The mechanism of ATP hydrolysis by the solubilized mitochondrial ATPase (MF₁) has been studied under conditions where catalytic turnover occurs at one site, uni-site catalysis (obtained when enzyme is in excess of substrate), or at two sites, bi-site catalysis (obtained when substrate is in excess of enzyme). Pulse-chase experiments support the conclusion that the sites which participate in bi-site catalysis are the same as those which participate in uni-site catalysis. Upon addition of ATP in molar excess to MF₁, label that was bound under uni-site conditions dissociates at a rate equal to the rate of bi-site catalysis. Similarly, when medium ATP is removed, label that was bound under bi-site conditions dissociates at a rate equal to the rate of uni-site catalysis. Evidence that a high affinity catalytic site equivalent to the one observed under uni-site conditions participates as an intermediate in bi-site catalysis includes the demonstration of full occupancy of a catalytically competent site during steady-state turnover at nanomolar concentrations of ATP.

Improved measurements of the interaction of ADP at a high affinity catalytic site have lead to the revision of several of the rate constants that define uni-site catalysis. The rate constant for unpromoted dissociation of ADP is equal to that for ATP (4 × 10⁻³ s⁻¹). The rate of binding ADP at a high affinity chaseable site (K₆ = 1 mM) is equal to the rate of binding ATP (4 × 10⁹ M⁻¹ s⁻¹). The rate of catalysis obtained when substrate binding at one site promotes product release from an adjacent site (bi-site catalysis) is up to 100,000-fold faster than unpromoted product release (uni-site catalysis).

MF₁ has a subunit structure of α₂βγδε. Catalytic sites are located on the β-subunits or at interfaces between α- and β-subunits. The catalytic sites show negative cooperativity in binding substrate and strong positive catalytic cooperativity. There are a total of six adenine nucleotide-binding sites on MF₁. Nucleotide bound at three of the sites exchanges rapidly with medium nucleotide during catalytic turnover. These sites are likely to have a catalytic function. The remaining three binding sites do not exchange readily with medium nucleotide. These sites are referred to as noncatalytic sites (for reviews see Boyer, 1987; Senior, 1988).

The fact that there are three exchangeable nucleotide-binding sites and three copies of the β-subunit/MF₁ have led a number of investigators to consider it likely that the enzyme has three functional catalytic sites. Evidence supporting this possibility comes from studies of the kinetic properties of the enzyme. In one such study (Grubmeyer et al., 1982; Cross et al., 1982), it was concluded that turnover can occur at one site (K₆ = 10⁻¹² M, uni-site catalysis), two sites (K₆ = 30 μM, bi-site catalysis), or three sites (K₆ = 150 μM, tri-site catalysis), depending on the substrate concentration.

Two laboratories have recently questioned the idea that the site responsible for uni-site catalysis is a normal site capable of multi-site catalysis at high substrate concentration (Bulough et al., 1987; Milgrom and Murataliev, 1987). Here, we have re-examined the properties of catalysis under uni-site and bi-site conditions and find that when medium ATP is removed, sites undergoing bi-site catalysis revert to uni-site rates. We also show that sites undergoing uni-site catalysis switch to bi-site rates when excess substrate is added. The results support the conclusion that the same sites participate in both activities. In the course of this work, we further characterized the interaction of ADP at a high affinity catalytic site. A preliminary report of some of this work has appeared (Cross et al., 1984, 1986).

Experimental Procedures

Preparation of MF₁—MF₁ was isolated from beef heart mitochondrial particles and stored as an ammonium sulfate suspension (Knowles and Penesky, 1972). Prior to each experiment, an aliquot of the suspension was centrifuged in a Beckman Airfuge. The supernatant was carefully removed, and the enzyme pellet was dissolved in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

MF₁ is prepared as follows. Step 1: native MF₁ is incubated at approximately 6 μM in SHPMg buffer with 5 mM MgATP for 2 min. Unbound nucleotide is removed by passage through a centrifuge.
column. Step 2: MF, is incubated at 3 μM with 4 mM MgGTP for 30 s, and the sample is passed through a centrifuge column equilibrated in SHPGm buffer with K'-P, added to adjust the P, concentration to 50 mM and the pH to 7.0. Step 3: after 10 min of incubation, free nucleotide is removed by passage through a third centrifuge column also equilibrated with SHPGm buffer adjusted to 60 mM P, and an additional 10-min incubation at high phosphate concentration, unbound ligand is removed on a fourth centrifuge column equilibrated in normal SHPGm buffer.

The nucleotide content of MF, at each step in this procedure has been measured using [3H]GTP and a luciferin-luciferase assay (Lund et al., 1986) of ROH-neutralized perchloric acid extracts treated with pyruvate kinase and phosphoenolpyruvate. In the first step, ADP produced by ATP cleavage fills the single unoccupied noncatalytic site. With passage through the first centrifuge column, MF, retains a total of 4.5 adenine nucleotides with three bound at noncatalytic sites and 1.5 bound at catalytic sites (MF,[3,1.5]). In the second step, adenine nucleotide bound at catalytic sites is chased during MgGTP cleavage. With passage through the second centrifuge column, MF, retains three adenine nucleotides at noncatalytic sites and one GDP at a catalytic site. In the third step, GDP, unlike ADP, is readily displaced from the catalytic site by incubating in the presence of high P, concentration to give MF,[3,0].

