Studies on the Kinetic Mechanism of Oxidative Phosphorylation*

SHELDON M. SCHUSTER,‡ GREGORY D. REINHART,§ AND HENRY A. LARDY

From the Department of Biochemistry and the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706

The kinetics of the synthesis of ATP from ADP and P i, by beef heart submitochondrial particles were examined. When P i, was the variable substrate positive cooperativity was observed, whereas if ADP was varied, linear double reciprocal plots were obtained. The analog of P i, thiophosphate, was a noncompetitive inhibitor of ATP synthesis with respect to ADP, while the analog of ADP, AMP(CH)P, was an uncompetitive P i, ATP exchange inhibitor. The kinetics of the initial velocity isotopic exchanges of oxidative phosphorylation were also examined. When the P i, ATP exchange was examined, it was found that if ADP concentration was held constant while ATP and P i, concentrations were varied at a constant ratio, linear double reciprocal plots were obtained. However, if P i, concentration was held constant and ADP and ATP concentrations were varied at constant ratio, apparent substrate inhibition was observed. The 2,4-dinitrophenol-sensitive ADP ATP exchange showed linear double reciprocal plots regardless of which components were varied. These results are interpreted to indicate that in the direction of ATP synthesis, the reaction is ordered, with P i, adding to the enzyme before ADP addition.

Studies of the mechanism of oxidative phosphorylation have recently been aimed at understanding the molecular mechanism of ATP synthesis. The purification and partial characterization of the ATPase molecule have greatly advanced this pursuit (reviewed in Ref. 1). The number of nucleotides bound to the ATPase molecule, their role in control of catalysis, and the affinity and specificity of the binding sites are active areas of investigation (2-5). Recent studies have begun to explore the chemical nature of the ATPase active site. Evidence for the presence of both a tyrosine (6, 7) and an arginine (8) in the ATPase active site has been presented.

An important aspect of the oxidative phosphorylation reaction that has not been investigated until recently is the kinetic mechanism. A most direct route for the determination of the amination order of binding and release of substrates and products for an enzymatic reaction has been outlined by Cleland (9). This technique involves the use of kinetic studies of the isotopic exchange reactions catalyzed by an enzyme, as first described by Boyer and Silverstein (10). Unfortunately, there is as yet no substantiated evidence indicating that the isolated ATPase catalyzes any of the isotopic exchanges normally associated with oxidative phosphorylation. Therefore, the problem must be approached in a submitochondrial particle system which is known to catalyze all of the relevant reactions. On the basis of results presented in this paper an ordered sequence is proposed.

Studies of the kinetic parameters of both the net synthesis of ATP and the P i, ATP exchange have previously been performed in several laboratories (11-13). Recently, Pedersen (14) has explored some of the kinetic properties of several of the reactions associated with electron transport and ATP synthesis. Some kinetic properties of the H 2O P i, exchange reaction have also been explored with submitochondrial particles prepared either by sonication (15) or by digitonin treatment (16). Only one of these studies (16) directly addresses the question of a mandatory binding order for ADP and P i, during ATP synthesis, and in this case no definite conclusion was reached. Consequently, workers in this field have depicted ATP synthesis as occurring with either ADP (17) or P i, (18) combining with the enzyme as the first substrate. The studies presented in this communication, therefore, are directed toward more precisely defining the order of addition of the substrates of ATP synthesis. A preliminary report of our conclusions has been published (19).

MATERIALS AND METHODS

Beef heart submitochondrial particles were prepared as described previously (5). The synthesis of ATP from ADP and P i, was measured in 1.0 ml in a medium containing the following: Bes/KOH, pH 7.0, 20 mM; succinate, 200 mM; glucose, 50 mM; potassium succinate, 20 mM; rotenone, 3 /µg; MgCl 2, 10 mM; hexokinase, 100 /µg/ml; and ADP and P i, (with about 10 6 cpm/µmol of 32P) as indicated. All reactions were performed at 30°. The reactions were carried out on vials so that rapid shaking in a water bath would insure aerobic conditions for the entire reaction period (generally, 3 min). The reactions were initiated by the addition of 2.5 mg of submitochondrial protein. After the incubations, the reaction was terminated by the addition of 0.1 ml of 60% perchloric acid. After chilling and centrifugation, a portion of the supernatant was extracted exhaustively to remove P i, as described by Pullman (20), and the remaining organic phosphate was counted.

