

Binding and Hydrolysis of TNP-ATP by *Escherichia coli* F₁-ATPase*

(Received for publication, September 29, 1995, and in revised form, November 14, 1995)

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It had previously been suggested that V_{\max} hydrolysis rate of 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate (TNP-ATP) by F₁-ATPase required filling of only two catalytic sites on the enzyme (Grubmeyer, C., and Penefsky, H. S. (1981) *J. Biol. Chem.* 256, 3718–3727), whereas recently it was shown that V_{\max} rate of ATP hydrolysis requires that all three catalytic sites are filled (Weber, J., Wilke-Mounts, S., Lee, R. S. F., Grell, E., and Senior, A. E. (1993) *J. Biol. Chem.* 268, 20126–20133). To resolve this apparent discrepancy, we measured equilibrium binding and hydrolysis of MgTNP-ATP under identical conditions, using β Y331W mutant *Escherichia coli* F₁-ATPase, in which the genetically engineered tryptophan provides a direct fluorescent probe of catalytic site occupancy. We found that MgTNP-ATP hydrolysis at V_{\max} rate did require filling of all three catalytic sites, but in contrast to the situation with MgATP, "bisite hydrolysis" of MgTNP-ATP amounted to a substantial fraction (~40%) of V_{\max} .

Binding of MgTNP-ATP to the three catalytic sites showed strong binding cooperativity ($K_{d1} < 1$ nM, $K_{d2} = 23$ nM, $K_{d3} = 1.4$ μ M). Free TNP-ATP (*i.e.* in presence of EDTA) bound to all three catalytic sites with lower affinity but was not hydrolyzed. These data emphasize that the presence of Mg²⁺ is critical for cooperativity of substrate binding, formation of the very high affinity first catalytic site, and hydrolytic activity in F₁-ATPases and that these three properties are strongly correlated.

ATP synthesis by oxidative phosphorylation is catalyzed by ATP synthase. The F₁ sector of this enzyme contains three catalytic nucleotide binding sites, located on the three β -subunits (Senior, 1988; Fillingame, 1990; Allison *et al.*, 1992; Capaldi *et al.*, 1994; Abrahams *et al.*, 1994). F₁ may be isolated in soluble form; it is an active ATPase (F₁-ATPase), which has proved valuable for studies of catalytic mechanism.

Since their introduction (Hiratsuka and Uchida, 1973), the trinitrophenyl (TNP)¹ derivatives of adenine nucleotides have been widely used to characterize nucleotide binding sites of proteins and enzymes. These analogs have the advantages that they are fluorescent and often bind with much higher affinity than the natural nucleotides. Grubmeyer and Penefsky (1981a, 1981b) showed that mitochondrial F₁ hydrolyzed MgTNP-ATP with K_m 1000 times lower and V_{\max} 600 times lower than for MgATP. Importantly, these workers demonstrated that MgTNP-ATP was hydrolyzed by (at least) two catalytic sites on the enzyme and further that there was strong positive catalytic

cooperativity between catalytic sites, such that hydrolysis of MgTNP-ATP pre-bound at a single site per F₁ was greatly accelerated in presence of excess nucleotide sufficient to fill an additional catalytic site(s) per enzyme molecule. Later, the same cooperative behavior was detected with the natural substrate MgATP, with acceleration factors of 10⁵–10⁶ on going from "unisite" to "multisite" catalysis, not only for the mitochondrial enzyme (Cross *et al.*, 1982) but also for F₁ from other sources (Senior, 1988; Penefsky and Cross, 1991).

A major question regarding the catalytic mechanism of F₁ has centered on whether occupation of two catalytic sites by substrate is sufficient to achieve V_{\max} in steady-state catalysis or whether occupation of all three sites is required. Recently, the development of a fluorescent probe in the form of a tryptophan residue specifically inserted into the catalytic sites, which directly monitors the degree of occupancy of the sites by nucleotide, has allowed us to answer this question. Using β Y331W mutant *Escherichia coli* F₁, we measured in parallel experiments both MgATPase activity and degree of occupancy of the catalytic sites as a function of MgATP concentration. A single K_m value was found adequate to describe the concentration dependence of MgATP hydrolysis, and this K_m value was very similar to K_{d3} , the dissociation constant for binding of MgATP to the third catalytic site (Weber *et al.*, 1993). Thus, steady-state hydrolysis of MgATP at physiological rate requires that all three catalytic sites are filled with substrate. Filling of only two sites was seen to generate at most a low and non-physiological activity.