MF,[2,0] was prepared as described above, except that the first step was omitted. [3H]Acetyl-MF, having a specific activity of 4 × 10⁶ cpm/nmol and 14 acetyl/molecule, was prepared by the method of Kagawa (1967).

Ligand Binding Measurements—Incubations were initiated by mixing equal volumes of radiolabeled nucleotide with MF,. Enzyme-bound ligand was separated from free ligand on Sephadex centrifuge columns. Each incubation time reported includes 20 s beyond the time of initiation of the centrifugation step. This allows for the time required to separate protein and unbound ligand on the centrifuge column (Grubmeyer et al., 1982).

A number of experiments in this study were conducted especially at very low MF, concentration or with a very large molar ratio of radiolabeled ligand to enzyme. The level of second binding of MF, and the resolution of bound from unbound ligand required in such experiments exceeded previously tested limits for the Sephadex centrifuge column technique. For example, to measure catalytic site occupancy during steady-state turnover it was necessary to use at least a 10-fold molar excess of substrate over enzyme. At the lowest ATP concentration tested, binding measurements were performed on samples containing 3 × 10⁻¹⁰ M MF, (see Fig. 2). Preliminary tests with [3H] acetyl-MF, demonstrated that at all MF, concentrations used, approximately 95% of the MF, was recovered in centrifuge column effluent when the application volume was between 80 and 130 μl, and defatted bovine serum albumin was included at 1 mg/ml in reaction mixtures and in the SHPGm buffer (SHPGmA) used to equilibrate the columns (Cross and Nalin, 1982). At high ATP concentrations, the MF, concentration was limited to 1 nM in order to avoid substrate depletion during the time required to mix the reaction and complete the binding assays. Binding measurements at 10 μM ATP and 1 nM MF, thus required a minimal resolution of 1 part bound from 10,000 parts unbound. As previously reported, the non-specific leakage of unbound ligand through a 1-ml column is approximately 1 part in 1,000 when the sample volume is 100 μl (Penevsky, 1977). However, we found that resolution improves considerably as the sample volume is decreased to 50 μl, where nonspecific leakage is less than 1 part in 200,000. Further reduction of the sample volume causes protein recovery to become variable and to drop below 85%.

Column effluents were collected directly in plastic liquid-scintillation vials and MF,-bound ligand was determined by scintillation counting. In order to obtain sufficient label for counting at very low MF, concentrations (10⁻¹⁰ to 10⁻¹¹ M), three 100-μl aliquots of each sample were applied to three separate 1-ml columns, and the effluents were collected in a single vial. Alternatively, a larger aliquot was applied to a 5-ml column. Corrections were made for recovery of MF, as determined by control experiments with [3H]acetyl-MF,. Additional corrections were made for the small amount of label in column effluents of controls lacking MF,. ATP.

**RESULTS**

**Catalytic Site Occupancy as a Function of ATP Concentration**—Site occupancy was measured during steady-state catalysis at various low [γ-²³P]ATP concentrations. The results shown in Fig. 1 demonstrate that at 12 nM [γ-²³P]ATP, one site/MF, remains fully occupied during multiple turnovers. Two observations support the identification of this site as a ⁰⁰°C containing 1% triethylamine, 1% bromine water, and 3% ammonium molybdate in 0.067 N HCl is added to an equal volume of acid-quinched sample containing 0.5 M perchloric acid, 1.0 mM Na⁺, and 5 mM ATP. The vials are stored 10 min on ice to allow complete precipitation of the phosphomolybdate complex. Samples are centrifuged for 10 min at 4000 × g in a Beckman J-6 centrifuge (⁰⁰°C) using a J-4.2 rotor with bucket adapters for 14-mm tubes. Supernatants are decanted, and pellets are dissolved in 150 μl of 1.0 N NaOH. Samples are then acidified by addition of 150 μl of 1.5 N acetic acid, and ³²P is determined by liquid scintillation counting as described above. Corrections were made for the recovery of ³²P, standards which ranged from 94 to 99%.

**Calculations**—The solid line given in Fig. 2 predicts the total number of moles of ³²P bound either as [γ-³²P]ATP or ³²Pi at catalytic sites/mole of MF, during steady-state hydrolysis. The line is calculated using the following equation:

\[ \frac{[E\cdot ³²Pi] / [E]}{[E]} = 1 - k_0 / ([ATP]k_0 + k_1) \]

where \([E\cdot ³²Pi] / [E]\) is the fraction of total enzyme with bound ³²P, \(k_0\) is the rate of ³²Pi release (1.2 × 10⁻⁶ s⁻¹; Grubmeyer et al., 1982), and \(k_1\) is the rate constant for ATP binding (4 × 10⁻⁸ M⁻¹ s⁻¹). \(k_3\) is the order of magnitude faster than the turnover rate at the highest ATP concentration shown in Fig. 2.

