Bi-site Catalysis in F₁-ATPase: Does It Exist?*

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The mechanism of action of F₁F₀-ATP synthase is controversial. Some favor a tri-site mechanism, where substrate must fill all three catalytic sites for activity, others a bi-site mechanism, where one of the three sites is always unoccupied. New approaches were applied to examine this question. First, ITP was used as hydrolysis substrate; lower binding affinities of ITP versus ATP enable more accurate assessment of sites occupancy. Second, distributions of all eight possible enzyme species (with zero, one, two or three sites filled) as fraction of total enzyme population at each ITP concentration were calculated, and compared with measured ITPase activity. Confirming data were obtained with ATP as substrate. Third, we performed a theoretical analysis of possible bi-site mechanisms. The results argue convincingly that bi-site hydrolysis activity is negligible, and may not even exist. Effectively, tri-site hydrolysis is the only mechanism. We argue that only tri-site hydrolysis drives subunit rotation. Theoretical analyses of possible bi-site mechanisms reveal serious flaws, not previously recognized. One is that, in bi-site catalysis, the predicted direction of subunit rotation is the same for both ATP synthesis and hydrolysis; a second is that infrequently occurring enzyme species are required.

F₁ is the catalytic sector of ATP synthase, the enzyme that synthesizes ATP in the last step of oxidative phosphorylation (for reviews, see Refs. 1 and 2; short reviews on individual topics can be found in Refs. 3–5). F₁ has a subunit composition of α₃β₃γ₃δε. The three catalytic nucleotide-binding sites are located at α/β interfaces, mainly on the β subunits (6). F₁ can be isolated in soluble form and is an active ATPase. It is frequently used as a model for catalysis by the holoenzyme ATP synthase (also called F₁F₀).

F₁ (and F₁F₀) can hydrolyze MgATP in different modes, depending on the substrate concentration. Two of these modes are clearly established and well characterized. At low, substoichiometric MgATP concentrations the enzyme binds MgATP with very high affinity, just to catalytic site one. A single turnover of MgATP hydrolysis ensues on this site, called “uni-site catalysis,” in which the chemical hydrolysis step is slow (0.1 s⁻¹ in Escherichia coli F₁, Ref. 1) and products release is even slower (0.001 s⁻¹). On the other hand, at cellular (millimolar) substrate concentrations, all three catalytic sites are filled and interact with each other, and the enzyme turns over rapidly (~100 s⁻¹). This “tri-site” or “multisite catalysis” is the physiologically relevant working mode of the enzyme (1). In multisite catalysis, MgATP hydrolysis on the β subunits drives rotation of the γε complex (7–9), possibly transmitted via α subunits (10). In contrast, uni-site catalysis can occur without subunit rotation (11).

The contribution to hydrolysis of enzyme molecules with two substrate-filled catalytic sites (“bi-site catalysis”) remains enigmatic. Originally, deviations from simple Michaelis-Menten kinetics (single K_m values gave a better fit, were often interpreted as indication for the coexistence of bi-site (lower K_m) and tri-site (higher K_m) catalysis. However, there are a number of reasons (1, 12–14) why reliance on enzyme kinetics alone to infer catalytic sites occupancy might lead to erroneous results in this enzyme with its three, interconverting catalytic sites. A breakthrough came in the form of an E. coli F₁ mutant (βY331W) where a Trp introduced into the adenine binding pocket could be used to directly monitor nucleotide occupancy of the catalytic sites. By comparing the MgATP concentration dependence of nucleotide binding and hydrolytic activity in βY331WF₁, we were able to determine that steady-state hydrolytic activity was at least largely due to enzyme molecules with all three catalytic sites occupied (1, 12). In a number of analyses using this method, the contribution of bi-site catalysis was estimated to be between 0 and 5% of V_max (12, 15, 16).

If bi-site catalysis were a true working mode of F₁ and could serve as an experimental model for multisite catalysis, it would clearly facilitate analysis of the enzymatic mechanism, particularly rotation. Thus, we decided to re-address the question of the extent of bi-site catalysis, putting emphasis on analyses in the substrate concentration range where enzyme species with two sites filled are most prevalent. A prerequisite for this analysis is the exact knowledge of substrate binding affinities of the three catalytic sites, to allow calculation of the distribution of all enzyme species at any given substrate concentration.

