Reaction Mechanism of the Membrane-bound ATPase of Submitochondrial Particles from Beef Heart*

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Submitochondrial particles from beef heart, washed with dilute solutions of KCl, so as to activate the latent, membrane-bound ATPase, F1, may be used to study single site catalysis by the enzyme. [γ-32P]ATP, incubated with a molar excess of catalytic sites, a condition which favors binding of substrate in only a single catalytic site on the enzyme, is hydrolyzed via a four-step reaction mechanism. The mechanism includes binding in a high affinity catalytic site, $K_a = 10^{12} \text{M}^{-1}$, a hydrolytic step for which the equilibrium constant is near unity, and two product release steps in which P, dissociates from catalytic sites about 10 times more rapidly than ADP. Catalysis by the membrane-bound ATPase also is characterized by a 106-fold acceleration in the rate of net hydrolysis of [γ-32P]ATP, bound in the high affinity catalytic site, that occurs when substrate is made available to additional catalytic sites on the enzyme.

These aspects of the reaction mechanism of the ATPase of submitochondrial particles closely parallel the reaction mechanism determined for solubilized, homogeneous F1 (Grumbeumer, C., Cross, R. L., and Penefsky, H. S. (1982) J. Biol. Chem. 257, 12092–12100). The finding that removal of the enzyme from the membrane does not significantly alter the properties of single site catalysis lends support to models of ATP synthesis in oxidative phosphorylation, catalyzed by membrane-bound F1, that have been based on the study of the soluble enzyme.

The highly active ATPase, F1, solubilized from the inner membrane of beef heart mitochondria is a multisubunit protein (1) that catalyzes the final transphosphorylation reaction in the synthesis of ATP from ADP and P, during oxidative phosphorylation (2). Studies with an ATP analog, trinitrophenyl ATP, provided direct evidence for negative cooperativity in nucleotide binding to catalytic sites on the enzyme for the presence of at least two catalytic sites on the protein and for catalytic site cooperativity (3, 4). Further studies on the mode of ATP hydrolysis by the soluble enzyme supported a four-step mechanism for hydrolysis in a single catalytic site (5, 6) as shown in Scheme 1.

$F_1 + ATP \rightarrow F_1 \cdot ATP \rightarrow F_1 \cdot ADP \cdot P_i \rightarrow F_1 \cdot ADP \rightarrow F_1 \cdot P_i$

**SCHEME 1**

ATP binds in the catalytic site to form the enzyme-substrate complex in the first step. The second step is the catalytic step. Hydrolysis takes place but products remain bound in the catalytic site. Steps 3 and 4 are product release steps. Both the individual rate constants and the equilibrium constants were determined for each of the steps in the mechanism. ATP binds in catalytic sites with very high affinity ($K_a = 10^{12} \text{M}^{-1}$). The equilibrium constant for the catalytic step ($K_2$) is near unity (5). These observations support proposals (5, 7) that ATP forms in catalytic sites on F1 from bound ADP and P; with little or no change in free energy and that the major requirement for energy in oxidative phosphorylation is for the release of product ATP from high affinity catalytic sites.

The proposed model for ATP synthesis in oxidative phosphorylation (5) was based directly upon the reaction mechanism (Scheme 1) elucidated for the soluble ATPase (5). However, the ATPase is membrane-bound in situ, and it is possible that there could be significant differences, both qualitatively and quantitatively, in the details of the reaction mechanism for ATP hydrolysis by the soluble versus the membrane-bound forms of the enzyme. The affinity constant for ATP in the catalytic site of membrane-bound F1 is the same as that of the soluble enzyme: $10^{12} \text{M}^{-1}$ (8). This paper describes experiments which show that it is possible to study the reaction mechanism of membrane-bound F1, that is, the ATPase of submitochondrial particles. It was found that membrane-bound F1, studied under the conditions of single site catalysis (a molar excess of catalytic sites over [γ-32P]ATP), exhibits rate constants and equilibrium constants similar to those demonstrated with the soluble enzyme. These observations lend added support to proposed models for the mechanism of action of the enzyme in oxidative phosphorylation (5, 7).