**Other Methods**—Adenine nucleotide concentrations were determined from the absorbances of solutions at 259 nm using an extinction coefficient of 15,400 cm⁻¹ M⁻¹. [³H]ADP was freed of contaminating ATP by treatment with hexokinase and glucose as described (Grubmeyer et al., 1982).

**FIG. 1. Catalytic site occupancy during steady-state turnover at 12 nM [γ-²³P]ATP.** MF,[2,0] at 1 nM was incubated with 12 nM [γ-²³P]ATP in SHPGmA buffer, pH 7.8. Bound ³²P/MF, (O) was measured at the times indicated by centrifuge column assays, and the number of catalytic turnovers was determined by measuring the optical density of acid-quenched samples as described under "Experimental Procedures." Times were selected to allow one or more turnovers without significant depletion of substrate. Where indicated by the arrow, 4 mM MgATP was added, and the noncachable ³²P (Δ) was measured 45 and 60 s later.

**TIME (sec)**

**BOUND ³²P/MF, TURNOVERS/F**
catalytic site. Addition of a large excess of unlabeled ATP results in rapid dissociation of greater than 95% of the bound \( ^{32}\text{P} \) (Fig. 1, triangles), and identical results are obtained to those shown in Fig. 1 when MF, [3,0] is used.

Experiments similar to the one shown in Fig. 1 were repeated over a wide range of \([\gamma^{32}\text{P}]\)ATP concentrations (3 × 10^{-10} to 10^{-5} M, Fig. 2, circles). The results demonstrate full occupancy of one site during steady-state hydrolysis at substrate concentrations down to 3 nM, well below published \( K_m \) values.

A model based on the binding change mechanism for \( F_1 \)-ATPases (Boyer, 1979) was used to derive an equation for predicting catalytic site occupancy as a function of ATP concentration (see “Experimental Procedures”). The solid line shown in Fig. 2 was generated by substituting values for the ATP concentration, and the rate constants for ATP binding (Fig. 3) and P, release (Grubmeyer et al., 1982). The model predicts that at ATP concentrations above 10^{-9} M, one catalytic site/MF, will be fully occupied by \( ^{32}\text{P} \)-labeled substrate and product. At these ATP levels, turnover results exclusively from bi-site catalysis, where substrate binding at one catalytic site promotes rapid release of product from an adjacent site. Since the highest concentration of ATP reported in Fig. 2 is still well below the \( K_m \) for bi-site catalysis, substrate binding will be rate limiting, and the enzyme will spend only a small fraction of its time in a form with two sites occupied. Hence, the stoichiometry is not expected to raise above 1.0.

With ATP concentrations at or below 10^{-9} M ATP, the model predicts (Fig. 2, solid line) that the rate of \([\gamma^{32}\text{P}]\)ATP binding at a second site will become sufficiently slow to allow time for spontaneous dissociation of \( ^{32}\text{P} \), by uni-site catalysis. Dissociation of ATP does not contribute to the decrease in level of bound \( ^{32}\text{P} \) since interconversion of bound substrate and product is rapid, and the rate of dissociation of ATP is three orders of magnitude slower than that of P, (Grubmeyer et al., 1982). The results not only demonstrate the existence of a high affinity site during steady-state bi-site catalysis, but the excellent agreement of the data with the calculated plot also provides independent confirmation of the rate constants for ATP binding and P, release.

It was previously reported in single turnover experiments that the rate of ADP dissociation is 10-fold slower than P,. This leads to the prediction that with decreasing concentrations of [\( ^{32}\text{P} \)]ATP, the drop in level of [\( ^{32}\text{P} \)]label bound/MF, should be shifted one log unit to the left of that shown in Fig. 2 for \( ^{32}\text{P} \). However, this prediction was not verified. The ATP concentration dependence of catalytic site occupancy during steady-state hydrolysis of [\( ^{32}\text{P} \)]ATP (Fig. 2, triangles) was identical to that detected using \([\gamma^{32}\text{P}]\)ATP (Fig. 2, circles). The results suggest that the rates of ADP and P, dissociation are equal. This is demonstrated directly by experiments reported in a later section.

The Rate of ATP Binding during Bi-site Catalysis—At ATP concentrations above 10^{-9} M (Fig. 2) but below 10^{-6} M (Cross et al., 1982), catalytic turnover will be due exclusively to bi-site catalysis. Since the rate-limiting step is ATP binding, the velocity should show a linear dependence on substrate concentration. A rate constant for ATP binding at a second site was previously determined over a narrow range of ATP concentrations (5-60 nM, Cross et al., 1982). Experiments reported in Fig. 3 extend these data to show a linear dependence of velocity over a 10-fold range in substrate concentration. A second-order rate constant of 4 × 10^{6} M^{-1} s^{-1} is calculated from the slope of the plot in Fig. 3. This value is in good agreement with the rate constant reported previously and is very similar to the rate of ATP binding to the first site on MF, (6 × 10^{6} M^{-1} s^{-1}, Grubmeyer et al., 1982).