In previous work (12, 15, 16), MgATP had been used as substrate. It has the technical disadvantage of having an overall high affinity for the catalytic sites of F₁, and so K_M and K_d are in a range where the true concentration of free substrate, knowledge of which is necessary to calculate the binding constants, is difficult to determine precisely. One problem is that the lower the concentrations of Mg²⁺ and ATP, the larger is the impact of uncertainty in the true dissociation constant for the metal-nucleotide complex. In addition, assessment of true Mg²⁺ concentrations becomes difficult in the submicromolar range, as does the determination of concentration of free (i.e. non-enzyme-bound) MgATP from total minus enzyme-bound ligand.

Previous studies (17–19) have indicated that the overall affinities of F₁ for MgGTP and MgITP are considerably lower than those for MgATP, while V_max(hydrolysis) appeared to be less affected. Both MgGTP and MgITP are competent at eliciting rotation (19) while MgGDP and MgIDP are good substrates in ATP synthesis (17). Unfortunately, GTP has significant absorbance at 295 nm, causing a large inner filter effect in Trp...
fluorescence experiments. Thus, we investigated here MgITP binding and hydrolysis in the catalytic sites of βY331F1, under different conditions. To interpret the experimental findings, we used a statistical analysis in which the probability of occurrence of all eight possible enzyme species (with zero, one, two, or three catalytic sites filled) was calculated at each substrate concentration and compared with the actual data. In light of the outcome, we also re-analyzed MgATP binding and hydrolysis. The results show that hydrolysis by enzyme molecules with two occupied catalytic sites plays an even lesser role than we had previously assumed. There is a strong possibility that bi-site catalysis as an independent working mode simply does not exist. Furthermore, as will be discussed, theoretical considerations indicate that even if bi-site catalysis does occur, it is most likely due to the activity of an enzyme species that accounts for maximally 3% of the total enzyme population.

**EXPERIMENTAL PROCEDURES**

**Enzyme Preparation and Analysis**—Wild-type F1 was from strain SWM1 (20), βY331 mutant F1 from strain SWM4 (12). Preparation of F1 was as described (18). Concentration of F1 was determined using the Bio-sense (21), with bovine serum albumin run as standard. Before use in activity and nucleotide binding assays, F1 was transferred into a buffer containing 50 mM Tris/H2SO4, pH 8.0, by passage through two consecutive 1-mL Sephadex G-50 centrifuge columns. This pretreatment produces enzyme with catalytic sites empty (22). To generate an enzyme population with homogeneous functional properties, the enzyme was incubated for 15 min with a 2-fold excess of isolated 16, and as the concentrations of F1 used in all experiments (≥80 nM) were 2 orders of magnitude higher than Kd for binding (about 1 nM; see Ref. 16), and as the concentrations of F1 used in all experiments (≥80 nM) were 2 orders of magnitude higher than Kd for binding (about 1 nM; see Ref. 16), the resulting enzyme was >99% e-replete. The isolated e subunit was a generous gift from Dr. Stanley D. Dunn (University of Western Ontario).

**Activity Assays**—ATPase activity was measured in 50 mM Tris/H2SO4, pH 8.0, at 23 °C. F1 concentration was 80–100 nM. The reaction was started by simultaneous addition of NaITP and MgSO4. In one set of experiments (Fig. 2, solid circles; Fig. 4a), ITP and Mg2+ were present in a 1:1 ratio, in a second set (Fig. 2, solid triangles; Fig. 4b) a 2.5 mM excess of Mg2+ over ITP was used. Samples were withdrawn at 10-, 20-, or 30-s intervals for a total time of 1, 2, or 3 min. Within this time, all hydrolysis rates were linear. Released P1 was determined colorimetrically (23, 24). ATPase activity was determined using the same technique, except that a Mg2+/ATP ratio of 1:2.5 was used. MgITP and MgATP concentrations were calculated using a dissociation constant of 20 μM, as determined for MgATP in Ref. 25. MgITP is expected to have the same dissociation constant (26). It should be noted that under the conditions of 2.5 mM Mg2+ excess virtually all ITP is complexed, thus uncertainties in the dissociation constant have no effect on the results.

**Fluorescence Measurements**—All fluorescence measurements were performed in a buffer containing 50 mM Tris/H2SO4, pH 8.0, at 23 °C, in a spectrofluorometer SPEX Fluorolog 2. Excitation wavelength was 295 nm. F1 concentration was 100–150 nM. For MgITP titration, two different conditions were used. In one series of experiments, NaITP and MgSO4 were added in a constant ratio of 1:1 to a solution of enzyme in buffer. Alternatively, MgSO4 was added in 2.5 mM excess over NaITP. Maximally two data points were acquired in an individual experiment to avoid interference by hydrolysis product MgIDP. For titration with uncomplexed ITP, buffer plus 0.5 mM EDTA was used, and NaITP was added. Background signals were subtracted, and inner filter and volume effects were corrected for by performing parallel titrations with wild-type F1. Nucleotide binding stoichiometries were determined from the decrease in β-Trp fluorescence at 360 nm (12). Nucleotide binding parameters were calculated by fitting theoretical binding curves to the measured data points. For MgITP binding a model with three sites of different affinity was used, for binding of uncomplexed ITP a model assuming one type of site was sufficient. For the fitting algorithm, see Ref. 27.

