Assessment of the Rate of Bound Substrate Interconversion and of ATP Acceleration of Product Release during Catalysis by Mitochondrial Adenosine Triphosphatase

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The oxygen exchange parameters for the hydrolysis of ATP by the F1-ATPase have been determined over a 140,000-fold range of ATP concentrations and a 5,000-fold range of reaction velocity. The average number of water oxygens incorporated into each Pi product ranges from a limit of about 1.02 at saturating ATP concentrations to a limit of about 3.97 at very low ATP concentrations. The latter value represents 400 reversals of hydrolysis of bound ATP prior to Pi dissociation. In accord with the binding change mechanism, this means that ATP binding at one catalytic site increases the off constant of Pi and ADP from another catalytic site by at least 20,000-fold, equivalent to the use of 6 kcal mol\(^{-1}\) of ATP binding energy to promote product release. The estimated rate of reversal of hydrolysis of F1-ATPase-bound ATP to bound ADP + Pi varies only about 5-fold with ATP concentration. The rate is similar that observed previously for reversal of bound ATP hydrolysis or synthesis with the membrane-bound enzyme and is greater than the rate of net ATP formation during oxidative phosphorylation. This adds to evidence that energy input or membrane components are not required for bound ATP synthesis.

Catalytic cooperativity of alternating sites is a prominent feature of the binding change mechanism developed in this laboratory (1, 2) to describe catalysis by mitochondrial and chloroplast ATP synthase and ATPase. An important manifestation of this cooperativity is the change in the nature and tightness of product binding at one catalytic site that results from substrate binding at another site, and, during synthase catalysis, from proton translocation across the coupling membrane. This paper concerns mitochondrial F1-ATPase, for which there are three principal types of evidence that binding of ATP at one site on the multisubunit enzyme promotes the release of products ADP and Pi from another site. One type of evidence is based on oxygen exchange measurements, including the demonstration of the increased water oxygen incorporation into each Pi released during net ATP hydrolysis at low ATP levels and measurements of the distributions of \([^{18}\text{O}]\Pi\), species formed from \([^{18}\text{O}]\text{ATP}\) both with submicronchondrial particles (3) and with purified F1-ATPase (4, 5). A second type of evidence is the retention at a catalytic site of bound ADP rapidly formed from medium ATP at concentrations far below that required for half-maximal velocity of net ATP hydrolysis (1). This observation and the oxygen exchange results are best explained by a continued reversal of hydrolysis of bound ATP to bound ADP and Pi at one catalytic site until ATP binds at an alternate site. A third type of evidence is the direct demonstration of the marked retention of trinitrophenyl ATP (6), or, with enzyme in excess of added ATP, of ATP, ADP, and Pi, at catalytic sites (7, 8) until sufficient substrate is added to bind at another catalytic site. Other related observations with F1-ATPase that agree with alternating site participation include the characteristics of efrapeptin (9) and substrate analog (24, 25) interactions, the inactivation of all catalytic subunits by derivitization of one subunit (see Ref. 13), the initial velocity behavior (1, 14–18), the removal of ADP inhibition by ATP binding with mitochondrial (19, 20) and chloroplast ATPase (21), the ability of ADP + P, to promote dissociation of a very tightly bound adenyl-5'-yl imidodiphosphate from F1-ATPase (22), the effects of cross-linking on activity (18), and the formation of bound ATP from medium P, by chloroplast ATPase (23).

In addition, alternating site participation for F1-ATPase agrees with the considerable evidence for such behavior of the ATP synthase during net ATP synthesis by chloroplasts and mitochondria (see Ref. 2 for summary of earlier data) including modulation of the extent of incorporation of water oxygens into ATP by changing the medium concentrations of either P, or ADP (24, 25) and recent observations on binding affinity changes (26).