**EXPERIMENTAL PROCEDURES**

32P (enzyme grade) was purchased from ICN and used without further purification. [γ-32P]ATP was prepared by enzymatic exchange (9) and purified on a short column of Dowex 1 chloride. Crystalline yeast hexokinase was purchased from Sigma and dialyzed to remove ammonium sulfate. Cellulose nitrate filters, Whatman type WCN, 0.45-μm pore size, were purchased from Arthur H. Thomas. Bovine serum albumin (fatty acid-free) was purchased from Boehringer Mannheim.

Submitochondrial particles, ETPH(Mg2+), were prepared from beef heart mitochondria (10) and activated by washing with buffered solutions of KCl as described (8). The specific ATPase activity of preparations of activated particles ranged from 6 to 10 units/mg of protein. Rapid mixing experiments were carried out in an Update Instruments apparatus (Madison, WI) apparatus as described earlier (5). All of the manipulations for aging of the enzyme-substrate complex in the apparatus were carried out at a constant flow velocity. The age of the reaction mixtures was varied by changing the length of the aging.

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1 The abbreviation used is: ETPH(Mg2+), submitochondrial particles prepared in the presence of ATP and Mg2+. 

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hose, or, in the push-push mode (5), by varying the time delay between the first and the second push. The amounts of $^{32}\text{P}$ and $[\gamma-^{32}\text{P}]\text{ATP}$ in reaction mixtures were determined by scintillation counting of aqueous and organic phases after extraction of perchloric acid-quenched samples with isobutyl alcohol/benzene (11). Aliquots of each of the phases were added to a scintillation mixture described earlier (12) and counted in a Beckman LS-350 liquid scintillation counter. Membrane-bound radioactivity was separated from the free form by filtration on nitrocellulose filters. Two filters, inserted one on top of the other, were mounted in a syringe filter holder (No. 4320 of Gelman Sciences, Inc., Ann Arbor, MI). The top of the filter holder was removed by machining so as to expose the entire available surface area of the filter. The filter holder, with a 5-cm length of polyethylene hose inserted in the outflow port, was mounted in a suction apparatus. The hose served to guide the filtrate into a receiving test tube that contained 20 $\mu$l of a solution consisting of 60% perchloric acid and 5 mM ATP. Filtration times averaged 15–25 s depending on the protein concentration of the reaction mixtures. At least 0.5 ml of each reaction mixture was passed through the filters, and approximately 0.8 ml of filtrate was collected. The filtrate was considered to be representative of the entire sample.

The amounts of ADP and ATP associated with activated submitochondrial particles were determined on the supernatants remaining after the particles were sedimented by centrifugation (free nucleotides) and on neutralized perchloric acid extracts of particle suspensions (total nucleotide). Bound nucleotide was calculated as the difference between total and free nucleotide in the sample. The assay for ATP and ADP was carried out as described (5) except that luminescence developed in the luciferin/luciferase mixtures was quantitated with an instrument constructed in this laboratory.

Protein was determined by a modified biuret procedure (5) using bovine serum albumin as standard. One unit of ATPase activity is defined as 1 $\mu$mol of ATP hydrolyzed per min. Specific activity is defined as units/mg of protein.