In contrast, for hydrolysis of MgTNP-ATP by mitochondrial F₁, it had been suggested previously that V_{\max} was reached upon occupation of only two of the three catalytic sites (Grubmeyer and Penefsky, 1981b). If this were the case, it would imply that the enzyme utilizes all three catalytic sites for ATP hydrolysis but only two sites for TNP-ATP hydrolysis. Considering that TNP-nucleotides are probably the most frequently used fluorescent analogs in studies of F₁-ATPases, this would constitute a serious discrepancy. The fact that V_{\max} for MgTNP-ATP hydrolysis is very low might be taken as consistent with the idea that MgTNP-ATP hydrolysis actually does occur by "bisite" catalysis, and indeed this low level of activity might indicate the general order of magnitude for bisite activity with other substrates. However, evidence from studies of isolated β -subunit (Rao *et al.*, 1988) and a catalytic site peptide fragment (Garboczi *et al.*, 1988) suggests that each catalytic site is potentially capable of binding MgTNP-ATP and therefore that in intact F₁ all three catalytic sites would be expected to bind MgTNP-ATP. Furthermore, intact F₁ was found to bind three MgTNP-ADP (mol/mol) at saturation (Grubmeyer and Penefsky, 1981a; Tiedge and Schäfer, 1986).

With the availability of the β Y331W mutant *E. coli* F₁ we are now able to measure MgTNP-ATP binding to catalytic sites directly. In the study presented here, we establish the relationship between catalytic site occupancy and MgTNP-ATP hydrolytic activity, and we report the K_d values for binding of MgTNP-ATP and free TNP-ATP to F₁ catalytic sites.

* This work was supported by National Institutes of Health Grant GM25349 (to A. E. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ The abbreviations used are: TNP, trinitrophenyl; TNP-ATP or TNP-ADP, 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate or -diphosphate, respectively.

MATERIALS AND METHODS

Wild-type and $\beta Y331W$ mutant F_1 were prepared from strains SWM1 (Rao *et al.*, 1988) and pSWM4/JP17 (Weber *et al.*, 1993), respectively, as described (Weber *et al.*, 1992). Before use, F_1 was equilibrated in 50 mM Tris/ H_2SO_4 , pH 8.0, by passing 100- μ l aliquots consecutively through two 1-ml Sephadex G-50 centrifuge columns; this treatment reduced the amount of nucleotide bound to catalytic sites to ≤ 0.2 mol/mol F_1 , as judged from the fluorescence signal of $\beta Y331W$ F_1 . Protein concentration of F_1 solutions was determined using the Bio-Rad protein assay (Bradford, 1976). The molecular mass of F_1 was taken as 382,000 Da (Senior and Wise, 1983). The F_1 concentration was 35–50 nM in all experiments unless stated otherwise.

TNP-ATP was purchased from Molecular Probes, Inc. (type T-7602, tri-sodium salt, supplied as 5 mg/ml solution in 0.1 M Tris, pH 9, purity 96% by high pressure liquid chromatography according to supplier) and was stored at $-20^\circ C$. Purity of this material was checked by thin layer chromatography as described by Grubmeyer and Penefsky (1981a) and showed a single spot with mobility equal to that of TNP-ATP. Phosphate analysis was carried out by the method of Taussky and Shorr (1953) after complete hydrolysis by calf intestinal alkaline phosphatase or in 12 M H_2SO_4 and gave values of 3.13 and 3.02 mol P_i per mol of TNP, respectively. Before hydrolysis, the P_i content was 0.02 mol/mol TNP. Concentration determinations for TNP-ATP were based on an extinction coefficient of 26,400 $M^{-1} cm^{-1}$ at 408 nm (Hiratsuka and Uchida, 1973). All experiments were performed at $23^\circ C$ in buffer containing 50 mM Tris/ H_2SO_4 , pH 8.0, with further additions as indicated. For measurements of hydrolytic activity, TNP-ATP (or ATP) and $MgSO_4$ were added in a concentration ratio of 2.5 to 1. MgTNP-ATP (and MgATP) concentrations were calculated according to Fabiato and Fabiato (1979), assuming that the TNP moiety did not affect Mg^{2+} complexation. Hydrolysis activities were calculated from the amount of P_i liberated; P_i was determined by a very sensitive colorimetric assay (van Veldhoven and Mannaerts, 1987).