We have applied improved \(^{18}\text{O}\) measurement techniques to the determination of how close to 1 water oxygen is incorporated into each Pi released when saturating concentrations of ATP are cleaved and how close to 4 water oxygens are incorporated into each Pi released as the ATP level is lowered until maximum water oxygen incorporation occurs. Previous measurements (4, 5) have not been sufficiently accurate to allow conclusion of whether slightly greater than one water oxygen may be incorporated into each Pi, formed at high ATP concentrations nor have they been extended to sufficiently low ATP concentrations to reach the expected upper limit in water oxygen incorporation. Measurements of the limits of water oxygen incorporation at high and low ATP, among other things, allows approximation of how much rate constants at one catalytic site may be changed by ATP binding at another. Such approximations are given herein. In addition,
measurements of the rate of bound ATP cleavage and reformation over a wide range of ATP concentrations and thus of the total catalytic turnover allow assessment of whether the capacity for catalysis of interconversion of bound ATP, ADP, and P_i is relatively independent of substrate concentration and membrane-associated events. Data reported here show independence of such catalytic capacity.

**EXPERIMENTAL PROCEDURES**

**Materials—** F-1-ATPase was isolated by the method of Penefsky (27) and stored at 4 °C as a suspension in 50 mM ammonium sulfate, pH 8.0, and in the presence of 0.125 M sucrose, 25 mM Tris-HCl, 1 mM EDTA, and 2 mM ATP. For experiments an aliquot of the suspension (0.1 ml) was pelleted, and the pellet was washed twice with the above buffer (ATP omitted), then dissolved at room temperature by addition of 0.005 ml of a buffered solution containing 50 mM Tris-sulfate, 60 mM K_2SO_4, and 1 mM EDTA at pH 8 (28) and centrifuged through a 1-ml syringe packed with Sephadex G-50 (80 mesh) previously equilibrated with the buffer solution (7). The protein concentration was estimated by the Lowry assay with bovine serum albumin (Sigma) as a standard. The concentration can be converted to a dry weight basis by multiplication by 1.16 assuming that the concentration as measured by the Lowry assay with bovine serum albumin as a standard can be converted to the dry weight basis by division by 1.16 (27). The specific activity of the ATPase was 110 units (moles Pi released per mg protein at 30 °C and 0.1 M lactate) during the time period over which these experiments were performed. Pyruvate kinase was purchased as the lyophilized powder (Sigma, rabbit muscle) and was dissolved in the experimental buffer (30 mM Tris-acetate, 30 mM K-acetate, pH 7.6) and freed of any salt or nucleotide present by centrifugation through a syringe as above. Any insoluble material was removed by centrifugation. The concentration of pyruvate kinase was determined from its absorbance at 280 nm using an absorption coefficient of 0.54 (mg/ml)_1. ATP was purified by column chromatography (30) and stored frozen at neutral pH. [3H]ATP and [γ-32P]ATP were products of ICN and Amersham Corp., respectively. [3H]ATP and [γ-32P]ATP were used during the assay of P_i conversion of ADP to [3H]ATP. The ATPase reaction was started with addition of F-1 ATPase in the amount indicated and continued for enough time to generate 50 to 100 nmol of P_i per ml of sample. The final sample volume was 2.5 ml. The reactions were quenched by vortexing with 2 ml of cold chloroform, and the samples were placed on ice. For each sample, an aliquot of 1 ml was processed for reaction by GC-MS using identical amounts of [32P]Pi into the absence of the diastereomeric P_i in the F-1 ATPase reaction system and for determination of the total amount of ATP and ADP present (see below), the remainder of the reaction mixture was diluted with water to about 13 or 23 ml with addition of 1 μmol each of carrier ATP and ADP and, in some cases, of 0.1 M Tris. The amount of contaminant P_i was determined by addition of a known amount of highly enriched [32P]Pi to a reaction mixture that had been quenched prior to addition of the enzymes with subsequent measurement by GC-MS of the dilution of the enrichment of the added [32P]Pi by the endogenous P_i. The amount of [32P]Pi, formed from any enzymic or nonenzymic hydrolysis of [32P]Pi, was determined by incubating duplicate samples in the absence of any nucleotide added at the indicated concentrations for enough time to generate 50 to 100 nmol of P_i per ml of sample. A known amount of 100 nmol of recrystallized natural abundance P_i, (KH_2PO_4) was added to 1 ml of each quenched reaction mixture, and the amount of [32P]Pi, was determined by GC-MS from the relative proportions of enriched and natural abundance P_i present. Typically, duplicate samples in the absence of F-1 ATPase were run for three different reaction times, and the amount of [32P]Pi, formed were fit to a straight line as a function of time after addition of pyruvate kinase. The amounts of [32P]Pi, present in other samples for which duplicate samples in the absence of F-1 ATPase were not run were calculated from this fit and the times these samples had been exposed to pyruvate kinase. While only 0.9 to 2.6 mmol of [32P]Pi, came from hydrolysis of [32P]Pi, P_i in the absence of ATPase the resulting correction to the P_i0 species was substantial at very low ATP concentrations. For example, the correction to this species constituted 89% of the observed value at 0.035 μmol ATP, 80% at 0.058 μmol ATP, and 50.2% at 0.16 μmol ATP (see Table 1). At higher ATP concentrations, this correction became negligible.