RESULTS

The specific ATPase activity of ETPH(Mg$^{2+}$) as prepared is normally in the range of 0.5–1 unit/mg of protein. In order to prove the catalytic sites of the membrane-bound enzyme with ATP, it is necessary to activate the ATPase. Washing with buffered solutions of potassium chloride (8) unmasks the enzyme, presumably by removing or displacing the ATPase inhibitor (13), and raises the specific activity of the resulting particles to 6–10 units/mg. As shown in Table I, however, the specific activity could be raised to 14 by further incubation at pH 10 for 2 h. If a value of 14 units/mg is taken to be the maximum attainable, then the bound enzyme in ETPH(Mg$^{2+}$) particles is 90–95% masked and the KCl-activated preparations used in the experiments described in this paper are 25–50% masked. Measurements of the maximal specific ATPase activity of submitochondrial particles are relevant to the absolute amount of F1 present on the membrane and to the turnover number of the membrane-bound enzyme. Unless the assumption is made that F1 is more active on the membrane than in solution (there being no evidence for such an assumption in reconstitution experiments (15)), it would appear that 10% of the protein of ETPH(Mg$^{2+}$) particles is F1, since the maximal specific activity of the soluble enzyme assayed under the same conditions is about 120 units/mg (14). Similar conclusions regarding the F1 content of submitochondrial particles were drawn from measurements with particles passed through a column of Sephadex (15). Using a $M_s$ of 347,000 for F1 (16), it is calculated that each milligram of ETPH(Mg$^{2+}$) contains 0.3 nmol of F1. In contrast, Harris, et al. (17) reported 0.42 nmol/mg of submitochondrial particles and Ferguson et al. (18) suggested that 8.5% of the submitochondrial particles is F1. Calculations in this paper requiring the amount of active F1 on submitochondrial particles assume a value of 0.3 nmol/mg of particle protein, corrected for the specific activity of the preparation. That is, if the specific activity is one-half maximum (for example, 7 units/mg), the preparation is considered to contain 0.15 nmol of enzyme with available catalytic sites. The turnover number of membrane-bound F1, calculated from a maximal specific activity of 14 units/mg, is 750 mol of ATP hydrolyzed per s/mol of F1 if the submitochondrial particles contain 0.3 nmol of F1/mg, and 560 if the submitochondrial particles contain 0.4 nmol/mg. Thus, the turnover number of membrane-bound F1 is in the same range (650–700) as that exhibited by the soluble enzyme (3).

Submitochondrial particles activated by washing with KCl were suitable for cold chase experiments (8), whereas nonactivated particles served poorly. Fig. 1A shows that ETPH(Mg$^{2+}$) with a specific activity of 0.5 units/mg bound relatively little $[\gamma-^{32}\text{P}]\text{ATP}$ (expressed as the difference be-

![Fig. 1. Cold chase experiments with activated and nonactivated submitochondrial particles.](image)

**TABLE 1**

<table>
<thead>
<tr>
<th>Protein activity of submitochondrial particles</th>
<th>Specific activity</th>
<th>microP, formed/min/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETPH(Mg$^{2+}$)</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>KCl-washed ETPH(Mg$^{2+}$)</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>KCl-washed ETPH(Mg$^{2+}$) incubated at pH 10 for:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 min</td>
<td>10.4</td>
<td></td>
</tr>
<tr>
<td>60 min</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td>13.8</td>
<td></td>
</tr>
</tbody>
</table>

Specific ATPase activities of submitochondrial particles activity was determined with a regenerating system for ATP (14). Submitochondrial particles were prepared as described under "Experimental Procedures." The reaction mixtures were divided into three aliquots. One aliquot was assayed for enzyme activity at 1:10 dilution of concentrated NH$_4$OH, and incubation was continued for the times indicated. The pH was adjusted after 10 min by adding 0.15 M MgATP and allowing the ATPase to turn over for an additional 5 s before stopping the reaction with 50 $\mu$l of perchloric acid/ATP stop solution. $^{32}\text{P}$ was separated from $[\gamma-^{32}\text{P}]\text{ATP}$ and counted as described under "Experimental Procedures." Hydrolysis is expressed as the per cent of the total $[\gamma-^{32}\text{P}]\text{ATP}$ added. The submitochondrial particles used were ETPH(Mg$^{2+}$) (specific activity = 0.53 units/mg) (A) and KCl-washed ETPH(Mg$^{2+}$) (specific activity = 0.5 units/mg) (B). The particles were prepared as described under "Experimental Procedures."
between the cold chase and the acid quench) even when large amounts of protein were present. However, activated particles bound substantial amounts of the radioactive substrate in catalytic sites (Fig. 1B). It may be seen that 1–2 mg of particles provided maximum binding under the experimental conditions used. In subsequent experiments, reaction mixtures contained amounts of submitochondrial particles which provided a molar excess of available catalytic sites over [γ-32P]ATP added. Such conditions favor binding of [γ-32P]ATP predominantly in the high affinity catalytic site (5).

Since cold chase-type experiments could be carried out with submitochondrial particles, it was possible to determine the rate at which ATP binds in the catalytic site. The forward rate constant \(k_{f}\) was \(4.6 \times 10^{6} \text{M}^{-1} \text{s}^{-1}\), and the reverse rate constant \(k_{r}\) for the rate of ATP dissociation was \(10^{6} \text{s}^{-1}\). These values provide a calculated value for the affinity constant \(K_{a} = k_{f}/k_{r}\) of \(10^{12} \text{M}^{-1}\) (8). Thus, the high affinity catalytic site found on the soluble enzyme is present in the membrane-bound enzyme as well.