Fluorescence experiments were performed as described in Weber *et al.* (1993). For MgTNP-ATP binding experiments, the buffer contained 50 mM Tris/ H_2SO_4 , pH 8.0, with nucleotide and $MgSO_4$ present in ratio of 2.5 to 1. For measurements of binding of TNP-ATP in the absence of Mg^{2+} , the buffer contained 0.5 mM EDTA instead of $MgSO_4$. It should be emphasized that the signal used to measure catalytic site MgTNP-ATP binding throughout this study was the tryptophan fluorescence of residue $\beta W331$. Even at the lowest concentrations used, binding of MgTNP-ATP was complete in less than 30 s; under these conditions, less than 5% of the analog was hydrolyzed. Parallel titrations of wild-type enzyme with MgTNP-ATP were used to correct for inner filter effects and/or resonance energy transfer from tryptophan residues other than $\beta W331$. Energy transfer from $\beta W331$ in one catalytic nucleotide binding site to MgTNP-ATP bound in another catalytic site, which might be considered a possible error source, is highly unlikely, as the critical transfer distance for the donor/acceptor pair tryptophan/TNP-ATP (~ 23 Å, Gryczynski *et al.* (1989)) is much smaller than the distance between catalytic sites (48 Å as calculated from the x-ray structure, Abrahams *et al.* (1994)). Enzyme prepared as described above contains noncatalytic sites essentially filled with endogenous adenine nucleotide.² In previous work with native *E. coli* F_1 , we showed that under the conditions used here for fluorescence measurements in the presence of Mg^{2+} , no release of noncatalytic site-bound nucleotide occurred over a period of 3 h (Weber and Senior, 1995). To test whether the presence of EDTA induced release of nucleotide from noncatalytic sites, native F_1 was incubated in 50 mM Tris- SO_4 , pH 8.0, 5 mM EDTA at $23^\circ C$, and nucleotide release was followed as described by Weber and Senior (1995) (the presence of EDTA prevents rebinding of released nucleotide, which is Mg^{2+} -dependent). The calculated $t_{1/2}$ for release of 1 mol of noncatalytic site nucleotide per mol of F_1 was 150 min. The fluorescence titrations were performed in 10-fold lower concentration of EDTA (above), and the signal was complete in ≤ 15 min. Therefore, no significant occupation of noncatalytic sites by TNP-ATP could occur during the time courses of the fluorescence experiments, and significant energy transfer between $\beta W331$ and noncatalytic site-bound TNP-nucleotide would not occur.

RESULTS

Fig. 1 shows hydrolysis of MgTNP-ATP by wild-type *E. coli* F_1 as a function of substrate concentration. At saturation, the MgTNP-ATPase activity was 0.16 units/mg, which is 1.4% of