ATPase was separated by anion exchange chromatography at 4 °C on 0.7 × 5 cm columns. Following sample application the columns were washed with 1 ml of 50 mM NaOH, 60 mM K_2SO_4, and 1 mM EDTA at pH 8.5 (33). [3H]ATP and [γ-32P]ATP were washed with 1 ml of 0.5 M H_2SO_4, 1 M K_2SO_4, and 1 mM EDTA at pH 2.5 (33). [3H]ADP, [γ-32P]ATP, and [3,32P]Pi, were washed with 1 ml of 10 mM HCl, ADP with 8 ml of 30 mM HCl, ATP with 8 ml of 60 mM HCl, and ATP with 8 ml of 1 N HCl. In most cases, the ATP and ADP fractions were lyophilized to dryness. The total amount of ATP present during the ATPase catalysis was calculated from the amount of H_2O added to the reaction column.

For isolation of the P_i formed by ATPase, and in controls without enzyme, aliquots of quenched reaction mixtures were adjusted to 1 ml with H_2O, 0.5 ml of liquidified phenol was added, and the protein removed along with the phenol by centrifugation. The phenol layer was washed with 1 ml of H_2O which was added to the organic aqueous layer. Phenol present in the combined aqueous layers was extracted into 2 ml of isobutanolbenzene (1:1, v/v). For most experiments molybdate was added, the phosphomolybdate was extracted with benzene, and the benzene was evaporated to an aqueous H_2O solution (30). The P_i was separated from the molybdate and converted to H_2PO_4 by column chromatography (30), the H_2PO_4 was lyophilized, converted to triethylphosphate with diazomethane (36, 32), and analyzed for [32P]Pi, species by GC-MS. The recovery of P_i by this method was quantitative and in some cases higher than 100%. In one of the latter cases the analysis by GC-MS was complicated by the presence of impurities in the diazoethane, and the results of such samples have been discarded. Other experiments were carried out as above, except that the P_i was not purified by the molybate extraction method. Instead the

The abbreviation used is: GC-MS, gas chromatography-mass spectrometry.
0.65- or 1-ml samples were quenched with 2 ml of chloroform, aliquots of the supernatant were diluted to about 13 ml with cold H2O and 5 ml of Mg-acetate, a trace of [y-32P]ATP, and the sample prepared containing 5 mM Mg-acetate, a trace of [y-32P]ATP, and the indicated amount of [18O]ATP at 30 °C in the Tris acetate buffer. The P1-ATPase, 6-7 mg/ml, was diluted 11-fold, and 5 μl were added to 0.05 ml of the above buffer at 30 °C. To initiate the reaction, 0.45 ml of the reaction mixture was added to the ATPase. After about 100 nmol of Pi had been produced, the reaction was quenched with 5 ml of cold chloroform. In one experiment, 5 μmol of Na2EDTA were added at this point. The aqueous layer was isolated and its volume adjusted to about 13 ml with cold H2O. Most of the nucleotide was removed by charcoal, and the P1 was purified by column chromatography with the use of columns (0.7 x 5 cm) as above. The amount of P1 formed was determined by comparison of the amount of 32P in the fraction with the total 32P present in the original sample. The amounts of contaminant P1 present and of [18O]P1 present in the [18O]ATP were determined as described above.

The 18O enrichment of the phosphoryl group of [18O]phosphoenolpyruvate was determined by analysis of the [18O]P1 produced by hydrolysis of the [18O]phosphoenolpyruvate catalyzed by glucose kinase in a coupled reaction (32). The enrichment of the γ-phosphoryl group of [18O]ATP was determined similarly.