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† The abbreviations used are: Bes, 2-(bis(2-hydroxyethyl)amino)ethylsulfonic acid; AMP(CH)P, adenosine-5'-[(α,β-methylene)-diphosphate.  

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The \( P_i \rightarrow ATP \) exchange reaction was measured as described by Pullman (20), except that the reaction mixture contained, in a 1-ml volume: sucrose, 200 mM; BES/KOH, pH 7.0, 20 mM; KCN, 1 mM; MgCl\(_2\), 10 mM; MgATP, MgADP, and P\(_i\) (with about 10^8 cpm/\mumol of \(^{32}\)P) as indicated in the figures. The reactions (at 30°) were initiated with 2.5 mg of submitochondrial protein. The reaction was allowed to run for 1 min and then was terminated by the addition of 0.1 ml of 60% perchloric acid. The reaction mixtures were chilled, centrifuged, and the organic phosphate counted as described above. The \( P_i \rightarrow ATP \) exchange was measured the same way as described for the \( P_i \rightarrow ATP \) exchange, except that ATP and IDP in the concentrations indicated replaced ATP and ADP. When the effect of AMP(CH\(_2\))P on the \( P_i \rightarrow ATP \) exchange was measured, the technique was the same as that described above except that the final reaction volume was 0.25 ml, and 1 mg of protein was used.

The exchange of label from \([^{14}C]ADP\) to ATP was determined using a reaction mixture similar to that described above for the \( P_i \rightarrow ATP \) exchange, except that \([^{14}C]ADP\) was included and \(^{32}\)P was not. After the reactions were stopped with perchloric acid, an aliquot of the supernatant was spotted on DEAE-paper. These spots were developed in a solvent system consisting of 0.6 M ammonium formate and 5 mM EDTA. The spots corresponding to ATP and ADP were cut out and counted in a standard toluene solution. In order to insure that initial velocity was being monitored, samples were taken for this exchange at 0, 0.5, 1.0, 2.0, and 5.0 min. In all cases, linear rates of incorporation of label from \([^{14}C]ADP\) to ATP were observed. All reactions were run with the \( P_i \), ADP, and ATP concentrations as indicated. A parallel sample was always run containing 0.5 mM 2,4-dinitrophenol (recrystallized from water), and the organic phosphate was counted as described above. The concentration of thiophosphate was as indicated.

protein was estimated by a biuret procedure (21). Nucleotide concentrations were determined spectrophotometrically as described previously (5). The following reagents were from these suppliers: hexokinase, Boehringer Mannheim; thiophosphate (recrystallized from water), Alfa Inorganics; \([^{14}C]ADP\) and \(^{32}\)P, from New England Nuclear; ADP, ATP, IDP, and ITP from P-L Biochemicals. All other reagents were purchased from commercial suppliers and were of the highest purity available.

**RESULTS**

The catalytic mechanism of ATP synthesis could most simply be viewed as the binding of ADP and Pi to form the basic components of the central complex of enzyme and transition state intermediates. To examine the kinetic parameters governing this process, both ADP and Pi were used as variable substrates in initial velocity and isotopic exchange studies of ATP synthesis, catalyzed by beef heart submitochondrial particles.

As seen in Fig. 1, when ADP is the variable substrate, straight line plots of 1/v versus 1/[ADP] were obtained throughout the range of \( P_i \) concentrations used (0.5 to 10 mM). Other experiments (not shown) demonstrate that this linear pattern remains unaltered with concentrations of up to 20 mM ADP. When \( P_i \) is the variable substrate (Fig. 2), there is a marked curvature to the double reciprocal plots, indicative of positive cooperativity. The Hill coefficients ranged from about 1.2 (at 200 \( \mu \)M ADP) to 2.3 at the low ADP concentrations.

Initial velocity studies were done using different energy sources such as NADH and \( N,N,N',N'- \)tetramethylphenylenediamine with ascorbate, and in all cases, the double reciprocal plots were qualitatively the same as those shown in Figs. 1 and 2. The Hill coefficients obtained with \( P_i \) as the variable substrate were independent of the energy source used. Further, when the initial velocity studies were done using rat liver submitochondrial particles prepared as described previously (5), the results were again similar to those reported in Figs. 1 and 2.