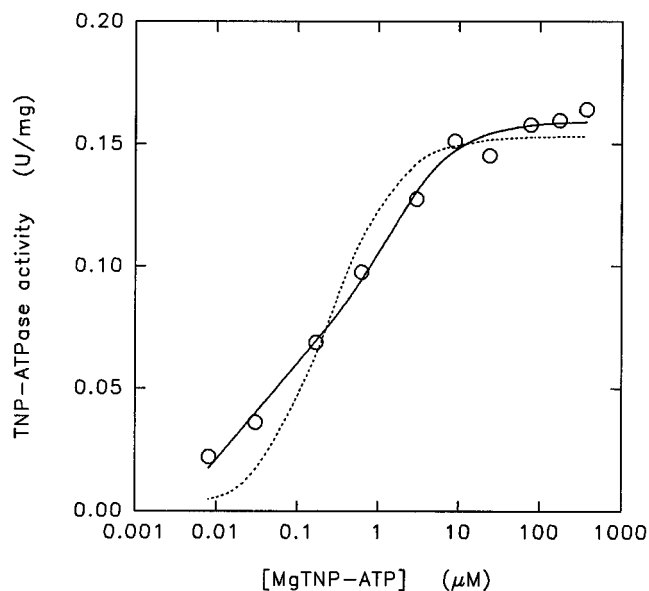


FIG. 1. **Hydrolysis of MgTNP-ATP by wild-type *E. coli* F_1 -ATPase.** Hydrolysis of MgTNP-ATP was measured at $23^\circ C$ and pH 8.0 as described under "Materials and Methods." The dashed line is a fit to the Michaelis-Menten equation assuming a single K_m value; the solid line is a fit assuming two K_m values. Each data point (open circles) represents the average of at least duplicate experiments.

the MgATPase activity under the same conditions ($23^\circ C$, pH 8.0). The dashed line in Fig. 1 is a fit to a model assuming Michaelis-Menten kinetics with a single K_m . However, it is evident that the fit could be improved significantly using a model with two K_m values (Fig. 1, solid line). The calculated K_m and V_{max} values obtained using both models are given in Table I. One point that is evident from these data is that *E. coli* F_1 hydrolyzes MgTNP-ATP relatively better than does mitochondrial F_1 , e.g. the ratio $V_{max}(MgTNP-ATP)/V_{max}(MgATP)$ is about 10-fold higher in *E. coli* F_1 as compared to mitochondrial F_1 .

In previous work we have used the $\beta Y331W$ mutant *E. coli* F_1 extensively to characterize catalytic site nucleotide-binding parameters (see Introduction). It should be noted that this enzyme has properties similar to wild type in both ATP hydrolysis and synthesis. Here, we found that the enzymatic characteristics of $\beta Y331W$ mutant F_1 with MgTNP-ATP as substrate (Fig. 2, open circles) were also very similar to those of the wild-type enzyme. At saturation, $V_{max}(MgTNP-ATP)$ (0.09 units/mg) was 1.5% of $V_{max}(MgATP)$. The dashed line in Fig. 2 represents a model with a single K_m . The fit could be improved considerably by using a model with two K_m values (Fig. 2, lower solid line). The calculated K_m and V_{max} values are given in Table I. It is evident from Table I that in both wild-type and mutant enzymes, for the model assuming two K_m values the hydrolytic mode represented by the lower K_m value contributed $\sim 40\%$ of the total activity.

Binding of MgTNP-ATP to $\beta Y331W$ mutant F_1 catalytic sites was measured using fluorescence of the genetically engineered tryptophan residue as signal. The results of titration experiments with MgTNP-ATP are shown in Fig. 2 (filled circles). At $\geq 10 \mu M$ MgTNP-ATP, the fluorescence of residue $\beta W331$ was completely quenched, indicating that all three catalytic sites were filled. Analysis of the MgTNP-ATP binding parameters using a non-linear least-squares fit gave the following: site 1, $K_d \leq 1$ nM; site 2, $K_d = 23$ nM; site 3, $K_d = 1.39 \mu M$. Compared to MgATP (Weber *et al.*, 1993, 1994a), binding of MgTNP-ATP was therefore 20–30-fold tighter, but the same pattern was evident, i.e. binding to three sites with widely different affinity

² J. Weber and A. E. Senior, unpublished data.

TABLE I
Binding and hydrolysis of MgTNP-ATP by *E. coli* F_1 -ATPase

F_1	Binding parameters			Model	Hydrolysis parameters			
	K_{d1}	K_{d2}	K_{d3}		K_{M1}	$V_{\max1}$	K_{M2}	$V_{\max2}$
		μM			μM	units/mg	μM	units/mg
Wild type	ND ^a	ND	ND	Single K_M	0.24	0.153		
				Two K_M	0.023	0.067	1.33	0.092
β Y331W	<0.001	0.023	1.39	Single K_M	0.32	0.089		
				Two K_M	0.032	0.035	1.33	0.056

^a ND, not determined.