**Calculation of Enzyme Species Distribution**—In the following, the occupancy status of the three catalytic sites of an F1 molecule is described by a three-digit number in binary code. A zero “0” indicates an empty site, a one “1” stands for an occupied site. The first digit gives the status of site one, which is in F1, in the presence of Mg2+, the high affinity site; the second digit gives the status of the medium affinity site two, and the third that of the low affinity site three. Thus, e.g. 110 describes an enzyme molecule where sites one and two are filled while site three is empty. In previous analyses (12, 15, 16), we had calculated the fraction of enzyme molecules with zero, one, two, or three sites filled. However, only in the cases of zero and three sites filled are the respective populations homogeneous, 000 and 111, respectively. An enzyme with one bound nucleotide can have this on site one (100), site two (010), or site three (001). An enzyme with two bound nucleotides can have site one, two, or three empty (011, 101, 110). There are in total eight possible species, a complexity which previous analyses did not take account of. While 100 and 110 are clearly the prevailing species with one site or two sites filled, respectively, some of the other species can reach a population of a few percent (Figs. 3–5), which depends on the ratio between the respective dissociation constants, Kd, at the three sites. The fraction θXX of a certain enzyme species, XX (with X = 0 or 1), contributes to the total population is given by the product of the probabilities, pX, of each individual site, n (with n = one, two, or three), being in the specified state.

For an occupied site,

\[ p_n = c_n(1 + K_d) \]

(Eq. 1)

where c is the concentration of free (i.e. not enzyme-bound) ligand and Kd is the thermodynamic dissociation constant of site n for the ligand under consideration.

For an empty site,

\[ p_n = 1 - c_n(1 + K_d) = K_d/(c_n + K_d). \]

(Eq. 2)

Thus, e.g. the fraction of enzyme molecules in state 110 can be calculated from,

\[ \theta_{110} = (c_n(1 + K_d)(c_n(1 + K_d))(1 - c_n(1 + K_d))). \]

(Eq. 3)

**RESULTS**

**ITP Binding to βY331F1**—Binding of ITP to the three catalytic sites of F1 was measured using the fluorescence of an introduced Trp residue, β-Trp-331 (1, 12, 22). We have documented previously that the βY331 mutant enzyme is fully competent in oxidative phosphorylation and ATP-driven proton pumping (12, 15). Titration with MgITP using an [Mg2+]/[ITP] ratio of 1:1 is shown in Fig. 1A. Titration was also carried out using a 2.5 mM [Mg2+] excess over [ITP] (data not shown) and with uncomplexed ITP in the presence of EDTA (Fig. 1B). For comparison, data for MgATP and uncomplexed ATP are also shown in Fig. 1, A and B. It was immediately obvious that ITP binding parallels ATP binding, identically with affinities that are lower by 1–2 orders of magnitude; similar behavior has been observed previously for IDP versus ADP (28). Also it was clear that in the presence of Mg2+, the enzyme displays a pronounced binding asymmetry for ITP, as it does for ATP, whereas in the absence of Mg2+, the enzyme behaves symmetrically toward both ITP and ATP. Calculated Kd values for

![Fig. 1. ITP binding to βY331F1. A, ●, MgITP binding under conditions [Mg2+] = [ITP]; ○, MgATP binding under conditions [ATP]/[Mg2+] = 5.5/1 (as used in previous work, Refs. 12, 16, and 22). B, ■, uncomplexed ATP binding, presence of EDTA; □, uncomplexed ATP binding, presence of EDTA. Lines are fits to the data points; binding models and resulting Kd values are given under “Experimental Procedures” and Table I, respectively.](image-url)
MgITP and uncomplexed ITP are given in Table I, where parallel values are also given for MgATP and ATP.