From these results one can eliminate the possibility of \( P_i \) and ADP additions being separated by an irreversible product-releasing step (i.e. a ping pong mechanism). However, further information is required to determine the existence of any mandatory binding order. If ADP is the obligatory first substrate in an ordered mechanism, an analog of phosphate should be an uncompetitive inhibitor of ATP synthesis when ADP concentration is varied. Thiophosphate has previously been shown to be an inhibitor of the \( P_i \rightarrow ATP \) exchange (22). To determine whether thiophosphate is a good analog of \( P_i \), the effect of this compound on the \( P_i \rightarrow ATP \) exchange was observed. This exchange yields a linear double reciprocal plot with respect to \( P_i \) concentration (Fig. 3, control line). This is in contrast to the curved double reciprocal plots obtained when net ATP synthesis is measured with \( P_i \) as the variable substrate (Fig. 2).

From the data in Fig. 3, it can also be seen that thiophosphate is a competitive inhibitor of the \( P_i \rightarrow ATP \) exchange. The effect of this analog on the net synthesis of ATP

![Fig. 1 (left).](image1) The effect of varying the concentration of ADP at fixed \( P_i \) concentrations on the rate of net ATP synthesis catalyzed by beef heart submitochondrial particles.

![Fig. 2 (center).](image2) The effect of varying the concentration of \( P_i \) at fixed ADP concentrations on the rate of net ATP synthesis catalyzed by beef heart submitochondrial particles.

![Fig. 3 (right).](image3) The effect of thiophosphate on the \( P_i \rightarrow ATP \) exchange catalyzed by beef heart submitochondrial particles. The concentration of ATP was 5 mM and of ADP was 1 mM. The concentrations of thiophosphate were as indicated.
catalyzed by beef heart submitochondrial particles is shown in Fig. 4. It can be concluded that thiophosphate is a noncompetitive inhibitor with respect to ADP concentration. Since these data do not support a mechanism wherein ADP is the first substrate to bind in an ordered kinetic mechanism, further kinetic studies were performed.

Using similar reasoning, a competitive inhibitor with respect to ADP should be an uncompetitive inhibitor of ATP synthesis when Pᵢ is the variable substrate. The uncompetitive inhibition experiment is difficult to interpret because of the curvature of the double reciprocal plot when Pᵢ is the variable substrate (Fig. 2). However, the isotopic exchange rate equations predict a similar uncompetitive inhibition pattern for the Pᵢ → ATP exchange when the ratio of variable substrate concentration to the ATP concentration is held constant (see "Discussion"). When adenosine-5′-(α,β-methylene)-diphosphate (AMP(CH₂)P) is used as an inhibitor of net ATP synthesis with ADP as the variable substrate, the competitive inhibition pattern shown in Fig. 5 is obtained. When this ADP analog is used as an inhibitor of the Pᵢ → ATP exchange when Pᵢ and ATP concentrations are kept at constant ratio, the pattern shown in Fig. 6A is obtained. The slope replot in Fig. 6B indicates that the lines are parallel. This implies that AMP(CH₂)P is an uncompetitive inhibitor of the exchange.

The data thus far presented are consistent with an ordered binding of the substrates of ATP synthesis, with Pᵢ adding prior to ADP addition. A sensitive technique consisting of monitoring isotopic exchanges at equilibrium for the determination of substrate binding order, if one exists, has been described (9, 10). The exchanges, however, need not be performed at equilibrium (see "Discussion"). Basically, this procedure involves varying the concentration of pairs of substrates in constant ratio and interpreting the results on the basis of the presence or absence of apparent substrate inhibition.

When the dinitrophenol-sensitive ADP → ATP exchange is measured with ATP concentration always equal to ADP, the results are as shown in Fig. 7. The double reciprocal plot is linear, showing no substrate inhibition. Similar results are obtained if the ADP → ATP exchange is monitored while ATP and Pᵢ concentrations are held at constant ratio (see Fig. 8).

