Supporting Text

A. Transfer of ΔG^0_{Enz} Between Enzymes.

Although the free energy simulations identifying the different sites were performed for MF_1, a complete experimental data set has not been published for this system. Consequently, we focus on the *E. coli* enzyme, EcF_1, for which the necessary data are available (1); in addition, we analyze experimental data for Thermophilic *Bacillus* PS3 F_1-ATPase (TF_1). This procedure requires the reasonable assumption that the essential features of EcF_1, TF_1, and MF_1 are similar although the quantitative aspects of some of the binding constants differ significantly. For example, the dissociation constant of ATP from the $\beta_{\rm TP}$-site of MF_1 is 10^{-12}M, whereas it is only 2×10^{-10}M in EcF_1. Nevertheless, the free energy of the reaction ATP+$H_2O \rightarrow$ ADP+Pi at the $\beta_{\rm TP}$ site is very similar in the two molecules [i.e., 0.4 kcal/mol for MF_1 (2) and -0.63 kcal/mol for EcF_1 (3)]. Also, the binding constants for ATP and ADP differ by similar magnitudes at each of the three sites for different species; e.g., the dissocation constants of ATP and ADP at the tight binding site are both about one hundred times higher in EcF_1 than that in MF_1 (1).

B. ATP Binding Constant at the $\beta_{\rm DP}$ Site.

Since the $\beta_{\rm DP}$ site is likely to be occupied by ADP,Pi during steady state hydrolysis (1), the binding constants for ATP at this site calculated using the total number of bound nucleotides under steady-state hydrolysis conditions are questionable. The mutant F_1-ATPase (4) with one β and the γ subunits crosslinked is likely to provide more reliable information on the binding constant for the $\beta_{\rm DP}$ site because for such a system no multisite hydrolysis can occur and the $\beta_{\rm DP}$ site is expected to be occupied by an ATP in the absence of medium ADP.

C. Additional Comparisons of the Present Results with Those of Wang and Oster.

Wang and Oster (WO) (5) assumed that the only source of free energy in F_1-ATPase is the binding free energy of ATP, which was assumed to be divided into two parts:

the first is used directly for rotation (the "power stroke") and the second is "stored" in a distorted form of the enzyme and becomes available as ADP is released (the "recoil power stroke"). The assumption of the separation of the two steps is based on the unisite measurements (see figure 3*a* in ref. 5), which show two drops in free energy during the reaction. However, this diagram is not valid for multisite catalysis, as we have pointed out in the main text. In the free energy diagram we have developed (see Fig. 1*a*), ATP binding is *one* of the sources of the free energy for the γ subunit rotation. This partial agreement with WO arises because the free energy of ATP binding in multisite catalysis involves the conformational change of β_{E} to β_{TP}, which is the site studied in unisite catalysis. However, unlike the model of WO, there is no storage of part of the binding energy in a distorted form of the enzyme in our model. Instead, a second source of free energy for the γ subunit rotation is provided by the stronger binding of the reaction products, ADP,Pi, to the β_{DP} site than to the β_{TP} site, as shown in Fig. 1a. The products ADP,Pi, according to our model, are released from a conformation close to the β_{E} site, which requires energy to be formed from the β_{DP} site through the rotation of the γ subunit. In the model of WO, from a corrected form of figure 3*b* of ref. 5, the β_{DP} site appears to have properties essentially identical to those of the β_{TP} site; specifically, WO seem to have assumed that the equilibrium constant for the hydrolysis reaction is near unity in the β_{DP} site, as it is in the β_{TP} site. This assumption disagrees with the experimental and simulation results and misses one of the essential aspects of the cooperativity involved in multisite catalysis.

D. Solution Conditions Used in the Kinetic Mechanism.

Although reversible interconversion between ATP and ADP+Pi occurs at the β_{TP} site, this interconversion corresponds to unisite hydrolysis with a rate constant on the order of 10^{-3} s^{-1} (3), much smaller than the lowest rate measured in the experiments of Yoshida and coworkers for TF_1 (>0.1 s^{-1} at the ATP concentration of 20 nM). Consequently, unisite hydrolysis is neglected in the present treatment. Moreover, no ADP or Pi was

added to the system in the experiments we analyze (1,7), so that the only source of ADP and Pi is the hydrolysis of ATP. The resulting concentrations of ADP and Pi (1,7) are sufficiently low during the hydrolysis reaction that the binding of ADP and Pi to the β sites can be neglected. Inhibition (8) (e.g., by MgADP) is also not considered in the present study because, in the experiments of Senior and coworkers, no inhibition was observed (1) and because the results from the Yoshida group (7) correspond to the "burst phase," before the occurrence of significant inhibition. A more detailed treatment including inhibition is required to explain these results.