The equilibrium constant for the catalytic step was determined by measuring the amounts of substrate and products bound in catalytic sites under the conditions of single site catalysis in two ways. The first exploited observations that the rate of dissociation of [32P]ATP (10−3 s−1) and of ADP (10−4 s−1; see below) from catalytic sites is slow. Consequently, during suitably short periods of incubation of [γ-32P]ATP and submitochondrial particles, very little product is released to the medium, and the amount of [32P] found in depurinized reaction mixtures is an appropriate measure of the amount of F1·ADP·[32P] formed. Thus, as shown earlier for soluble F1, the amount of [32P] in acid-quenched reaction mixtures provides a measure of the amount of bound products, while the difference between the amount of [32P] formed in the cold chase and the acid quench provides a measure of the amount of F1·ATP formed. The ratio of these values is \(K_{a}\). The results of an experiment carried out in the rapid mixing apparatus are shown in Fig. 2. It may be seen that the values obtained tend at longer time periods to a value of about 0.4. In other experiments (not shown), values between 0.3 and 0.6 were observed.

The second method of measuring \(K_{a}\) utilized filtration to separate membrane-bound from free radioactivity. The results of several experiments with varying concentrations of [γ-32P] ATP and submitochondrial particles are shown in Table II. It may be seen that the values obtained for \(K_{a}\) within each experiment were reasonably constant over the 8-min time period of the incubations. At the end of the incubations, the total amount of radioactivity bound to submitochondrial particles in each of the three experiments had decreased to about 80% of the original. Thus, the reaction mixtures behaved as though they were at equilibrium. The presence of carbonyl cyanide m-trifluoromethoxyphenylhydrazone in reaction mixtures (Table II, column C) did not appear to have any effect on the values observed. There was, however, variation in the actual value of \(K_{a}\) found in different experiments (Table II) as well as a small difference in the numbers obtained by the two types of measurements (compare Table II and Fig. 2).

The rate of dissociation of [32P] from catalytic sites on submitochondrial particles was determined in filtration experiments to be 1.4 × 10−8 s−1 (8). The rate of dissociation of ADP from catalytic sites also was determined by filtration. Activated submitochondrial particles were incubated with [H,γ-32P]ATP so as to form the enzyme-substrate complex. The amounts of free radioactivity were determined by separating submitochondrial particles from soluble components on nitrocellulose filters (Fig. 3). Similar to observations made with soluble F1, ADP dissociated at a rate \(k_{d} = 1.9 \times 10^{-6} \text{s}^{-1}\) (about 10 times slower than [32P], \(k_{d} = 2 \times 10^{-6} \text{s}^{-1}\)) in this experiment (Fig. 3). It is noteworthy that the amount of free radioactive ATP detected was not influenced by the filtration procedure. That is, the total amount of [γ-32P]ATP determined as [32P]glucose 6-phosphate in the reaction mixtures in the presence of glucose and hexokinase (2–4%) was the same as the total amount of [γ-32P]ATP found in filtrates. In separate experiments (not shown), the experiment of Fig. 3 was repeated with hexokinase and glucose added to reaction mixtures after formation of the enzyme-substrate complex. During long periods of incubation (20–80 min), [3H]ADP dissociated from catalytic sites is subject to the action of adenylate kinase, and [3H]AMP may be found in the reaction mixtures (2). The presence of the hexokinase trap prevents any [3H]ATP that might arise via the action of adenylate kinase from rebinding to F1. The apparent rate of dissociation of [3H]ADP under these conditions did not differ significantly from that shown in Fig. 3.