Replacement of Tightly Bound \( ^{32}\text{P} \)-Labeled Ligand by Medium Substrate during Steady-state Bi-site Catalysis—An alternating site mechanism predicts that under steady-state conditions at substrate concentrations well below the \( K_m \) for bi-site catalysis, bound \( ^{32}\text{P} \)-labeled product will dissociate at a rate equal to the rate of ATP binding. Results presented in Fig. 4 verify this prediction. When enzyme having one fully occupied site during turnover at 10 nM \([\gamma^{32}\text{P}]\)ATP is diluted 11-fold into medium containing the same concentration of unlabeled ATP, bound \( ^{32}\text{P} \) chases rapidly (Fig. 4A, open circles). In a control, where enzyme is diluted into buffer containing \( ^{32}\text{P} \)-labeled substrate at the same specific activity, bound \( ^{32}\text{P} \) remains constant at 1 mol/mol as expected (A, triangles). When another sample is diluted 33-fold into buffer containing nonradioactive ATP at 3-8 nM, 85% of the \( ^{32}\text{P} \) label chases in a pseudo-first-order fashion (B). In each case, dividing the observed rate of loss of bound label by the ATP concentration gives a value equal to the rate constant for ATP binding (5-6 × 10^{6} M^{-1} s^{-1}). The results demonstrate that release of product from a very high affinity

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**Fig. 2. Catalytic site occupancy during steady-state turnover at low substrate concentrations.** Experiments similar to the one described in Fig. 1 were run at varying concentrations of \([\gamma^{32}\text{P}]\)ATP (○) or [\( ^{32}\text{H} \)]ATP (Δ). The MF, concentration was 1 nM when ATP concentrations were at or above 10^{-3} M and one-tenth the ATP concentration at substrate levels below 10^{-8} M. The value plotted was calculated as the average \( ^{32}\text{P} \) or \( ^{32}\text{H} \) bound/MF, at three to four different times during steady-state catalysis, as shown by the solid line in Fig. 1.

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**Fig. 3. Rate of ATP binding to MF, during bi-site catalysis.** MF, was incubated with \([\gamma^{32}\text{P}]\)ATP as described in Figs. 1 and 2. Aliquots were removed at various times and quenched by addition of an equal volume of 1 M perchloric acid containing 1 mM P, and 10 \( \mu \)M ATP. The amount of \( ^{32}\text{P} \) formed was determined as described under “Experimental Procedures.” Initial reaction velocities are plotted against ATP concentration.

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**Fig. 4.** Replacement of tightly bound \( ^{32}\text{P} \)-labeled ligand by medium substrate during steady-state bi-site catalysis. An alternating site mechanism predicts that under steady-state conditions at substrate concentrations well below the \( K_m \) for bi-site catalysis, bound \( ^{32}\text{P} \)-labeled product will dissociate at a rate equal to the rate of ATP binding. Results presented in Fig. 4 verify this prediction. When enzyme having one fully occupied site during turnover at 10 nM \([\gamma^{32}\text{P}]\)ATP is diluted 11-fold into medium containing the same concentration of unlabeled ATP, bound \( ^{32}\text{P} \) chases rapidly (Fig. 4A, open circles). In a control, where enzyme is diluted into buffer containing \( ^{32}\text{P} \)-labeled substrate at the same specific activity, bound \( ^{32}\text{P} \) remains constant at 1 mol/mol as expected (A, triangles). When another sample is diluted 33-fold into buffer containing nonradioactive ATP at 3-8 nM, 85% of the \( ^{32}\text{P} \) label chases in a pseudo-first-order fashion (B). In each case, dividing the observed rate of loss of bound label by the ATP concentration gives a value equal to the rate constant for ATP binding (5-6 × 10^{6} M^{-1} s^{-1}). The results demonstrate that release of product from a very high affinity site promotes rapid release of product from an adjacent site. Since the highest concentration of ATP reported in Fig. 2 is still well below the \( K_m \) for bi-site catalysis, substrate binding will be rate limiting, and the enzyme will spend only a small fraction of its time in a form with two sites occupied. Hence, the stoichiometry is not expected to raise above 1.0.
Enzyme—As mentioned above, our previous attempt underestimated its true value. When the MF, to measure the rate of catalysis and unpromoted release from steady-state bi-site-dium substrate at the same rate as bi-site catalysis. ATP concentration yields a value equal to the rate constant for bi-site catalysis. The results show that when 20 nM [γ-32P]ATP for ATP binding (4-5 initial loaded under uni-site conditions is promoted by the binding of substrate at an adjacent site.