**ITPase Activity of βY331W F₁**—ITPase assays at all ITP concentrations were linear, with no lags or bursts, and there was no indication of any inhibition by product MgIDP. It should be noted that all the activity assays, and the binding data above, were performed on fully ε-subunit-replete enzyme. As expected from the binding affinities, much higher concentrations of MgITP than of MgATP were required to achieve significant hydrolysis rates (Fig. 2). On the other hand, Vₘₐₓ(MgITP) for βY331W F₁ was found to be significantly higher than Vₘₐₓ(MgATP), a feature not observed with wild-type enzyme (18). Precise determination of Kₘ(MgITP) and Vₘₐₓ(MgITP) for βY331W F₁ was hampered by a decrease in activity at high (−10 mM) MgITP concentrations. This decrease was observed under all tested experimental conditions, also when using an excess of ITP over Mg²⁺ (data not shown), and is likely due to substrate inhibition. When all data points shown in Fig. 2 (top) were taken into account, under conditions where [Mg²⁺] = [ITP] (filled circles), Kₘ was determined to be 700 μM and Vₘₐₓ = 6.5 units/mg (fit shown as dashed line). For the data obtained under 2.5 mM Mg²⁺ excess (filled triangles), Kₘ was 800 μM and Vₘₐₓ = 4.5 units/mg (fit not shown). Clearly, these fits underestimated the true Kₘ and Vₘₐₓ values.

Better fits were obtained when the two highest data points in each curve (at 10 and 15 mM ITP) were excluded from the evaluation (fit to the filled circles in Fig. 2, top, shown as solid line). For the [Mg²⁺] = [ITP] condition, the resulting Kₘ was 1500 μM, Vₘₐₓ = 9.9 units/mg. For the data obtained under 2.5 mM Mg²⁺ excess, Kₘ was 1800 μM, Vₘₐₓ = 6.8 units/mg. (The lower Vₘₐₓ under conditions of excess [Mg²⁺] is consistent with established observations for ATP hydrolysis, and is referable to inhibition by Mg²⁺ ions.) In comparison, Kₘ(MgATP) was 29 μM, Vₘₐₓ(MgATP) = 3.0 units/mg (Fig. 2, lower, open circles and dotted line). As is obvious from the tabulation of results in Table I, Kₘ(MgITP) values agreed well with Kₘ(MgITP). This was also the case for MgATP, as was noted previously (12, 16). It should be noted that, for the evaluation described below, knowledge of Kₘ was not required, but Vₘₐₓ values were used to calculate relative specific activities (v/Vₘₐₓ). However, errors in Vₘₐₓ(MgITP) due to the inhibition at high MgITP concentrations were too small to have a significant impact on the outcome of the present study. It is important to note that in none of the cases did a fit with a second Kₘ value result in any improvement.

**Enzyme Species Distribution**—Based on the Kₐ values meas-

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**TABLE I**

| Conditions | Binding constants | Kinetic constants |
|------------|------------------|------------------|
| MgITP      |                  |                  |
| [Mg²⁺] = [ITP] |                 |                  |
| Kₛ₁ = 0.33 | Kₘ = 1500        | Vₘₐₓ = 9.9⁺      |
| Kₛ₂ = 62  |                  |                  |
| Kₛ₃ = 1400|                  |                  |
| [Mg²⁺] = 2.5 mM excess over [ITP] | |                  |
| Kₛ₁ = 0.33 | Kₘ = 1600        | Vₘₐₓ = 6.8⁺      |
| Kₛ₂ = 41  |                  |                  |
| Kₛ₃ = 1400|                  |                  |

ITP (plus EDTA)  

Kₛ₁, Kₛ₂, Kₛ₃ = 2600  
(N = 2.6)

MgATP  

[ATP]/[Mg²⁺] = 2.5⁺  

Kₛ₁ = 0.01  
Kₛ₂ = 0.8  
Kₛ₃ = 28  
Kₘ = 29  
Vₘₐₓ = 3.0

[Mg²⁺] = 2.5 mM excess over [ATP]  

Kₛ₁ = 0.02  
Kₛ₂ = 1.4  
Kₛ₃ = 23  
Kₘ = ND⁺  
Vₘₐₓ = ND

ATP (plus EDTA)  

Kₛ₁, Kₛ₂, Kₛ₃ = 53  
(N = 2.8)

⁺ Calculated from the fits in which the two highest data points were omitted, see "Results."
⁺⁺ This concentration ratio was used to be consistent with our previous work on MgATP binding and hydrolysis (12, 16, 27). Note that very similar data were obtained when [Mg²⁺] = 2.5 mM excess over [ATP].
⁺⁺ ND, not determined.
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Fig. 3. Distribution of enzyme species with different catalytic site occupancy as a function of MgITP concentration. Distributions (lines) were calculated based on $K_{r1} = 0.33 \mu M$, $K_{r2} = 62 \mu M$, $K_{r3} = 1400 \mu M$ (values for [Mg$^{2+}$] = [ITP] conditions) as described under “Experimental Procedures.” Species designations (100, 111, etc., as defined under “Experimental Procedures”) are located closely below the maximum of the respective curve. Note the logarithmic scales.