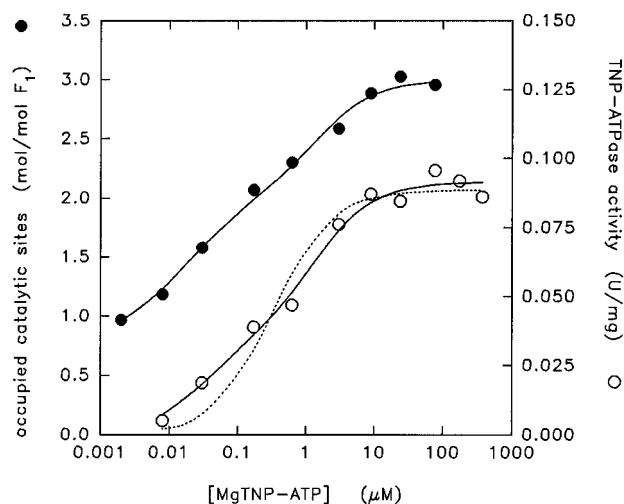


FIG. 2. Binding and hydrolysis of MgTNP-ATP by β Y331W mutant *E. coli* F_1 -ATPase. Hydrolysis of MgTNP-ATP was assayed at 23 °C and pH 8.0 as described under "Materials and Methods." The dashed line is a fit to the Michaelis-Menten equation assuming a single K_m value; the lower solid line is a fit assuming two K_m values. Each data point (open circles) represents the average of at least duplicate experiments. Binding of MgTNP-ATP (filled circles) was measured under the same conditions as hydrolysis, using the fluorescence of the β W331 residue as signal (see "Materials and Methods"). Each data point represents the average of at least duplicate experiments. The upper solid line is a fit to the binding data assuming a model with a different K_d value for each of three binding sites.

at each site.

The agreement between K_{M1} and K_{d2} on the one hand and K_{M2} and K_{d3} on the other is remarkable (Table I). The results therefore show that only enzyme molecules that have all three catalytic sites filled with MgTNP-ATP are able to hydrolyze it at maximum rate. However, it is apparent also that F_1 molecules that have just two catalytic sites occupied by substrate do have significant MgTNP-ATPase activity. This point is further examined in Fig. 3, where the MgTNP-ATPase activity (filled circles) is plotted versus the fraction of catalytic binding sites occupied by MgTNP-ATP. This latter parameter is of course an average for all the enzyme molecules in the population. The solid line in Fig. 3 shows the calculated activity expected if only enzyme molecules with three substrate-filled sites are catalytically active (*i.e.* bisite activity = zero). The dashed line in Fig. 3 shows the calculated activity expected if enzyme molecules with two sites filled show 38% of the activity exhibited by enzyme molecules that have all three sites filled. It is clear from Fig. 3 that a model that ascribes partial (38%) activity to enzyme molecules with two substrate-occupied sites results in a good fit to the actual hydrolysis data.

We had previously shown that, in absence of Mg^{2+} , ATP bound to all three catalytic sites of β Y331W mutant F_1 with the same affinity ($K_d = 76 \mu\text{M}$) (Weber *et al.*, 1994a). Binding of TNP-ATP was studied here under the same conditions. As can be seen from Fig. 4, TNP-ATP also filled all three catalytic sites in absence of Mg^{2+} . A model assuming n identical, independent