Determination of [18O] in P1—The lyophilized samples of H3PO4 were derivatized with diazomethane in ether (30) using appropriate precautions. After careful evaporation of excess diazomethane and ether, the resulting triethylphosphate was dissolved in dichloromethane at a concentration of about 1 nmol/μl. An aliquot containing about 1 nmol of sample was analyzed for 18O with a Hewlett-Packard 5995A GC-MS. With the injection port at 250 °C, isothermal elution at 160 °C, and a helium flow rate of 30 ml/min the triethylphosphate eluted in about 1 min from a glass column (6 feet x 2 mm) packed with 3% OV-275 on 40-60 Chromosorb T. Using selective ion monitoring, the diethylphosphate ion fragment of m/z 155 and its corresponding 18O counterparts of m/z 157, 159, 161, and 163 (designated as P18O, where i = 0, 1, . . . , 4) were analyzed by scanning up to 70 times/analysis.

Calculations from Oxygen Incorporation Measurements—The observed distributions of 18O in [18O]P1 were corrected for, in order, spillover from a fragment of m/z 153 (no 18O) that contained four oxygen atoms with the same distribution of 18O as the diethylphosphate fragment, its size being determined by analysis of a natural abundance sample, contamination from unenriched P1, and, where necessary, the presence of [18O]P1, produced by hydrolysis of [18O]ATP or [18O] phosphoenolpyruvate in the absence of P1. The resulting distributions are referred to as the observed distributions throughout this paper.

If no oxygen exchange accompanies the hydrolysis, the enrichment, the distribution of the phosphoryl groups of [18O]ATP or of [18O]phosphoenolpyruvate can be calculated from Equation 1,

\[ E_{P1} = \frac{[3(18O) + 2(18O_2) + 18O_4] - E_{4O}}{3} \]

where \( E_{4O} \) is the observed fractional enrichment (0 ≤ \( E_{4O} \) ≤ 1), \( 18O_4 \) (0 ≤ \( 18O_4 \) ≤ 1) is the relative abundance of the diethylphosphate species containing i atoms of 18O (0 ≤ i ≤ 4), and \( E_{4O} \) is the fractional 18O content of the solvent which is 0.00198 for natural abundance 18O in H2O.

If oxygen exchange occurs, the distribution of the [18O]P1 product of the F1-ATPase reaction will depend on the rate of exchange and the enrichments of both the substrate ([18O]phosphoenolpyruvate if an ATP-regenerating system is used) and the water because the observed exchange is the sum of the exchange out of oxygen from the substrate and exchange in of water oxygen enriched with natural abundance 18O. Exchange in of 18O from water oxygen will increase the size of the \( 18O_4 \) species so that, for instance, if all of the oxygen of [18O]Pi had undergone exchange with water oxygen, the amount of the \( 18O_4 \) species would be 0.79% for natural abundance water and not zero. Thus, the observed amount of exchange will be less than the calculated exchange lost if the observed enrichment can be significant if a very large amount of exchange has occurred and the P1-ATPase is not very much greater than 0.79%. The observed enrichment may be viewed as a sum of two terms as in Equation 2,

\[ E_{P1} = E_{ATP} + (1 - \alpha)E_{4O} \]

where \( E_{P1} \) is the observed enrichment, \( E_{ATP} \) and \( E_{4O} \) are the enrichments of the substrate ([18O]ATP or [18O]phosphoenolpyruvate) and the H2O, respectively (the former being calculated from the data using an equation analogous to Equation 1), (0 ≤ \( E_{4O} \) ≤ 1) is the fraction of the [18O]P1 product that arose from exchange out of the 18O of the substrate, and (1 - \( E_{4O} \)) is the fraction of the [18O]P1 product that arose from the exchange in of the 18O in the water. Values of \( \alpha \) are readily obtained from Equation 2 because the values of all other terms are known.