Measurement of the Pᵢ → ATP exchange results in a linear double reciprocal plot when ATP and Pᵢ concentrations are varied together as shown in Fig. 9. However, if the ratio of ATP to ADP is held constant over the same ATP concentration range, as in Fig. 9, the double reciprocal plot shows definite substrate inhibition at two different Pᵢ concentrations (see Fig. 10A). That this substrate inhibition is complete and not just a partial inhibition can be seen from the plot of log [ATP] versus exchange rate (Fig. 10B), which appears symmetrical at both Pᵢ concentrations tested. Since we have previously noted that different nucleoside triphosphate substrates are hydrolyzed by mitochondrial ATPases with markedly different kinetics (5), it was essential to examine an alternate substrate exchange with Pᵢ. When the Pᵢ → ITP exchange is measured, a linear double reciprocal plot is obtained when the concentration of ITP and Pᵢ are varied at constant ratio (Fig. 11). This is
The use of isotopic exchange to determine the order of substrate addition in a Bi Bi mechanism has been described by Royer and Silverstein (10) and by Cleland (9):

\[
\begin{array}{cccccc}
 & A & B & P & Q \\
\downarrow & \downarrow & \uparrow & \uparrow & \\
E & EA & ( & EP & E \\
\end{array}
\]

At equilibrium, raising the concentration of \( B \) to infinity (while keeping \( B \) in constant ratio with either \( P \) or \( Q \)) will inhibit the \( A \rightarrow P \) exchange (if one exists) as well as the \( A \rightarrow Q \) exchange. The same argument should hold, therefore, if there is no \( Q \) produced by the reaction, i.e. a Bi Uni reaction:

\[
\text{DISCUSSION}
\]

not the case, however, if \( \text{ITP} \) and \( \text{IDP} \) concentrations are varied at constant ratio, as seen in Fig. 12. From the data of Fig. 12, it is clear that substrate inhibition is observed at high \( \text{ITP} \) and \( \text{IDP} \) concentrations. This is similar to the results obtained with the \( \text{P}_1 \rightarrow \text{ATP} \) exchange measurements (Figs. 9 and 10).

Fig. 7 (left). The kinetics of the 2,4-dinitrophenol-sensitive ADP → ATP exchange catalyzed by beef heart submitochondrial particles. The concentrations of ADP and ATP were always kept equal, while the concentration of \( \text{P}_1 \) was 5 mM.

Fig. 9 (right). The kinetics of the \( \text{P}_1 \rightarrow \text{ATP} \) exchange catalyzed by beef heart submitochondrial particles. The concentrations of \( \text{P}_1 \) and ATP were kept equal, while the concentration of ADP was 1 mM.

Fig. 8 (center). The kinetics of the 2,4-dinitrophenol-sensitive ADP → ATP exchange catalyzed by beef heart submitochondrial particles. The concentration of ADP and ATP were always kept equal, while the concentration of \( \text{P}_1 \) was 5 mM.

Fig. 10. \( A \), the kinetics of the \( \text{P}_1 \rightarrow \text{ATP} \) exchange catalyzed by beef heart submitochondrial particles. The concentrations of ADP and ATP were kept equal, while the \( \text{P}_1 \) concentration was as indicated. \( B \), the rate of \( \text{P}_1 \rightarrow \text{ATP} \) exchange versus the \( \log[\text{ATP}] \) at the \( \text{P}_1 \) concentrations indicated. The data are from \( A \).

Fig. 11. The kinetics of the \( \text{P}_1 \rightarrow \text{ITP} \) exchange catalyzed by beef heart submitochondrial particles. The concentrations of \( \text{P}_1 \) and ITP were kept equal, while the concentration of IDP was 1.5 mM.

Fig. 12. The kinetics of the \( \text{P}_1 \rightarrow \text{ITP} \) exchange catalyzed by beef heart submitochondrial particles. The concentrations of IDP and ITP were kept equal, while the concentration of \( \text{P}_1 \) was 5 mM.
As the concentration of B approaches infinity (while kept in constant ratio with P), the A → P exchange will become inhibited.

Studying the Bi Uni production of ATP from ADP and P,

by the above method to determine the order of substrate addition (if there is one) would be a practical impossibility if it were absolutely necessary to conduct the study at equilibrium. However, careful examination of the rate equations governing the isotopic exchange in a Bi Uni mechanism reveals that the same reciprocal plot patterns are predicted regardless of whether or not the reaction is at equilibrium.