The rate at which ATP binds in promoter sites was determined by exploiting observations that nonhydrolyzable as well as hydrolyzable nucleotides promote the hydrolysis of trinitrophenyl ATP and of ATP bound in high affinity catalytic sites (4). Fig. 4A shows the time course of ADP-promoted hydrolysis of bound [γ-32P]ATP. As found earlier, the rate of promoted hydrolysis is slower with nonhydrolyzable nucleotides than with the hydrolyzable forms (4). The rate of ADP binding is calculated by assuming that, under the conditions

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2 H. S. Penefsky, unpublished experiments.
of the experiment, the rate-limiting step in the promotion of [$\gamma^{32P}$]ATP hydrolysis in the high affinity catalytic site is the rate of binding of ADP. A second order plot of the data is shown in Fig. 4B. The data fit second order kinetics for two concentrations of submitochondrial particles (1 and 2 mg/ml) and two concentrations of ADP (10 and 20 $\mu$M). The calculated bimolecular rate constants were 1.2 x $10^5$ M$^{-1}$ s$^{-1}$ and 1.4 x $10^5$ M$^{-1}$ s$^{-1}$, respectively.

The maximum rate of hydrolysis of ATP bound in the high affinity catalytic site was determined in the rapid mixing apparatus (Fig. 5). The enzyme-substrate complex was formed by incubating (in the aging hose of the instrument) mixed equal volumes of [$\gamma^{32P}$]ATP and activated submitochondrial particles. A second push on the syringe plungers mixed the enzyme-substrate complex with a large excess of nonradioactive ATP. Under these conditions, ATP hydrolysis proceeds at maximum rates. The mixtures were allowed to react for periods of time shown on the abscissa. The point at zero time represents the extent of hydrolysis expected from an equilibrium distribution of substrate and products in the high affinity catalytic site. However, within 5 ms after mixing the enzyme-substrate complex with nonradioactive ATP, substantial hydrolysis of the bound radioactive substrate occurred (70–80% in most experiments). Since at least two reaction half-times must have elapsed during the initial 5-ms time period, a maximum rate constant of 300 s$^{-1}$ is calculated for this rapid phase. The acceleration in the rate of net hydrolysis when membrane-bound ATPase is shifted from hydrolysis under conditions of single site catalysis (10$^{-4}$ s$^{-1}$) to multisite catal-
FIG. 4. Rate of ADP binding to promoter sites on membrane-bound ATPase. The reaction mixture contained 1 mg of activated ETPH(Mg++) (specific activity = 9 units/mg), 20 mM Tris-
SO4, pH 8, 0.5 mM MgSO4, and 4 mM P. The particles were allowed to equilibrate in the buffer for 1 min before adding 40 µl of [γ-32P] ATP stock solution (2.3 µM, 5.5 × 10^6 cpm/nmol). After 5 s of further incubation to permit formation of the enzyme-substrate complex 20 µl of 1 mM ADP was added (A) and the reaction was allowed to continue for the times indicated on the abscissa. The final volume was 1.0 ml. The reaction was stopped by adding 50 µl of a solution containing 60% perchloric acid and 5 mM ATP. 32Pi formed was separated from [γ-32P]ATP as described under "Experimental Procedures." The stock solution of ADP was incubated before use with 50 mM glucose, 1 mM MgSO4, and 20 units of hexokinase for 20 min to remove any ATP that might have been present. The amount of ADP bound (in nanomoles) was set equal to the difference between the nanomole of 32Pi formed in the absence of ADP, acid quench and 32Pi formed in the presence of ADP and membrane-bound ATPase. The stock solution of ADP was incubated before use with 50 mM glucose, 1 mM MgSO4, and 20 units of hexokinase for 20 min to remove any ATP that might have been present. The amount of ADP bound (in nanomoles) was set equal to the difference between the nanomole of 32Pi formed in the presence of ADP and in the absence of ADP, acid quench (B). The acid-quench values at zero time (○) and at 20 s (■) were obtained by adding 50 µl of perchloric acid/ATP solution to the reaction mixture at 5 and 15 s, respectively, after addition of [γ-32P]ATP. Per cent hydrolysis is calculated as the per cent of the total amount of [γ-32P]ATP added. B, second order plot of two experiments. ADP0 and P0 are the initial concentrations of ADP and membrane-bound ATP, respectively. F1, 32P is the concentration of the enzyme-substrate complex determined in the promotion experiment (cold chase minus acid quench in A) and is set equal to F1, ADP. •, 10 µM ADP and 2 mg of submitochondrial particles, (left ordinate); ■, 20 µM ADP and 1 mg of submitochondrial particles (right ordinate).