E. Initiation vs. Trisite Hydrolysis.

Initiation and trisite ATP hydrolysis involve the same conformational changes of the subunits and use the changes in the binding free energies resulting from the hydrolysis of one ATP molecule as the driving force. One species included in the kinetic scheme, $\mathrm{S}(\beta_{\mathrm{DP}}, \mathrm{T})$, does not participate directly in the trisite cycle, but it does contribute to the occupation of the catalytic sites (see Supplementary Text, Section F). The contributions of the various species depend on the ATP concentration, which is the externally controlled variable and is always in excess of the concentration of F_1-ATPase.

F. Example of Steady State Approximation and Rate Constant Analysis.

For example, for $\mathrm{S}(\beta_{\mathrm{E}}, \mathrm{T}; \beta_{\mathrm{DP}}, \mathrm{D})$, one has in the trisite catalysis

$$\frac{\mathrm{d}[\mathrm{S}(\beta_{\mathrm{E}}, \mathrm{T}; \beta_{\mathrm{DP}}, \mathrm{D})]}{\mathrm{dt}} = k_1[\mathrm{T}][\mathrm{S}(\beta_{\mathrm{DP}}, \mathrm{D})] - (k_{-1} + k_t + k_{-2})[\mathrm{S}(\beta_{\mathrm{E}}, \mathrm{T}; \beta_{\mathrm{DP}}, \mathrm{D})] = 0 \qquad [1]$$

The values used for the rate constants are given in the legend to Fig. 3*a*. They are directly measured or are obtained from the equilibrium constants given in the thermodynamic section; e.g., k_{-1} is obtained from the ATP binding free energy at the β_{E} site and the value of k_1. As stated in the legend, the rate constant k_{r}, which determines the rate of the 120^o rotation of the γ subunit when hydrolysis takes place with only two β sites (the β_{TP} and β_{E} sites) occupied, is the only fitting parameter used in the present analysis. There are no direct measurements of its value. Its value, in principle, can be obtained

by analyzing the time dependence of the ATP hydrolysis rate of a nucleotide-depleted F_1-ATPase and would serve as a test of our analysis. For EcF_1, k_r was obtained to be around 0.04 s^{-1} by fitting the occupation of β sites obtained from experiments; this value was used subsequently to calculate the ATP concentration dependence of the hydrolysis rate constant. In the case of TF_1, it was found that if k_r is greater than 4 s^{-1}, both the hydrolysis and occupation results are explained. The larger k_r obtained for TF_1 than that for EcF_1 indicates that a nucleotide-depleted TF_1 should show a more prominent initial burst phase in the time-dependent rate of ATP hydrolysis than does EcF_1. The current analysis also provides detailed information on the ATP concentration dependence of various species as shown in Figs. 4 and 5.

We note, as described in the text, that the process involving k_r and that involving k_t share the same free energy source, namely the change in the free energy of ATP binding in the β_E to β_{TP} transformation and the change in the free energy of ADP,Pi binding in the β_{TP} to β_{DP} transformation. Nevertheless, the present analysis of the experimental results yielded a smaller value of k_r, relative to k_t, in particular for EcF_1. The main difference between the two processes is that in the reactant species for k_t [i.e., $S(\beta_E, T; \beta_{DP}, D)$, which occurs in trisite catalysis] all three β sites are occupied, whereas in the reactant species for k_r (i.e., $S(\beta_E, T)$, which occurs in the initiation of the enzyme activity for a nucleotide-depleted F_1-ATPase), the β_{DP} is empty. One would expect that the interactions between the β_{DP} site and the γ subunit and that between the β_{DP} and β_{TP} sites depend on whether the β_{DP} is occupied or not. This dependence suggests that, although the thermodynamics for the chemical processes of k_r and k_t are similar, the detailed potential energy surfaces that control the reaction rate are different. Enzymes from different species may respond differently to the occupation of the β_{DP} site, which would lead to different k_r and thus a different dependence of the rate of the ATP hydrolysis on the ATP concentrations.