Promoted Release of Product from a Site Loaded under Uni-site Conditions—When MF, is mixed with a substoichiometric amount of [γ-32P]ATP, rapid binding occurs at a single high affinity site (Ks = 10^-12 M, Grubmeyer et al., 1982). Binding is followed by a rapid equilibration between bound substrate and products and a slow rate-limiting release of products (uni-site catalysis). The experiments reported in Fig. 5 were performed to determine whether the high affinity site loaded under uni-site conditions is capable of participating in normal bi-site catalysis. The results show that when 20 nM [γ-32P]ATP is mixed with 100 nM MF, and the resulting complex is diluted 400-fold with buffer containing 3-8 nM nonradioactive ATP. Results are expressed as the fraction of the initial 32P bound/MF, remaining bound. Second-order rate constants for ATP binding were calculated from the data to be 6-6 x 10^6 M^-1 s^-1 for each experiment.

Unpromoted product dissociation rates during uni-site catalysis and from sites initially loaded under bi-site conditions. Reactions were initiated by mixing equal volumes of MF, and [γ-32P]ATP in SHP MgA buffer, pH 8.0. Ligand binding was measured using centrifuge column assays. A, enzyme loaded under uni-site conditions was prepared by mixing either 49 nM [H]ATP (A) or 40 nM [γ-32P]ATP (C) with 100 nM MF. After 30 s to allow formation of the enzyme-substrate complex, reactions were diluted 500-fold with buffer, and subsequent dissociation of label was monitored. At the time of dilution, 68% of the added [H] or 70% of the added [γ-32P]ATP was bound. Results are expressed as the fraction of these zero time values remaining bound. B, enzyme loaded under steady-state bi-site conditions was prepared by incubating 1 nM MF, with either 10 nM [H]ATP (A) or 10 nM [γ-32P]ATP (C) for 80 s. Unbound ligand was removed on centrifuge columns. Column effluents were collected directly in SHP MgA buffer to elute the enzyme-ligand complex 11-fold and the subsequent dissociation of label was monitored. At the time of removal of unbound ligand, 0.97 mol of [H] or 0.94 mol of [γ-32P] were bound/mol MF, results are expressed as the fraction of these zero time values that remains bound.

Fig. 4. Replacement of tightly bound nucleotide during bi-site turnover. Reactions were initiated by mixing 1 nM MF, and 10 nM [γ-32P]ATP in SHP MgA buffer, pH 8. Bound 32P/MF, was measured by centrifuge column assays as described under "Experimental Procedures." A, 32P/MF, was measured in aliquots removed at various times after mixing (●). At the time indicated by the arrow, aliquots were diluted 11-fold into either 10 nM ATP (○) or 10 nM [γ-32P]ATP (△) and bound label was monitored. B, after an 80-s preincubation to load sites under bi-site conditions, aliquots were diluted 33-fold into 3 nM (●), 4 nM (○), 5 nM (△), or 8 nM (△) nonradioactive ATP. Results are expressed as the fraction of the initial 32P bound/MF, remaining bound. Second-order rate constants for ATP binding were calculated from the data to be 4-5 x 10^6 M^-1 s^-1 for each experiment.

Fig. 5. ATP-promoted release of 32P initially bound under uni-site conditions. Reactions were initiated by mixing equal volumes of MF, and [γ-32P]ATP in SHP MgA buffer, pH 8.0, to give final concentrations of 100 and 20 nM, respectively. After 30 s to allow complex formation, aliquots were diluted 400-fold into 3 nM (●), 4 nM (○), 5 nM (△), or 8 nM (△) unlabeled ATP. The dissociation of 32P was measured by centrifuge column assays. At the time of dilution, 75% of the added [γ-32P]ATP was bound to MF,. Results are expressed as the fraction of this zero time value that remains bound. Second-order rate constants for ATP binding were calculated from the data to be 4-5 x 10^6 M^-1 s^-1 for each experiment.
ess which occurs during reversible binding of ADP at a site having a much higher affinity than previously suspected. This explanation is consistent with the demonstration in a later section of ADP binding at a catalytic site having a $K_a = 1$ nM.

Results presented in Fig. 6A also show that under the more dilute conditions, $[^3H]ADP$ dissociates at the same rate as $[^3P]Pi$ (circles). Dissociation of ATP does not contribute to the observed rates since it is three orders of magnitude slower (Grubmeyer et al., 1982).

If bi-site catalysis occurs by substrate-promoted product release from a tight site characteristic of uni-site catalysis, then removal of medium substrate should result in product dissociation rates returning to those observed for uni-site catalysis. This prediction is verified by the experiments reported in Fig. 5B. MF$_s$ was loaded during bi-site catalysis with $[^3H]ATP$ or $[^p-32P]ATP$. Following removal of medium substrate and dilution to $0.09 \text{ nM MF}_s$, the rates of dissociation of ADP and Pi are measured as the rates of dissociation of $[^3H]$ (triangles) and $^3P$ (circles). These rates are found to be equal at $2.2 \times 10^{-4} \text{ s}^{-1}$. A similar slowing of the rate of $P$ dissociation upon removal of medium ATP was observed in studies with the chloroplast enzyme, CF$_s$, (Wu and Boyer, 1986).