By employing the steady state assumption for the enzyme forms E, EA and ( ), where ( ) represents the EAB = EP central complex, the following relationships with respect to the rate constants defined by Diagram II are obtained.

\[
\frac{d[E]}{dt} = -k_1[E][A] + k_2[EA] + k_3[\text{central complex}] - k_4[E][P] = 0 (1)
\]

\[
\frac{d[EA]}{dt} = +k_1[A][E] - k_2[EA][A] - k_3[\text{central complex}] - k_4[EA][P] = 0 (2)
\]

\[
\frac{d[\text{central complex}]}{dt} = +k_5[B][EA] - k_7[\text{central complex}][A] - k_8[\text{central complex}][P] + k_9[A][B] = 0 (3)
\]

Using the empirical method of King and Altman (23) for solving these simultaneous equations in the following relationships which describe the concentration of enzyme forms E and EA.

\[
[E] = \frac{k_2k_3 + k_1 + k_9k_9[1]}{\text{denominator}} (4)
\]

\[
[EA] = \frac{k_3k_1 + k_9[1] + k_9k_9[P]}{\text{denominator}} (5)
\]

where denominator = \(k_2k_3 + k_1 + k_9k_9[A] + k_9k_9[B] + k_9k_9[P] + k_9k_9[3][P] + k_9k_9[A][B]\) and \([Ef] = \text{total enzyme concentration} - [E] + [EA] + [1] \).

The initial rate of the \(A^* \rightarrow P^*\) exchange is given by the relationship:

\[
v_{A^* \rightarrow P^*} = -k_2[1] (6)
\]

where ( ) denotes central complex which is isotopically labeled, and A* and P* denote labeled A and P, respectively. When following the A* → P* exchange, steady state amounts of EA and central complex will become labeled, whereas E will not. The steady state assumptions governing these labeled enzyme forms are given by:

\[
\frac{d[EA^*]}{dt} = k_2[E][A^*] - k_3[EA^*][A] - k_4[EA^*][P] + k_5[A][B] = 0 (7)
\]

\[
\frac{d[1^*]}{dt} = k_7[1][EA^*][B] - k_8[1^*][A^*] - k_9[1^*][P] = 0 (8)
\]

Solving these simultaneous equations results in the distribution equation for labeled central complex to be of the following form:

\[
[e] = \frac{k_2k_3}{k_1 + k_9} + k_9k_9[1] \] (9)

where \(\sigma_2 = \text{specific activity of } [A]\). Substituting in Equation 4, we get Equation 8.

By inspection, it is obvious that if B and P are kept at constant ratio, i.e. \([B] = x[P]\), the resulting squared term in the denominator will make the function go to zero as B approaches infinity. By contrast, if A and P are kept at constant ratio, i.e. \([A] = x[P]\), the resulting function predicts no substrate inhibition at high levels of A. During the derivation of the above equations, at no time was an equilibrium assumption made.

Similar equations can be derived for the \(B^* \rightarrow P^*\) exchange. In this case, only central complex becomes labeled.

\[
\frac{d[1^*]}{dt} = k_3[E][B][A^*] - k_4[1^*][A^*] - k_5[1^*][P] = 0 (10)
\]

or

\[
[1^*] = \frac{\sigma_1k_1k_9[A][B][E]}{k_2k_3 + k_1 + k_9k_9[1]} (11)
\]

where \(\sigma_1 = \text{specific activity of } B\). The rate of the \(B^* \rightarrow P^*\) exchange is given by:

\[
v_{B^* \rightarrow P^*} = \frac{k_2k_9k_9[A][B][E]}{k_1 + k_9} (12)
\]

Substituting Equation 5 and 11 into 12, we get Equation 13

\[
v_{B^* \rightarrow P^*} = \frac{\sigma_kk_9k_9[A][B][E]}{k_1 + k_9} (13)
\]

By inspection, we can see that if B is varied at constant ratio with P, i.e. \([B] = x[P]\), the resulting equation contains squared terms in both numerator and denominator, and no complete inhibition at high B is predicted. Similarly, if A is varied at constant ratio with P, \([A] = x[P]\), the resulting equation predicts no inhibition at high A. Once again, no equilibrium assumptions have been made.