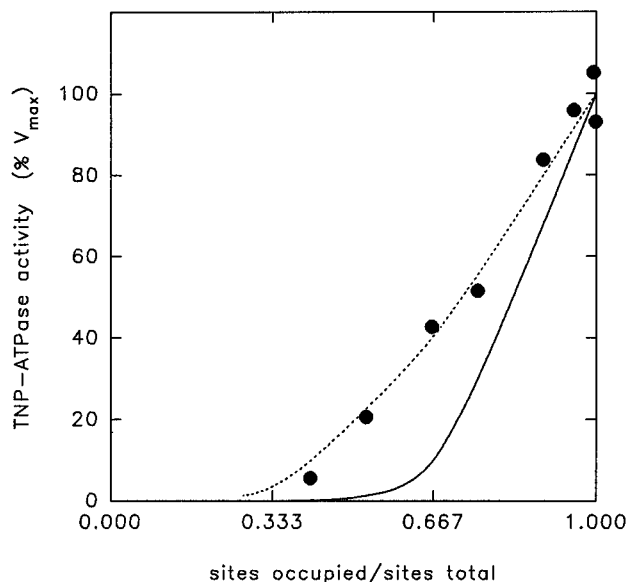


FIG. 3. Estimation of the extent of bisite hydrolysis of MgTNP-ATP by β Y331W mutant *E. coli* F_1 -ATPase. Data for MgTNP-ATP hydrolysis (from Fig. 2) were plotted against the fraction of catalytic sites occupied (solid circles). The latter parameter was calculated from the binding data of Fig. 2. The solid line is the calculated hydrolysis activity expected if bisite activity equals zero; the dashed line is the calculated hydrolysis expected if bisite activity equals 38% of V_{\max} (note: bisite activity is defined as $(V_{\max1}/(V_{\max1} + V_{\max2}))$, and the figure 38% was derived from the data of Table I, line 4).

binding sites gave a reasonable fit (Fig. 4, solid line) with calculated $K_d = 4.1 \mu\text{M}$ at 2.8 sites. However, a better fit was obtained using a model with three different independent binding sites, with $K_{d1} = 1.3 \mu\text{M}$, $K_{d2} = 4.1 \mu\text{M}$, and $K_{d3} = 32 \mu\text{M}$ (Fig. 4, dashed line).

We found that in absence of Mg^{2+} , TNP-ATP was not a hydrolysis substrate. After incubation of 400 nM β Y331W mutant F_1 with 100 μM TNP-ATP in presence of 0.5 mM EDTA for 3 h at 23 °C, pH 8.0, the amount of released P_i was found to be below the detection limit of the assay (100 pmol). From the data, we estimate that the TNP-ATP hydrolysis rate in absence of Mg^{2+} is below 10^{-5} units/mg, *i.e.* <0.01% of V_{\max} (MgTNP-ATP). Similar data were seen previously for ATP hydrolysis in absence of Mg^{2+} (Weber *et al.*, 1994a).

DISCUSSION

The major objective of this study was to determine whether maximal rates of MgTNP-ATP hydrolysis by F_1 -ATPase are achieved when just two of the three catalytic sites on the enzyme are occupied by substrate, as had been suggested previously (Grubmeyer and Penefsky, 1981b), or whether maximal rates are achieved only when all three catalytic sites are filled, as had been demonstrated to be the case for MgATP hydrolysis (Weber *et al.*, 1993, 1994a). The results established that V_{\max} rates of MgTNP-ATP hydrolysis are achieved only when all three catalytic sites are filled.

An important difference between MgTNP-ATP and MgATP

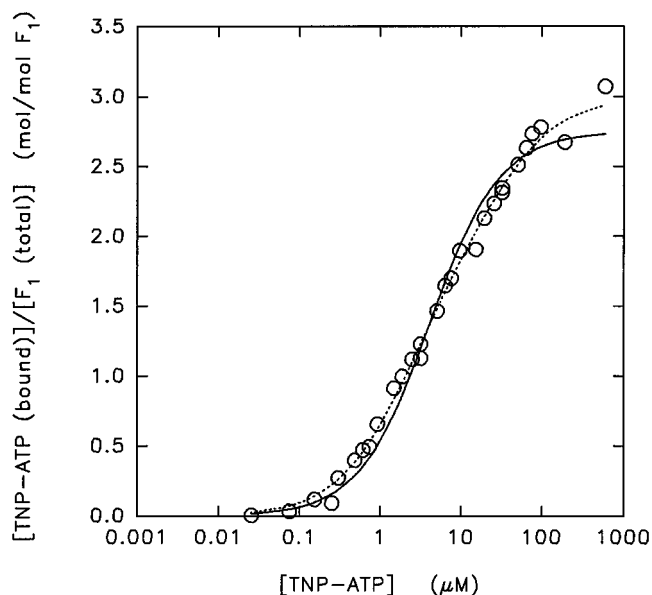


FIG. 4. Binding of TNP-ATP to $\beta Y331W$ mutant *E. coli* F_1 -ATPase in the absence of Mg^{2+} . Free TNP-ATP binding in absence of Mg^{2+} was measured as described under "Materials and Methods" (open circles). The F_1 concentration was 90 nM. The solid line is a fit assuming n independent and equivalent sites. The dashed line is a fit assuming three sites of differing affinity (see text for details).