Fig. 4. Comparison of relative MgITPase activity and enzyme species distributions. A, [Mg$^{2+}$] = [ITP] conditions; B, [Mg$^{2+}$] = 2.5 mM excess over [ITP] conditions. Symbols (■ and ▲) show measured relative ITPase activity (right-hand scale). Solid lines show species distribution (left-hand scale). Thick solid lines stand for species 111, thin solid lines for remaining species, with assignment exactly as described in the legend to Fig. 3. (Note that in B, species 001 and 011 are off-scale.) The dotted lines represent predicted activity of sum of species 101 plus 111, if both have the same specific activity. The dashed lines represent predicted activity of the sum of species 110 plus 111 if both have the same specific activity. For further details, see “Results.”

to overall hydrolysis would indeed be very small (<0.15% of $V_{\text{max}}$). One might argue that such small differences between the thick solid line and the actual data points, if they exist at all, are due to activity of species 110, not species 101. However, calculation shows that as both species have the same concentration dependence, but species 110 is 25 to 35 times as populous (Figs. 3 and 4), the specific activity of species 110 would be less than 0.2% of that of species 111 (see also Footnote 1).

$K_r$ values and enzyme species distribution determined here are based on the assumption of three different, independent sites. This is the most likely scenario in F₅-ATPase, where positioning of the γ subunit likely determines binding affinity at each site, thus we believe our approach is well supported. However, cooperativity in binding of nucleotides could, in theory, affect calculation of relative distributions of enzyme species. For example, binding at site one might reduce the affinity at sites two and three. Klotz and Hunston, and others (42, 43), have developed a “stoichiometric formulation” to deal with such cases, in which “stoichiometric constants” are used to describe ligand binding, without any assumption of a specific binding model. This approach allows differentiation only between enzyme species with zero, one, two, or three filled catalytic sites, i.e., species 100, 010, and 001 are lumped together, as are 110, 101, and 011. When we applied this approach, we found stoichiometric dissociation constants very close to the $K_r$ values given in Table I. The resulting calculated enzyme species distributions were very similar to those for 000, 100, 110, and 111 in Figs. 3–5. While we cannot estimate individual activities of species 110 or 101 by this approach, we can state with confidence that the sum of their activities is ≤0.15% of $V_{\text{max}}$. (Species 100, 010, 001, and 011 may be ignored, because of negligible activity and/or very low frequency.) Therefore the major conclusions of this paper are not affected by cooperativity in the nucleotide binding parameters.

**Experimental Procedures.**
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Fig. 5. Comparison of relative MgATPase activity and enzyme species distributions. Symbols (C) show measured relative ATPase activity (right-hand scale), solid lines show species distribution (left-hand scale). The thick solid line stands for species 111, thin solid lines for remaining species, with assignment exactly as described in the legend to Fig. 3. (Note that species 001 and 011 are off-scale.) The dotted lines represent predicted activity of sum of species 101 plus 111, if both have the same specific activity. The dashed lines represent predicted activity of sum of species 110 plus 111 if both have the same specific activity. For further details, see “Results.”

ATPase Activity of Individual Enzyme Species—Fig. 5 shows the corresponding analysis for the MgATP data. The data points very clearly follow the species 111 distribution curve, for tri-site catalysis, and fall far from the dotted line (species 101 plus 111) or the dashed line (species 110 plus 111), showing that the bi-site species do not contribute to hydrolysis.

DISCUSSION

The goal of this study was to determine the possible role of bi-site catalysis during ATP hydrolysis by F\textsubscript{1}-ATPase. To this end, we analyzed nucleotide occupancy of the catalytic sites and hydrolysis rates as a function of substrate concentration, using two different substrates, MgITP and MgATP, under varied experimental conditions ([Mg\textsuperscript{2+}] = [nucleotide], Mg\textsuperscript{2+} excess, or nucleotide excess). We combined the results with an extensive statistical analysis of all eight possible species of enzyme that can exist, in regard to catalytic sites occupancy, calculating the distribution of each species as a fraction of the total population at each nucleotide concentration and comparing it with the hydrolysis activity seen at that concentration. Taken together the results present overwhelming evidence that the contribution of bi-site catalysis is negligible. All measurable steady-state hydrolytic activity is due to enzyme molecules with three occupied catalytic sites, and the data cast serious doubts on the existence of bi-site catalysis as an independent working mode of the enzyme. As we discuss below, it is evident also that occupancy of all three sites is a pre-requisite for ATP-driven subunit rotation, which apparently occurs exclusively by a tri-site mechanism.