FIG. 5. Maximum rate of hydrolysis of [γ,32P]ATP bound in catalytic sites on submitochondrial particles. The experiment was carried out in an Update Instrument rapid mixing apparatus with three syringes and two mixers in the push-push mode as described earlier (5). Each syringe contained the following buffer: 20 mM Tris-
SO4, pH 8, 0.5 mM MgSO4, and 4 mM P. The syringes also contained the following additional components: enzyme syringe, 4 µg/ml KCl-washed ETPH(Mg++) (SMP); substrate syringe, 0.197 µM [γ,32P] ATP; cold chase syringe, 20 mM MgATP. The first push on the syringe pistons mixed equal volumes (190 µl) of submitochondrial particles and [γ,32P]ATP via mixer 1. The reaction mixture was allowed to age for 2.3 s in hose A in order to form the enzyme-substrate complex. The second push mixed the enzyme-substrate complex in hose A with an equal volume (190 µl) from the ATP (cold chase) syringe via mixer 2. The push rates were 1 cm/s for push 1 and 2 cm/s for push 2. The variations in the age of the chased reaction mixtures shown on the abscissa were obtained by changing the length of hose B. The chased reaction mixtures were collected in receiving vessels held at the nozzle on the end of hose B. The vessels contained 0.2 ml of 60% perchloric acid and 1.0 ml of a solution identical with that placed in the cold chase syringe. 32P was formed was separated from [γ-32P]ATP as described under "Experimental Procedures." Hydrolysis is calculated as the per cent of the 32P in reaction mixtures that is present as 32P after correction for the small amount of 32P (1–5%) in the original [γ,32P]ATP. The value for zero time of incubation with the cold chase (30%) was obtained by disconnecting mixer 2 and injecting the enzyme-substrate complex formed in hose A directly into a vessel containing 1.0 ml of cold chase and 0.2 ml of 60% perchloric acid.

The affinity constant for ATP in the catalytic site (K) is 10^8 M^-1 for both forms of the enzyme (5, 8), and the equilibrium constant for the catalytic step (k2) is near unity for soluble F1 (5) and the ATPase of submitochondrial particles (this paper). In addition, product P; dissociates (k3) about 10 times more rapidly than product ADP, and the rate-limiting step in net hydrolysis of ATP during single site catalysis (the rate of dissociation of ADP (k4 = 10^-4 s^-1)) is the same (Ref. 5 and Fig. 3). An attempt was made to determine k3 by incubating an enzyme-substrate complex formed from 1 mg of activated submitochondrial particles and 0.1 nmol of non-radioactive ATP with 0.6 µM 32P (6 × 10^6 cpm/nmol). After subtraction of the control without particles, no radioactivity was found in the ATP remaining in the high affinity catalytic site. ATP hydrolysis by membrane-bound F1 also is characterized by the large rate acceleration that occurs when ATP is made available to additional catalytic sites on the soluble enzyme (6). That is, the rate of net hydrolysis is increased about 10^3-fold when the enzyme is shifted from conditions of single site catalysis to multisite catalysis (Fig. 5). Moreover,
the rate of binding of substrate ATP to additional catalytic sites on membrane-bound F1 is the same as the rate of binding in the high affinity catalytic site. Two conclusions may be drawn from these considerations. First, the high affinity catalytic site on submitochondrial particles, like that on soluble F1 (5, 6), has the properties of a normal catalytic site on the molecule. Second, it would appear that significant changes do not take place, at least with respect to single site catalysis, when the mitochondrial ATPase is removed from the inner membrane. These observations support the assumption, implicit in many studies, that the soluble ATPase is an appropriate model system for analysis of the mechanism of action of F1, in oxidative phosphorylation. Thus, the model for ATP synthesis in oxidative phosphorylation catalyzed by F1 (5), which was based upon the presence of high affinity catalytic sites on the soluble enzyme and on the finding that the equilibrium constant for the catalytic step was near unity, also is applicable to the membrane-bound ATPase of submitochondrial particles. The model proposes that ATP is formed in oxidative phosphorylation from bound ADP and P_i with virtually zero change in free energy, and that the major requirement for energy in oxidative phosphorylation is for the release of product ATP from high affinity catalytic sites (5).