For all ATP concentrations the oxygen exchange parameters were calculated by comparing the enrichments of the [18O]P1 product and the phosphoryl group of the substrate ([18O]ATP or [18O]phosphoenolpyruvate). The enrichment of the [18O]P1 due to exchange out of the 18O of the substrate is given by \( a_{4O} \). The exchange parameters (30) are given by Equations 3-6 and are further defined under "Results."

\[ P_r = 4(1 - 1/(P/O)) \]

\[ R = P_r/(1 - P_r) \]

\[ \alpha = R \]

Values of \( P_r \) were used along with the transition probability functions of Hackney (34) and the distribution of 18O in the phosphoryl group of [18O]phosphoenolpyruvate to calculate the theoretical distributions expected if all P1 released was produced by one reaction pathway to give the total amount of oxygen exchange observed. For these calculations when exchange was extensive an appropriate correction was made for the contribution of 18O from the natural abundance of water (see above). Calculations were made with aid of a Hewlett-Packard 9825A computer.

RESULTS

The Use of [18O]Phosphoenolpyruvate for γ-[18O]ATP Regeneration—For the experiments at lower ATP concentrations it was advantageous to use phosphoenolpyruvate labeled in the phosphoryl group with 18O and pyruvate kinase to maintain a nearly constant low γ-[18O]ATP concentration. This also serves to prevent any slow ADP-dependent medium P1 = HOH exchange (4). It was necessary to check if the catalytic rate remained constant over a sufficient time period to collect ample P1 for 18O analyses. An ATP-regenerating system consisting of 0.2 mM phosphoenolpyruvate and 0.05 mM pyruvate kinase was found to be sufficient to maintain a linear time course of hydrolysis until more than 100 nmol of P1 were accumulated. Similar linearity was also observed with use of 0.5 mg/ml of pyruvate kinase (data not shown). An ATP-regenerating system was used for all exchange experiments except for those done at ATP concentrations above 0.5 mM.

ATP Dependence of Water Oxygen Incorporation into P1, Released—The results of a number of 18O incorporation experiments are given in Fig. 1. Three parameters are plotted as a function of the total ATP concentration. One is \( r \), the average total rate of oxygen exchange, which is a measure of the rate of formation of bound ATP from bound ADP and P1. Another is the O/P ratio, the average number of water oxygens present in each P1 released. This can vary between one (no oxygen exchange) and four (infinite oxygen exchange). The third parameter shown is the steady state velocity, which is estimated from single-point measurements from the same reaction mixtures, or for ATP concentrations greater than 0.5 mM, by steady state velocity measurements carried out in the presence of an ATP-regenerating system (1).

Analysis of the [18O]P1, by GC-MS gives the distribution of the 18O in this product. These distributions are given in Table I along with the final total concentrations ATP and values of \( P_r \), the partition coefficient, and \( R \), the average number of
reversals of bound ATP hydrolysis for each net ATP cleavage.
The reactions leading to oxygen incorporation into tightly bound reactants (depicted by <) during ATP hydrolysis can be depicted as in Equation 7

\[
\text{HOH} + E < \text{ATP} \rightleftharpoons E < \text{ADP} + \text{Pi},
\]

The value of the hydrolysis constant, \(k_h\), governs the total rate of water oxygen incorporation into Pi. One water oxygen must appear in each Pi for the net hydrolysis reaction. The rate constant for formation of bound ATP from bound ADP and Pi, \(k_r\), governs the rate at which Pi oxygens form water oxygens and thus the rate at which water oxygens are incorporated into Pi by exchange during net hydrolysis. The rate of an irreversible step-terminating exchange is governed by \(k_t\). The partitioning of bound Pi between exchange and release is given by the partition coefficient, \(P\), which is the ratio \(k_r / (k_r + k_t)\). Pi can range from 0 (no exchange) to 1 (infinite exchange) (30). The parameter \(R\) is a measure of the number of reversals of bound ATP hydrolysis prior to Pi release (\(R = k_t / k_r\)). Also given in Table I are the theoretical distributions corresponding to the observed \(^{18}\text{O}\) enrichment of the Pi produced (see under "Experimental Procedures").