Analysis of catalytic site occupancy was based on quenching of the fluorescence of a Trp residue, \(\beta\)-Trp-331, in the catalytic site. MgITP was selected as ligand/substrate, to shift the dissociation constants for catalytic sites one and two (\(K_{d1}\) and \(K_{d2}\)) into an experimentally better accessible concentration range. However, it also served a second purpose, which was to refute criticism of the fluorescence technique itself. Since introduction of the technique (12), a number of arguments had been presented questioning the validity of the results (29–31). Questions regarding the rapidity of the fluorescence response and effects of dissociation of \(\varepsilon\)-subunit were rebutted in Refs. 15 and 16. One further point raised was that the presence of “inhibitory MgADP” would interfere with the site occupancy analysis during MgATP hydrolysis (30, 31). So far, we failed to detect MgADP inhibition in \(E. coli\) F\textsubscript{1} under the conditions used to measure MgATP hydrolysis.\(^2\) It is nevertheless important to note that for enzymes and/or under conditions where MgADP inhibition can be observed, MgIDP inhibition is much less pronounced or absent (32–34). The ITPase assays reported in this work showed no deviations from linearity at any [ITP]. Thus, the close resemblance of the results obtained with MgATP and MgITP corroborated the absence of inhibitory MgADP in the MgATP binding/hydrolysis experiments.

Although our data appear to reject bi-site catalysis, we cannot rule out that it actually contributes some activity in the order of <0.15% of \(V_{max}\) and it is interesting to discuss in detail the theoretical bi-site catalysis mechanism, because this too is germane to the question of whether it exists or not. Specifically, the question is whether species 101 (with site two empty) or species 110 (with site three empty), or a combination of both, could be responsible for this activity. A possible answer to this question comes from consideration of subunit rotation directions. Fig. 6A shows our mechanism for tri-site ATP hydrolysis (1, 35, 36). Each individual site changes its binding affinity in the sequence H (high) → M (medium) → L (low) → H →. While a number of modifications will be required to describe tri-site ATP synthesis adequately (for a speculative model, see Fig. 3 in Ref. 36), as far as the “binding change” is concerned, the scheme in Fig. 6A can be readily reversed. Thus, in tri-site ATP synthesis the affinity sequence for any one site would be H → L → M → H → (see Fig. 3 of Ref. 36), i.e. opposite to the sequence in tri-site hydrolysis. Therefore, since the binding affinity changes at any one site are determined by the direction of subunit rotation, in the tri-site mechanism the direction of rotation in synthesis is predicted to be opposite to that in hydrolysis, as generally accepted.

Fig. 6B shows the widely cited model for reversible bi-site ATP synthesis and hydrolysis (37), which we have redrawn in the same format as the model in Fig. 6A. In synthesis direction (counterclockwise in Fig. 6B, solid arrowheads), the sequence of binding affinity changes at each site is H → L → M → H →, the same sequence as in tri-site synthesis. The sequence for hydrolysis (clockwise in Fig. 6B, open arrowheads), is H → M → L → H →, i.e. opposite to synthesis and predicting again rotation in opposite directions for synthesis versus hydrolysis. In hydrolysis, the incoming MgATP binds to the L site (site three), while the M site (site two) is empty. Obviously, this scenario generates species 101, which, as we calculated above, contributes maximally only 2–3% to the total enzyme population. Otherwise, the mechanism itself appears feasible. If the incoming MgATP molecule were to bind more conventionally to site two yielding the more populous species 110, and the binding change sequence were again H → M → L → H →, after the binding change the high-affinity site one would be empty, which would (a) interrupt the mechanism, (b) generate a species with very low probability (011), and (c) most likely lead to the release of MgATP from the (now) L site.

It is possible to write a bi-site hydrolysis mechanism based on species 110 which does not entail these problems, as shown in Fig. 6C. Here incoming MgATP binds conventionally to the M site (site two) rather than to the L site (site three). However, the resulting sequence of binding affinity changes at any one