It is assumed throughout these experiments that the high affinity catalytic site identified on submitochondrial particles is in fact the same high affinity catalytic site identified on the solubilized ATPase (5). In view of the broad similarity in the properties of the two forms of the enzyme when tested under the conditions of single site catalysis, it would appear that the assumption is valid.

Recently, Wise et al. (19) reported that the ATPase solubilized from Escherichia coli plasma membranes, an enzyme closely similar to the mitochondrial ATPase with regard to both structure and function (reviewed in Ref. 1), has an apparent high affinity catalytic site, and, under conditions of single site catalysis, an equilibrium constant near unity and a rate of dissociation of 32Pi from catalytic site of about 10^3 s^{-1}. These observations suggest that a high affinity catalytic site and other characteristics of single site catalysis are common to the ubiquitously distributed energy transducing ATPase.

This paper presents reasonable evidence that the equilibrium constant for the catalytic step is near unity for membrane-bound ATPase (Table II and Fig. 2). However, the actual values observed are not as invariant as those exhibited by the soluble enzyme (5), nor has it been possible to obtain unambiguous measurements of the actual rate of approach to equilibrium. Such measurements permit an estimation of the individual rate constants for the catalytic step (5). As discussed earlier, the distribution of substrate and products in the catalytic site during single site catalysis (that is, K_i) is a factor in the calculation of k_a, k_b, and k_c (5).

Although the observed rate of ADP binding to the promoter site on membrane-bound F1 (10^8 M^{-1} s^{-1}) is the same as the rate of ADP binding to the soluble ATPase (5) (k_a = 10^8 M^{-1} s^{-1}), these values are low for a bimolecular rate constant. While the available evidence suggests that the promoter site is a catalytic site on F1 (4, 6), the rate of ADP binding as measured in the experiment of Fig. 4 cannot properly be assigned as the value of k_a for submitochondrial particles. Recently, Cross et al. (20) reported a revised k_a of 10^8 M^{-1} s^{-1} for soluble F1. It may be that the promotion experiments of Fig. 4 reflect two rate constants: a more rapid binding step characterized by the faster bimolecular rate constant and a slower conformational change that leads to the observed acceleration of hydrolysis.

Activated submitochondrial particles contain considerable amounts of bound adenine nucleotides, about 800 pmol of ATP and 400 pmol of ADP/mg of protein (see "Experimental Procedures"). Virtually no free ADP or ATP is detected in the preparations using the luciferin/luciferase assay as described under "Experimental Procedures." If submitochondrial particles contain 0.3–0.4 nmol of F1/mg of protein, the adenine nucleotide bound in the tight, nonexchanging sites, presumably located on subunit a (21), could account for much of the adenine nucleotide associated with the particles. This point is of some importance to experiments on the type described in this paper, since it is required that the catalytic sites be substantially free of adenine nucleotides when radioactive ATP is added to a suspension of particles. However, the cold chase experiments clearly show that a substantial fraction of the added [γ-32P]ATP binds to high affinity catalytic sites.

None of the parameters of single site catalysis measured in these experiments was influenced by uncouplers of oxidative phosphorylation such as carbonyl cyanide m-trifluoromethoxyphosphorylhydrazine. Similarly, neither the affinity constant in the catalytic site nor the rate of dissociation of 32Pi, from the catalytic site was affected by the uncoupler (8). However, the net rate of ATP hydrolysis during single site catalysis was not influenced by the concentration of P_i in the external medium. For these reasons, it is unlikely that the appearance of 32Pi in the medium during studies of single site hydrolysis with submitochondrial particles was the result of an energy-dependent exchange between free P_i and the γ-phosphoryl of bound [γ-32P]ATP. The failure of uncouplers to affect the reactions of single site catalysis may reflect the extensive washing of the particles during the activation of the ATPase (8). Such washing would remove endogenous substrates. It is also unlikely that the mitochondrial membrane would be significantly energized by the small amount of ATP added in experiments. However, when submitochondrial particles are deliberately energized by addition of oxidizable substrates, large, uncoupler-sensitive changes in the properties of the high affinity catalytic site are observed (22).
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REFERENCES