Since bound substrate and products are in rapid equilibrium (Grubmeyer et al., 1982), the fraction of label present as product must be taken into account in calculating the intrinsic dissociation rate constants for product release. Results presented in Table I show that the ratio of product to substrate for enzyme loaded under bi-site conditions is slightly higher than that for enzyme loaded under uni-site conditions (1.4 compared with 0.53). When the appropriate corrections are made, the dissociation rate constants for unpromoted ADP and $P_i$ release from both uni-site- and bi-site-loaded enzyme are identical at $4 \times 10^{-4} \text{ s}^{-1}$.

**Tight Binding of ADP at Catalytic Sites**—The need to dilute MF$_s$ to subnanomolar concentrations in order to accurately measure the rate of dissociation of ADP suggests that ADP binding at a single catalytic site on MF$_s$ is much tighter than previously reported. Scatchard analysis of direct binding measurements using $[^3H]ADP$ and MF$_{[3,0]}$ shows that 0.7 mol of ADP/mol of MF$_s$ at a rapidly chaseable site having a $K_a$ of 1 nM (Fig. 1B) and 0.4 mol of ADP/mol of MF$_s$ at a site resistant to rapid chase having a $K_a$ of 7 nM (C). Release of ligand from the chaseable site is promoted by nanomolar concentrations of ATP at the same ATP-concentration-dependent rate as bi-site catalysis ($5 \times 10^{-4} \text{ s}^{-1}$, data not shown). Dissociation rates for the chaseable and nonchaseable sites in the absence of medium nucleotide were determined to be $2 \times 10^{-4} \text{ s}^{-1}$ and $6 \times 10^{-4} \text{ s}^{-1}$, respectively (data not shown).

The chaseable ADP site appears to be the same site as that which binds ATP tightly under conditions for uni-site or bi-site catalysis. Medium ATP promotes ADP release from the sites at identical rates, and the rate of unpromoted ADP dissociation from each site is the same. The nonchaseable site shown in Fig. 7C may be the same fractional nonchaseable catalytic site as that reported previously by Kironde and Cross (1986).

Association rate constants for the high affinity ADP sites can be calculated from their measured $K_a$ values and dissociation rate constants to be $2 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ and $10^{-3} \text{ M}^{-1} \text{ s}^{-1}$ for the chaseable and nonchaseable sites, respectively. Attempts to measure the association rates directly using native MF$_s$ or MF$_{[3,0]}$ were unsuccessful. Second-order rate constants varied with ADP concentration, decreasing with increasing ADP.

The results suggest that ADP binding is a two-step process with initial binding at a loose site followed by a conformational change that increases the stability of bound ligand and allows its retention through a centrifuge column. Attempts to measure the first-order process indicate that it proceeds at less than $10^{-2} \text{ s}^{-1}$.

Experiments were performed to determine the cause of heterogeneity in the ADP-binding site. It was found not to be dependent on the initial nucleotide content, since MF$_{[3,0]}$, MF$_{[2,1]}$, and MF$_{[2,0]}$ all gave similar Scatchard plots. The results with MF$_{[3,0]}$ suggest that a noncatalytic site is not involved. In addition, the $K_a$ for binding at the vacant non-catalytic site on MF$_{[2,1]}$ is 50-fold higher than that for the chaseable ADP site characterized in Fig. 7 and the association rate is a 100-fold slower (Kironde and Cross, 1987). The heterogeneity was also found not to result from a time-dependent denaturation of MF$_s$ at very dilute concentrations. MgSO$_4$ at 20 mM completely eliminated the nonchaseable fraction and only slightly decreased the chaseable fraction. Conditions reported by Senior (1981) to remove heterogeneity of Mg$^{2+}$ content of MF$_s$, i.e. catalytic turnover with ATP in excess to Mg$^{2+}$, resulted in an increase in chaseable ADP (to 0.8 mol/mol) with a near complete elimination of the nonchaseable fraction.