Simulation studies (W. Y., Y. Q. G., and M. K., unpublished results) of ADP,Pi

binding at the $\beta_{\rm DP}$ site show that there is a conformational difference between the empty and occupied $\beta_{\rm DP}$ site and that the interaction between the $\beta_{\rm DP}$ site and the γ subunit is strongly influenced by the ADP,Pi binding to the β site. We note also that a structure of the F_1-ATPase from the spinach chloroplast with apparently no bound nucleotides, has all β sites in "closed" forms and that the γ subunit is partly disordered (9). This structure indicates that the overall conformation of F_1-ATPase, as well as the interaction between the β and γ subunits, depends on the nucleotide occupation. Further analyses (simulations and experiments) are necessary to test the above suggestion.

G. Equation for the Occupation of the β Sites.

From Fig. 2, during steady-state ATP hydrolysis, the total occupation of the catalytic sites, N, is

$$\begin{aligned} N &= \{[\mathrm{S}] + 2([\mathrm{S}(\beta_{\rm DP}, \mathrm{D})] + [\mathrm{S}(\beta_{\rm E}, \mathrm{T})] + [\mathrm{S}(\beta_{\rm DP}, \mathrm{T})]) \\ &\quad +3([\mathrm{S}(\beta_{\rm DP}, \mathrm{D}; \beta_{\rm E}, \mathrm{T})] + [\mathrm{S}(\beta_{\rm DP}, \mathrm{T}; \beta_{\rm E}, \mathrm{T})])\}/[\mathrm{E}]_0, \end{aligned} \qquad [2]$$

where the square brackets are used to indicate steady-state concentration of each species and $[\mathrm{E}]_0$ is the total concentration of the F_1-ATPase.

H. [ADP], [Pi] and [ATP] Dependence of the Speed of Rotation.

In the trisite hydrolysis mechanism developed here, hydrolysis occurs at one β site during its transition from $\beta_{\rm TP}$ to $\beta_{\rm DP}$ and there is a simultaneous transition of a $\beta_{\rm DP}$ site to a $\beta_{\rm E}$ site. Once the products are in the $\beta_{\rm E}$-like site, it is expected that they can be released without significant conformational change of the other two sites. Therefore, there is no rotation of the γ subunit during the release of ADP,Pi. The two 1-ms waiting times (7) in the γ subunit rotation at an intermediate rotational angle of γ (about 90°) then correspond to Pi and ADP release, respectively. This behavior would suggest that there exist two barriers in Fig. 1 at the 90° rotation angle of the γ subunit (not shown). Another possibility (7) for the two waiting times was suggested to be the hydrolysis reaction and the release of Pi (7); this possibility is not consistent with the present

mechanism because the hydrolysis is expected to occur before the β site actually reaches β_{DP}. Further, because the release of Pi and ADP does not provide a driving force in the present mechanism and occurs at a nearly constant rotation angle (about 90° in Fig. 1) of the γ subunit, neither the rotation rate of γ subunit nor the hydrolysis rate are expected to depend on the concentrations of ADP and Pi. Single molecule experiments at different concentrations of Pi yielded essentially the same rotation rate of the γ subunit, as long as the applied frictional load was the same (6).

The binding of ATP to the empty β site is also largely decoupled from the γ subunit rotation (the 0° to $\approx$ 0° region in Fig. 1) in the present mechanism. One would expect that the time for the γ subunit to finish a 120^o rotation once an ATP binds to the empty site is also independent of the ATP concentration. This independence was observed experimentally (6).

At low concentrations of ATP, the rate-limiting step for hydrolysis is the binding of ATP to the empty site. According to the present mechanism, the hydrolysis rate depends linearly on the ATP concentration for a wide range of [ATP] (in the nM to μM range, Fig. 3) in the case of TF_1. As [ATP] increases to the submillimolar range, however, the release of ADP and Pi becomes the rate-limiting steps, since they are independent of the concentration of ATP and have rate constants on the order of 1 ms^{-1} (7).

It is interesting to consider also the occupations observed in the crystal structure from the viewpoint of the proposed mechanism, although other factors might be involved in determining them. From the dissociation constants given in Table 1, the β_{TP} site would be occupied by an AMPPNP (an ATP analog) and the β_{DP} site by an ADP, if the crystal is prepared with [AMPPNP] = 250 μM and [ADP] = 5 μM. None of the sites could bind Pi. This conclusion is in agreement with the crystal structure (10). However, if the crystal is prepared with sulphate in the solution, β_{E} could be occupied by an ADP and a sulphate in the presence of 660 μM ADP and 20 mM $MgSO_4$, thus transforming it to a β_{HC} site, as found in a crystal structure (11).

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