The results of Fig. 1 and Table I allow several conclusions. The observed upper limit of the O/P ratio at low ATP concentrations is 3.97 ± 0.01 (S.D., n = 4). This corresponds to an \(R\) value of 410 ± 63. The O/P values decrease markedly as the ATP concentration is raised to 20 \(µ\text{M}\). The net reaction velocity increases only slightly over this range, however. The total rate of synthesis of enzyme-bound ATP from ADP + Pi, about 58 to 70 mol of ATP (moles of F\(_1\)-ATPase\(^{-1}\) s\(^{-1}\)), varies little over the range of ATP concentrations where values of O/P and \(R\) vary greatly. The distributions of \(^{18}\text{O}\)Pi, in Table I are all homogenous within experimental error. This means that each molecule of \(^{18}\text{O}\)ATP bound has the same probability of undergoing the average amount of exchange observed for each Pi released.

For an alternating site mechanism the maximum values of O/P and \(R\) would be expected to characterize turnover of only one catalytic site per three present, e.g. with conditions of excess enzyme, Grubmeyer et al. (7) have determined the rate constants for individual reaction steps under conditions of excess enzyme. Their results, in harmony with our data, predict a limiting O/P value at very low ATP concentrations of very close to 4. However, the extremely tight binding of ATP for single-site catalysis demonstrated by Grubmeyer et al. (7) makes it likely that at the lowest ATP concentrations employed in the studies presented here (Fig. 1) and in those of Gresser et al. (1) appreciable two-site catalysis occurred. Even so, the value for the rate of reversal of bound ATP hydrolysis obtained by Grubmeyer et al. (7) was within the range of values obtained in this work (Fig. 1) taking into account that the equilibrium constant for hydrolysis of bound ATP with very low medium ATP concentration is near unity (7).

As discussed under "Experimental Procedures," the distributions labeled observed in Table I have been corrected. One necessary correction arose because pyruvate kinase was found to catalyze the hydrolysis of phosphoenolpyruvate. The rate was about 0.04 \(\mu\text{M}/\text{min}\) in the pH 7.4 buffer at 30 °C in the presence of 2.4 mm phosphoenolpyruvate and 0.5 mg/ml of pyruvate kinase and was increased about 2-fold by inclusion of 5 mm Mg\(^{2+}\)-acetate. Correction for the \(^{18}\text{O}\)Pi produced by this reaction was the greatest source of error in the distributions at low ATP concentration (Table I). This is the reason for the use of a less than optimal concentration of pyruvate kinase, 0.05 mg/ml, in our experiments. A few experiments performed using 0.5 mg/ml of pyruvate kinase gave essentially the same exchange parameters (Table I) but slightly greater velocities. These velocity values were the ones used to calculate values of \(r\) at very low ATP concentrations (Fig. 1). At higher ATP concentrations where the amount of \(^{18}\text{O}\)Pi species was reduced, the greatest source of error was in correction for contaminant unlabeled Pi.

At saturating ATP concentrations, the O/P and \(R\) parameters have values of 1.02 ± 0.01 (estimated uncertainty, n = 3) and 0.02 ± 0.01, respectively. Table II gives values of \(R\) and \(P\), and, in addition, the distributions of the \(^{18}\text{O}\)Pi product and of the starting \(^{18}\text{O}\)ATP. As noted above, no \(^{18}\text{O}\)ATP-regenerating system was employed in these experiments. The O/P values determined at lower ATP concentrations in the presence of the ATP-regenerating system agree...
the extensive oxygen exchange that occurs with less than micromolar concentrations of ATP. The change in extent of the text. The F₁-ATPase ranged from those obtained by Choate et al. (4) in the absence of an ATP-regenerating system and with 5 μM or greater ATP present.

