomycin, aurovertin, and As_i. (3) It rationalizes the mechanistic independence of electron transfer and ATP synthesis. These three conditions implicitly define an extremely restricted class of ATP synthesis mechanisms. At the present time the pseudorotation mechanism is the *only* mechanism that has been shown to belong to this restricted class. In the absence of a demonstration showing that other mechanisms can also meet these three conditions, it is conceivable that these three conditions implicitly define a unique mechanism of ATP synthesis in oxidative phosphorylation.

Note added in proof: De Master and Mitchell [*Biochemistry* **12**, 3616 (1973)] have recently reported an As_i-insensitive $P_i \rightleftharpoons H_2O$ exchange accompanying ATP-driven NAD^+ reduction which appears analogous to the As_i-insensitive intermediate $P_i \rightleftharpoons H_2O$ exchange accompanying myosin catalyzed ATP hydrolysis. Both of these As_i-insensitive exchanges can be rationalized by the same reaction mechanism as the ATP $\rightleftharpoons$ H_2O exchange except that the ^{18}O appears in the P_i cleaved from ATP rather than in ATP itself. An interpretation of the muscle oxygen exchanges in terms of the pseudorotation mechanism will be presented elsewhere.

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