**DISCUSSION**

The data presented support the conclusion that the catalytic sites of MF$_s$ which participate in bi-site catalysis are the same sites as those which participate in uni-site catalysis. The mode of catalysis exhibited by these sites appears to be strictly dependent on the availability of medium ATP to accelerate product release. This is supported by the following observations: 1) the existence of a high affinity site during steady-state bi-site catalysis is demonstrated by the full-site occupancy attained at nanomolar concentrations of ATP (Figs. 1 and 2). These concentrations are far below the $K_a$ for bi-site catalysis (30 $\mu$M, Cross et al., 1982). 2) When enzyme undergoing steady-state bi-site catalysis at nanomolar ATP concentrations is subjected to pulse-chase measurements, bound label dissociates at a rate predicted by the rate of ATP binding (Fig. 4). This shows that the high affinity site detected under these conditions (Fig. 2) is kinetically competent for bi-site catalysis. 3) Upon addition of medium ATP in molar excess to MF$_s$, uni-site-loaded label dissociates at a rate predicted by the rate of ATP binding (Fig. 5). This demonstrates that sites loaded under conditions for uni-site catalysis are capable of switching to bi-site catalysis when medium substrate is available. 4) The intrinsic rate constants for unpromoted product release are identical for both uni-site

### Table I

**Equilibrium distribution for interconversion of bound substrate and products during uni-site and bi-site catalysis**

| Time after mixing s | Bound P/bound ATP mol/mol |
|---------------------|---------------------------|
| Uni-site            |                           |
| 30                  | 0.54                      |
| 45                  | 0.62                      |
| Bi-site             |                           |
| 55                  | 1.3                       |
| 75                  | 1.4                       |
| 95                  | 1.4                       |

MF$_s$ and [(γ-$32P$)]ATP were incubated as described in Fig. 6 for uni-site and bi-site catalysis. At the times indicated, $[^32P]Pi$ was removed on a centrifuge column. Column effluents were collected directly in perchloric acid containing carrier $P_i$ and ATP. An aliquot of the quenched effluent was counted for total $^{32P}$ and another was assayed for $[^3PP]Pi$ as described under "Experimental Procedures." Bound ATP was calculated as the difference between total bound $^{32P}$ and bound $[^3P]Pi$.
and bi-site loaded enzyme (Fig. 6, Table I, and text). This again indicates that the same sites participate in both activities.

Two laboratories have disputed the idea that the catalytic site characterized under uni-site conditions is capable of participating in multi-site catalysis when excess ATP is added (Rullough et al., 1987; Milgrom and Murataliev, 1987). Under the conditions used by these investigators, uni-site-loaded label did not chase at a sufficiently rapid rate or to a sufficient extent. Recently, Penefsky (1988) has repeated some of these experiments. In contrast, he finds that 80% of the uni-site-loaded label chases in a kinetically competent manner. In the experiments reported here, we have used enzyme and substrate concentrations that are far below those used in the other studies. We find that at least 85% of uni-site-loaded label chases in a kinetically competent manner (Fig. 5). Penefsky (1988) has offered several explanations for the failure to observe rapid chase of uni-site-loaded label under certain conditions. To these, we would add the possibility that differences in the Mg²⁺ content of MF, purified by the different laboratories might contribute to the differences noted. This suggestion is based on the effect that Mg²⁺ has on the ability of medium ATP to chase tightly bound ADP (see "Results").

 Several previous studies have suggested that uni-, bi-, and tri-site catalysis all occur in the micromolar concentration range for ATP. Hatefi's laboratory reports three Kₐ values (1 μM, 100 μM, and 1 mM, Wong et al., 1984) while Boyer's laboratory reports two Kₐ values (1.7 and 250 μM) plus a K₉, (20 μM) which characterizes a transition between high and low activity for the intermediate P = H₂O oxygen exchange reaction (Gresser et al., 1982). From the data presented in Fig. 2, it would appear that uni-site catalysis will occur only at picomolar concentrations of ATP when substrate is added in excess of enzyme. Thus, all observations made by Wong et al. (1984) and Gresser et al. (1982) likely resulted from bi-site and tri-site catalysis. One of the three Kₐ values measured by Wong et al. (1984) might reflect a transition between low and high activity forms of MF, obtained with the filling of the single vacant noncatalytic site that is present on native enzyme (Kironde and Cross, 1986). The K₉ values measured by Gresser et al. (1982) for the oxygen exchange reaction might reflect a kinetic partitioning between reassembly of ATP at the catalytic site, which is required for exchange, and substrate-promoted product release, which terminates the exchange.

A second contribution of the current studies is the improved measurements of the interaction of ADP at a high affinity catalytic site. Scheme 1 summarizes the rate constants for uni-site catalysis. Values for steps 1 and 2 are unchanged from those reported previously (Grubmeyer et al., 1982). For step 4, an accurate measurement of the dissociation rate constant for ADP (k₄ = 4 x 10⁻⁴ s⁻¹, Scheme 1) was made possible by the use of subnanomolar concentrations of MF₁. In previous studies (Grubmeyer et al., 1982), MF₁ was diluted to 14 nM. Unbeknownst at the time, this concentration was well above the Kₐ for binding at the high affinity chaseable site. Thus, under these conditions, a small amount of medium nucleotide would have been in equilibrium with bound ligand. The slow rate of loss of bound label measured under these conditions (1.2 x 10⁻⁴ s⁻¹, Grubmeyer et al., 1982) apparently reflects some process other than the rate of dissociation of ADP from the catalytic site. One such process might be a slow dissociation of endogenous nucleotide which would reduce the specific activity of medium [³H]ADP. This explanation is consistent with the rate of dissociation of ADP (4 x 10⁻⁴ s⁻¹) from a noncatalytic site having a Kₐ of 50 nM (Kironde and Cross, 1987).