**DISCUSSION**
The experimental approaches used in this paper allow a better measurement than previously reported of both the very small amount of oxygen exchange that occurs during hydrolysis by F₁-ATPase of millimolar concentrations of ATP and the extensive oxygen exchange that occurs with less than micromolar concentrations of ATP. The change in extent of exchange is strikingly large. As noted in Table I the P₁ formed at high ATP concentrations acquires only about 0.02 oxygens from water by exchange, while at low ATP concentrations about 99% of the oxygens of the P₁ released are derived from water. The medium ATP concentration thus modifies markedly one or more of the rate constants governing the oxygen exchange of P₁ bound at the catalytic site prior to its release. We have interpreted these findings on the basis that the oxygen exchange arises from the reversal of hydrolysis of ATP bound at the catalytic site, as indicated by Equation 7. It has been shown for pyrophosphatase (35), sarcoplasmic reticulum ATPase (36), alkaline phosphatase (37), and myosin ATPase (38, 39) that the rate of oxygen exchange correlates with the rate of formation of a covalent compound of ATPase at the catalytic site. The ATP → HOH exchange of chloroplast thylakoids has been shown to be accompanied by covalent bond cleavage (40). Such results and the known characteristics of phosphoryl transfer reactions (41) give assurance that interpretation of the oxygen exchange of P₁-ATPase as resulting from reversible hydrolysis of bound ATP is justified. The estimated numbers of reaction reversals prior to release of P₁ R (Table I), are minimal values, as it is assumed that the rotation or tumbling of the bound P₁ is sufficiently rapid that all phosphate oxygens are equivalent. Such a rapid tumbling of bound P₁ occurs during myosin ATPase-catalyzed hydrolysis of ATP (42). Also, any definite preference for more rapid exchange of 3' out of the 4 oxygens (a plausible suggestion if the γ-P₉O₅ portion of a bound P₁, rotated around one fixed O−P bond) would have been reflected by an heterogenous distribution of labeled oxygens in the product P₁. This was not observed.

The participation of tightly coupled alternating sites offers a satisfying explanation for the modulation by medium ATP of the extent of reversal of cleavage of bound ATP. The extensive exchange at low ATP concentrations reflects the properties of an enzyme species containing tightly bound substrates and products undergoing reversible hydrolysis on
only one of three catalytic sites. At high ATP concentrations, the rate of bound ATP synthesis from bound Pi and ADP, \( r \), remains high even though the probability of reversal of the hydrolysis step is only about 2% because the flux across this step is now very rapid, and many bound Pi molecules are exposed to possible exchange in a short time period. This is as expected if both the rate constant for reversal of this step, \( k_\text{r} \), and the concentration of the exchanging species are not greatly affected by ATP concentration. We are thus led to suggest that whenever the medium ATP molarity exceeds the F1-ATPase molarity, the enzyme retains one and only one catalytic site that continues to catalyze reversible cleavage of bound ATP. Other catalytic sites may be filled with ATP or ADP plus Pi, but be inactive, i.e. not catalyzing interconversion of these reactants. Fig. 2 depicts such behavior at high ATP concentrations where all three sites are occupied.

A continued reversible cleavage of tightly bound ATP at a rate suggested by the data of Grubmeyer et al. (7) and the results of Fig. 1 make unnecessary the postulation, as in a previous scheme (1), of a species with bound ATP at a catalytic site that does not undergo oxygen exchange. However, the active catalytic site must change in properties as ATP adds to the transitionally inactive catalytic sites. The rapid net rate of catalysis at saturating medium ATP concentrations requires that the rate of hydrolysis of tightly bound ATP must be faster during one or more steps of the sequence than during single-site catalysis. One possibility is that the important conformational changes during the rapid binding change step are accompanied by an equally rapid cleavage of bound ATP. Coordinated and not single steps would allow such a possibility to be included in a reaction scheme and would require appropriate modification of the three-site scheme presented earlier from this laboratory (1). That scheme followed a usual pattern for enzyme reaction mechanisms, namely a sequence of steps that may form the principal pathway, with recognition that branched pathways with sequential steps may also occur. With the type of site-site cooperation and properties of the mitochondrial ATPase that are being revealed, catalytic pathways involving a single series of steps may not provide an adequate description. With the ATPase, interconversion of substrates at the tight catalytic site may continue while binding and release steps occur at other catalytic sites (see Fig. 2).

An alternative explanation for the high rate of oxygen exchange; \( r \), at higher ATP levels is that when two or three catalytic sites are occupied a more rapid ATP cleavage is not limited to the binding change step but occurs at the active site during all steps of the catalytic sequence. At high ATP concentrations the rate constant for cleavage of bound ATP could approach the turnover rate, about 600 s\(^{-1}\) (Fig. 1) which is considerably above the rate constant obtained at very low ATP levels, 12 s\(^{-1}\) (7). A continued oxygen exchange at high ATP concentrations would be expected if the rate constant for reversal remained about the same. The equilibrium at the active site would, of course, greatly favor bound ADP + Pi.