Therefore it can be concluded that the reciprocal plot patterns described by Cleland (9) as diagnostic for an ordered addition of substrates will qualitatively be the same regardless of whether or not the experiments were performed at equilibrium.

The isotopic exchange data presented here are entirely consistent with an ordered addition of the substrates of ATP synthesis. The ADP → ATP exchange follows the patterns predicted by the \(B^* \rightarrow P^*\) exchange equations (see Figs. 7 and 8). The \(P → ATP\) exchange patterns follow those predicted by the \(A^* → P^*\) exchange equation. Most notably, the \(P → ATP\) exchange with the concentration of ADP varied at constant ratio to the concentration of ATP shows total substrate inhibition (Fig. 10A) while the \(P → ATP\) exchange with the concentrations of P and ATP varied at constant ratio produces a linear double reciprocal plot (Fig. 9).

Other predictions of these model isotopic exchange rate equations are also borne out by the data presented. If a dead-end inhibitor binds to the EA enzyme form, the resulting rate equation describing the rate of \(A^* → P^*\) exchange, when written in reciprocal form, would be as follows in Equation 14.
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\[
\frac{\sigma_I}{u_{i \to f}} = \left( \frac{k_i + k_o}{k_i k_o P} \right) \left( 1 + \frac{[I]}{K_i} \right) + \frac{k_d k_o + k_o}{k_i k_o A[A]B} + \frac{k_d k_o + k_o}{k_i k_o A[B]} + \frac{k_d k_o + k_o}{k_i k_o A[A][B]}
\]

where \([I]\) = inhibitor concentration and \(K_i = \) inhibition constant. From this equation, it can be seen that the inhibitor, \(I\), would be an uncompetitive inhibitor when \(A\) is the variable substrate if and only if the concentration of \(A\) is varied at constant ratio with the concentration of \(P\). If the experiment is performed at a fixed concentration of \(P\), the inhibition would appear noncompetitive.

The effect of AMP(\(CH_2\))P on the \(P_i \rightarrow \) ATP exchange conforms to this predicted behavior exactly. When the concentration of \(P_i\) is varied in constant ratio to the ATP concentration, AMP(\(CH_2\))P acts as an uncompetitive inhibitor (see Fig. 6A). If the same experiment is performed at a fixed ATP concentration, a noncompetitive pattern results. Since the competitive initial velocity pattern obtained in Fig. 5 implies that AMP(\(CH_2\))P and ADP bind to the same enzyme form, these data suggest that ADP binds to the ES complex of the ordered model, i.e. ADP is the obligatory second substrate.

Also consistent with this hypothesis is the observation that thiophosphate is a noncompetitive inhibitor of initial velocity when ADP is the variable substrate. It should be noted at this point that the rate equation for the \(P_i \rightarrow \) ATP exchange predicts that thiophosphate will be competitive versus the exchange when \(P_i\) and thiophosphate bind to the same enzyme form (see Fig. 3).

Although we have considered the reactions of ATP synthesis in terms of a Bi Uni mechanism, the involvement of \(H_2O\) can be examined because it is a reaction product. Since \(H_2O\) is always present in saturating amounts, one would expect that high concentrations of ADP would inhibit the \(P_i \rightarrow \) H2O exchange reaction only if ADP was the second substrate to bind in an ordered mechanism. The published data of Jones and Boyer (15) and Cooper (16) show that high concentrations of ADP do indeed inhibit the \(P_i \rightarrow \) H2O exchange reaction. These data are consistent with our results indicating an ordered addition of \(P_i\) and ADP during the synthesis of ATP. However, these data do not indicate the order of \(H_2O\) release with respect to ATP release.

On the basis of these considerations, we propose the following model describing the simplest kinetic mechanism consistent with the data presented:

\[
P_i \quad MgADP \quad MgATP
\]

The distinguishing characteristic of this model is the mandatory order of addition of substrates to the enzyme, with inorganic \(P\) being the first substrate to bind in the direction of ATP synthesis. It should be noted that no definitive conclusions can be made at this time with regard to the order of \(P_i\) and ADP release from the isolated ATPase.

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