Measurement of the rate of ADP binding is complicated by the fact that it appears to be a two-step process (see "Results"). Instead, equilibrium binding of ADP was measured, yielding a Kₐ of 1 nM at a chaseable catalytic site (Kₐ in Scheme 1). This is more than 100-fold lower than a value measured previously (Hilborn and Hammes, 1973). An association rate constant can be calculated using the measured Kₐ and the dissociation rate constant (Kₐ and K₈, Scheme 1). This gives a value of 4 x 10⁻⁴ M⁻¹ s⁻¹ for k₄ (Scheme 1). This is essentially the same rate as that for binding ATP (k₄, Scheme 1). The difference in Kₐ values for ATP and ADP binding (1/Kₐ and K₈, Scheme 1) is due to a 1000-fold slower rate of dissociation of ATP from the catalytic site (k₈ versus k₈, Scheme 1).

The binding of P, at a catalytic site containing bound ADP is not measurable with MF₁ in aqueous solution. However,
using the relationship \( K_{\text{r}} = K_{s} \cdot K_{s} \cdot K_{s} \text{ where } K_{s} \text{ is the overall equilibrium constant for ATP hydrolysis (} 4 \times 10^{3} \text{)} \) and assuming that \( K_{s} \text{ is not larger than } 10^{-7} \text{ M}^{-1} \text{, a value of } 8 \times 10^{-4} \text{ M} \text{ can be calculated for } K_{s}. \text{ From } K_{s} \text{ and } k_{s}, \text{ a value of } 5 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1} \text{ is calculated for the rate of } P_\text{b} \text{ binding at a site containing bound ADP } (k_{b}, \text{ Scheme 1}).

During ATP synthesis, ADP and P, must bind readily at the same site. Overall, the two substrate-binding steps would be thermodynamically favored if they were linked \((K_{s} \cdot K_{s} = 8 \times 10^{-7} \text{ M})\). This may occur on the energized membrane but not with de-energized membrane or soluble MF. Several studies suggest that energization of the membrane is necessary to bind both ADP and \( P_\text{b} \) at the same site (Rosing et al., 1977; Shoshan and Strotmann, 1980). It is also possible that ATP must be bound at an adjacent catalytic site for rapid binding of both ADP and P (Nalin and Cross, 1982).

In light of the revised value for the rate-limiting step in uni-site catalysis, the rate enhancement obtained when substrate binding promotes product release is 100,000 rather than 1 million as reported earlier (Grubmeyer et al., 1982). The revised value is closer to the values obtained for Escherichia coli BF (Wise et al., 1984) and from oxygen exchange measurements (O’Neal and Boyer, 1986). It should also be noted that since the dissociation rate constants for \( P_\text{b} \) and ADP are now known to be equal, product release may not be ordered as shown in Scheme 1.

Final comment should be made regarding the question of whether two or three catalytic sites on MF, participate during multisite catalysis at saturating substrate concentration. The simultaneous participation of three sites might normally be assumed for an enzyme having three copies of the catalytic subunit. However, it has been suggested that the interaction of the single-copy subunits with one \( \alpha \beta \) pair might render one catalytic site nonequivalent or nonfunctional (Amzel et al., 1982). Data presented here strongly supports the presence of at least two interacting sites. Over a wide range of ATP concentrations, the rate of product release is equal to the rate of ATP binding at an adjacent site (Figs. 3–5). This feature of catalysis was detected earlier in oxygen exchange studies (Kayalar et al., 1977; Choate et al., 1979) and in measurements with TNP-ATP (Grubmeyer and Penefsky, 1981). Triphasic kinetic behavior observed in the micromolar concentration range for ATP was originally interpreted as support for the participation of three catalytic sites (Gresser et al., 1982; Wong et al., 1984). As noted above, this evidence may now warrant reinterpretation. This would leave the kinetic properties which we have referred to as uni-, bi-, and tri-site catalysis as the main support for participation of three sites (Cross et al., 1982). Evidence presented here confirms turnover of a single site at picomolar concentrations of ATP \((K_{s} = 10^{-12} \text{ M}, \text{ Grubmeyer et al., 1982})\) and turnover of two sites at nanomolar to micromolar concentrations \((K_{s} = 30 \mu \text{M}, \text{ Cross et al., 1982})\). A second \( K_{s} \) observed at 150 \( \mu \text{M ATP} \) (Cross et al., 1982) could reflect half-maximal turnover at three sites. However, nucleotide binding at an exchangeable site, perhaps a “non-equivalent” catalytic site which functions as a regulatory site, might be responsible for the 2-fold rate enhancement obtained in going from bi-site to tri-site concentrations of ATP. Although we favor the view that three sites can function simultaneously (Cross, 1981, 1984, 1988), this alternative remains a viable possibility.

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