Measurement of the equilibrium constant for the reversible hydrolysis of bound ATP at high ATP concentrations would be useful but does not appear to be readily accessible experimentally. Hence, although main patterns and consequences of coordinated three-site interactions are becoming apparent, there are clearly a variety of possible intermediate states and subtle influences of the events at one subunit on events at another that need further study. More information and penetrating evaluations appear essential for an adequate understanding of the mechanism of action of the complex enzyme. Nevertheless, the striking demonstration that the ATPase continues to catalyze the formation of the covalent bond structure of ATP over a wide range of ATP concentrations sufficient to change the steady state velocity over 5000-fold adds to the evidence that ATP formation at the catalytic site is independent of membrane binding and any associated proton motive force.

It is of interest to compare the rates of interconversion of bound ATP and bound ADP + Pi, observed with the isolated F1-ATPase to those of the membrane-bound form active as part of ATP synthase or for ATPase preparations intermediate in complexity between the F1-ATPase and the ATP synthase. Values of the rate of formation of bound ATP during ATP hydrolysis (i.e. values of \( r \)) by submitochondrial particles were estimated from literature results (43) using a value of 0.147 mg of F1 per mg of submitochondrial particle protein (44). These values and those estimated from oxygen exchange studies of the oligomycin-sensitive ATPase (4) and a reconstituted ATPase (45) are given in Table III. No dependence on ATP concentration was evident over the range 20 \( \mu \text{M} \) to 5 \( \text{mM} \). The values of \( r \) fall in the range of 13 to 79 s\(^{-1}\) and are all similar within a factor of 5 or less to those found in this work with the isolated F1-ATPase (Table I). The rate of formation of bound ADP + Pi, from bound ATP during ATP synthesis by submitochondrial particles under energized conditions can be calculated from the results of Hackney and Boyer (24). The value of this rate is proportional to the activity of the particles (Table III). The rate of bound ATP hydrolysis during net ATP synthesis by submitochondrial particles shows no obvious dependence on the concentrations of ADP or of Pi, and the values of \( r \) are similar to those obtained under conditions of net ATP hydrolysis. Also, the rate of medium ATP formation by the ATP synthase is less than the rate of bound ATP formation by the F1-ATPase (Table III). To summarize, the rate of reaction reversal at the active site of the F1-ATPase or the ATP synthase is largely independent of the presence of phospholipid, membrane-bound proteins, and components including the proton-con-
dunting apparatus, protonmotive force, and whether net synthesis or hydrolysis is occurring. The characteristics of Fig. 2 thus pertain to both net ATP synthesis and hydrolysis.

The constancy of the rate of synthesis of bound ATP from bound ADP + P\textsubscript{i} supports the reaction model in which the pronounced effect of the increase in medium ATP concentration in decreasing the extent of water oxygen incorporation into each P\textsubscript{i} does not arise from variation in the exchange step. Such promotion is readily understood if part of the energy of binding of ATP to P\textsubscript{i} is reflected in conformational changes in F\textsubscript{1}, transmitted by subunit interaction to an alternate catalytic site so as to drastically increase off constants for both P\textsubscript{i} and ADP. If, as indicated by the data, the k\textsubscript{i} remains about constant, the 20,000-fold change in the value of k\textsubscript{i} would correspond to the use of about 6 kcal of binding energy to promote product release. Such use of binding energy to promote catalytic steps concurs with views advanced by Jencks (46). During net formation of ATP by ATP synthase catalysis, an input of about 13 to 14 kcal/mol of ATP is required to reach the (ATP)/(ADP)(P\textsubscript{i}) ratios attainable by mitochondria (47). If a quarter of this energy input is used for export of ATP from the matrix space, then 10 to 11 kcal is used for formation of ATP by the synthase. The estimate of use of 6 kcal for conversion of tight to loose ADP and P\textsubscript{i} is compatible with about one-half of the energy input for the binding change during ATP synthesis being used to promote competent binding of ADP and P\textsubscript{i}, and the other half to promote release of tightly bound ATP.

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