\(^2\) \(E. coli\) cells in vivo, under anaerobic conditions, use F\textsubscript{1}F\textsubscript{0}-ATP synthase to hydrolyze MgATP to generate their membrane potential. Thus MgADP inhibition would be physiologically debilitating, and its absence is expected. In contrast, in mitochondria, chloroplasts, and \(Bacillus\) PS3 (which does not grow anaerobically), ATP synthesis is the only physiological role of F\textsubscript{1}F\textsubscript{0}-ATP synthase, and MgADP inhibition, which has been seen in these species, could be beneficial.
site is H → L → M → H →. This is the same order as for the bi-site synthesis mechanism in Fig. 6B or for the tri-site synthesis mechanism (Fig. 3 in Ref. 36), and, note, it is opposite to the order of binding affinity changes for the tri-site hydrolysis (Fig. 6A). Thus, we are forced to the conclusions that a conventional bi-site hydrolysis based on species 110 would require rotation in the same direction as synthesis, which is extremely unlikely, and that upon increasing substrate concentrations...
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during hydrolysis (from bi-site to tri-site) the enzyme should reverse the rotation direction, which of course was not observed. Hence, if a low level of bi-site hydrolysis does exist, it is probably due to species 101, not species 110. While hydrolysis by species 101 is formally bi-site catalysis, only the (very doubtful) hydrolysis by species 110 would be consistent with the bi-site mechanism as previously described (13, 30, 31). In general terms, this theoretical analysis shows that it is not possible to devise a bi-site mechanism which can describe reversible synthesis/hydrolysis without invoking less probable enzyme species, or unlikely scenarios such as a reversal of the order of affinities between the three sites due to presence of a proton gradient. These theoretical considerations concerning bi-site catalysis appear not to have been fully explored by previous investigators.

As to the question of whether ATP synthesis occurs by a bi-site or a tri-site mechanism, the above analysis shows that either is theoretically feasible. However, because there are literally no existing reported measurements of catalytic sites occupancy, obtained under equilibrium conditions during steady-state ATP synthesis, the question cannot yet be answered. The fact that the enzyme is required to operate in the tri-site mode during steady-state ATP synthesis, the question cannot yet be answered. The fact that the enzyme is required to operate in the tri-site mode during physiological synthesis also.

Finally, some discussion should be devoted to a recent paper where improvements in technique allowed analysis of subunit rotation in *Bacillus* PS3 enzyme on a submillisecond time scale (39). The fact that ATP-driven rotation apparently occurs by the same mechanism from MgATP concentrations as low as 20 mM up to saturation caused the authors to claim it as being due to bi-site catalysis. However, the authors also show (Fig. 3 of Ref. 39) that rotation at 20 mM MgATP is a very slow (−0.1% of $V_{\max}$) manifestation of rotation at substrate saturation, and that the $K_m$ for rotation is 15 μM. The latter value is very close to the $K_{d2}$ of 21 μM determined for the enzyme from the same source using the fluorescence technique described here (40). On the other hand, $K_{d2}$ in this enzyme appears to be very low. At 20 mM MgATP, site two is already half-filled (see Fig. 2 of Ref. 40). Thus, it is likely that the rotation observed at 20 mM MgATP is indeed due to enzyme molecules which have, transiently, all three sites filled. The facts that (a) only a single mechanism for rotation was observed from 20 mM up to 2 mM MgATP (39), and (b) it has now been established that, at 2 mM MgATP, hydrolysis in *Bacillus* PS3 enzyme occurs by a tri-site mechanism (40), argues convincingly that only tri-site catalysis drives rotation. Interestingly, using a fluorescent ATP analogue to study catalytic site occupancy simultaneously with rotation, the same group came to the conclusion that F$_1$ appears to operate in a tri-site mode (41). The fluorescent product was released only in the third 120° rotation step after binding, as predicted by our tri-site mechanism (1).

Acknowledgment—We thank Cori Ringholz for excellent technical assistance.