hydrolysis was found, however. In previous work we showed that, for MgATP hydrolysis, the rate of "bisite" activity (*i.e.* the rate of hydrolysis manifested by an enzyme molecule having just two catalytic sites filled) was but a small fraction of V_{max} , if it occurred at all (Weber *et al.*, 1993). The previous data allow an estimate that bisite hydrolysis of MgATP could range from zero to $\leq 2\%$ of V_{max} . Here, we saw that bisite hydrolysis of MgTNP-ATP amounted to a substantial fraction (38%) of V_{max} . It may be noted that in absolute terms this rate of bisite hydrolysis is very slow, and indeed that V_{max} for MgTNP-ATP hydrolysis by *E. coli* F_1 was only 1.4% of V_{max} (MgATP). Nevertheless, the fact that there is real bisite hydrolysis of MgTNP-ATP may well imply that the rate of bisite hydrolysis of MgATP is greater than zero.

We wish to emphasize that our conclusion that *E. coli* F_1 shows bisite hydrolysis of MgTNP-ATP is based on both kinetic experiments showing the appearance of two K_m values and on equilibrium binding data taken under the same conditions and that it is the agreement between K_m values and K_d values that supports our conclusion. In our opinion, it would be unjustified to conclude that bisite hydrolysis occurred on the basis of kinetic data alone, and for this reason we believe that this is the first real demonstration of bisite hydrolysis by an F_1 enzyme.

Previously, the association rate constants for binding of MgATP at the first and third catalytic sites of *E. coli* F_1 have been calculated, and both are close to $10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Weber *et al.*, 1994b). However the rate constant for binding to site 2 is not yet known. From the data presented here, we can calculate k_{cat}/K_m values for MgTNP-ATP hydrolysis by wild-type *E. coli* F_1 in both bisite and trisite hydrolysis, which are, respectively, $1.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $0.76 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. This indicates that the rate of binding of substrate to site 2 was 1 order of magnitude faster than binding to site 3. This is a significant finding in view of the fact that the enzyme is required to have all three sites filled to achieve physiological rates of MgATP hydrolysis. It means that, should nucleotide happen to dissociate from site 2 during the catalytic cycle, thus temporarily disabling the enzyme, it would tend to rebind very rapidly and restore the

enzyme molecule to the fully active state. Dissociation of nucleotide from site 1 is much less likely to occur because the dissociation rate constant at this site is extremely slow (Senior, 1988; Penefsky and Cross, 1991).

The equilibrium binding data reported here showed that generally TNP-ATP mirrored ATP in its behavior and was therefore a good analog for catalytic site ATP binding. MgTNP-ATP was bound with 20–30-fold higher affinity than MgATP but showed the same pattern of three sites with different affinities. The presence of Mg^{2+} was seen to cause a huge increase in affinity for TNP-ATP at catalytic site one, as was previously seen to be the case with ATP (Weber *et al.*, 1994a). The K_d for binding of MgTNP-ATP at catalytic site 3 was of similar magnitude to that for MgTNP-ATP binding to isolated β -subunit (Rao *et al.*, 1988) and also for binding of free TNP-ATP at each of the catalytic sites in absence of Mg^{2+} . Similar behavior was noted previously with MgATP (Weber *et al.*, 1994a). Therefore, the data presented here provide additional evidence to support our previous conclusion that the presence of Mg^{2+} is critical for manifestation of strong substrate binding cooperativity, formation of the very high affinity site one, and presence of catalytic activity and that these three properties are strongly correlated.

In previous studies of binding of free ATP (in absence of Mg^{2+}), we found that all three catalytic sites bound the nucleotide with the same K_d value, and thus it appeared that all three sites were equivalent under these conditions (Weber *et al.*, 1994a). The new data reported here with free TNP-ATP suggest, however, that the three sites showed somewhat different affinities toward the analog, although not to anywhere near the extent seen with MgTNP-ATP. Therefore, it is apparent that even in the absence of Mg^{2+} , the catalytic sites of F_1 -ATPase show some degree of apparent "asymmetry," in agreement with recent chemical modification experiments (Haughton and Capaldi, 1995).

Acknowledgment—We thank Cheryl Bowman for excellent technical assistance.

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