REFERENCES
1. Weber, J., and Senior, A. E. (1997) Biochim. Biophys. Acta 1319, 19–58
2. Nakamoto, R. K., Ketchum, C. J., and Al-Shawi, M. K. (1999) Annu. Rev. Biophys. Biomol. Struct. 28, 205–234
3. Special Issue: Proton-Motive ATPases (2000) J. Exp. Biol. 203(1)
4. Special Issue: The Mechanisms of F$_1$–ATPases (2000) Biochim. Biophys. Acta 1458(2/3)
5. Special Issue: ATP Synthesis in the Year 2000 (2000) J. Bioenerg. Biomembr. 32(4/5)
6. Abramhs, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Nature 370, 621–628
7. Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K., Jr. (1997) Nature 386, 299–302
8. Kato-Yamada, Y., Noji, H., Yasuda, R., Kinosita, K., Jr., and Yoshida, M. (1998) J. Biol. Chem. 273, 19275–19277
9. Sambongi, Y., Ikuo, Y., Tanabe, M., Omote, H., Iwamoto-Kihara, A., Ueda, I., Yanagida, T., Wada, Y., and Futai, M. (1999) Science 286, 1722–1724
10. Weber, J., Nadanaciva, S., and Senior, A. E. (2000) FEBS Lett. 483, 1–5
11. Garcia, J. J., and Capaldi, R. A. (1998) J. Biol. Chem. 273, 15840–15845
12. Weber, J., Wilke-Mounts, S., Lee, R. S. F., Grell, E., and Senior, A. E. (1993) J. Biol. Chem. 268, 20126–20133
13. Boyer, P. D. (1993) Biochim. Biophys. Acta 1140, 215–250
14. Jencks, W. P. (1997) Annu. Rev. Biochem. 66, 1–18
15. Lobos, S., Weber, J., and Senior, A. E. (1998) Biochemistry 37, 10846–10853
16. Weber, J., Dun, S. D., and Senior, A. E. (1999) J. Biol. Chem. 274, 19124–19128
17. Perlin, D. S., Latchney, L. R., Wise, J. G., and Senior, A. E. (1984) Biochemistry 23, 4988–5003
18. Weber, J., Lee, R. S. F., Grell, E., Wise, J. G., and Senior, A. E. (1992) J. Biol. Chem. 267, 1712–1718
19. Noji, H., Bald, D., Yasuda, R., Itoh, H., Yoshida, M., and Kinosita, K., Jr. (2001) J. Biol. Chem. 276, 25480–25486
20. Rao, R., Al-Shawi, M. K., and Senior, A. E. (1988) J. Biol. Chem. 263, 3648–3656
21. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
22. Weber, J., Wilke-Mounts, S., and Senior, A. E. (1994) J. Biol. Chem. 269, 20462–20467
23. Tauskow, H. H., and Sherr, E. (1953) J. Biol. Chem. 202, 675–685
24. van Veldhoven, P. P., and Mannaerts, G. P. (1987) Anal. Biochem. 161, 45–48
25. Pecoraro, V. L., Hermes, J. D., and Cleland, W. W. (1984) Biochemistry 23, 5262–5271
26. Dawson, R. M. C., Elliot, D. C., Elliot, W. H., and Jones, K. M. (eds) (1979) *Daw for Biochemical Research*, 2nd Ed
27. Lobos, S., Weber, J., Wilke-Mounts, S., and Senior, A. E. (1997) J. Biol. Chem. 272, 3648–3656
28. Nadanaciva, S., Weber, J., and Senior, A. E. (2000) Biochemistry 39, 1883–9590
29. Murata, T., and Boyer, P. D. (1994) J. Biol. Chem. 269, 15431–15439
30. Boyer, P. D. (1997) Annu. Rev. Biochem. 66, 717–749
31. Milgrom, Y. M., Murataliev, M. B., and Boyer, P. D. (1998) Biochemistry 37, 1037–1043
32. Vasilyeva, R. A., Minkov, I. B., Fitin, A. F., and Vinogradov, A. D. (1982) Biochem. J. 202, 9–14
33. Hyndman, D. J., Milgrom, Y. M., Bramhall, E. A., and Cross, R. L. (1994) J. Biol. Chem. 269, 28871–28877
34. Jault, J. M., Paik, S. R., Grodsky, N. B., and Allison, W. S. (1993) Biochemistry 32, 14979–14985
35. Weber, J., Bowman, C., and Senior, A. E. (1996) J. Biol. Chem. 271, 18711–18718
36. Weber, J., and Senior, A. E. (2000) Biochim. Biophys. Acta 1458, 300–309
37. Cross, R. L. (1991) Annu. Rev. Biochem. 60, 681–714
38. Boyer, P. D. (2000) Biochim. Biophys. Acta 1458, 252–262
39. Yasuda, R., Noji, H., Yoshida, M., Kinosita, K., Jr., and Itoh, H. (2001) Nature 410, 898–904
40. Ren, H., and Allison, W. S. (2000) J. Biol. Chem. 275, 10057–10063
41. Nishizaka, T., Adachi, K., Itoh, H., Kinosita, K., Jr., Noji, H., Oiwa, K., and Yasuda, R. (2001) Biochim. Biophys. Acta 1498, 158–164
42. Klotz, I. M., and Hunston, D. L. (1979) Arch. Biochem. Biophys. 193, 314–328
43. Peters, F., and Lücken, U. (1986) Methods Enzymol. 126, 733–740

3 Recently, a revised bi-site mechanism tried to overcome the problem by suggesting a hydrolysis sequence parallelling that shown in Fig. 6C (38). This, however, only shifts the problem to the synthesis direction, where ADP and Pi bind to the low affinity site three while leaving the medium affinity site two empty (38).