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

Jochim Weber and Alan E. Senior

From the Department of Biochemistry, University of Rochester Medical Center, Rochester, New York 14642

It had previously been suggested that $V_{\text{max}}$ 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 $V_{\text{max}}$ 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 yj331W 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 $V_{\text{max}}$ 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 $V_{\text{max}}$.

Binding of MgTNP-ATP to the three catalytic sites showed strong binding cooperativity ($K_{d1} < 1 \text{ nM}$, $K_{d2} = 23 \text{ nM}$, $K_{d3} = 1.4 \text{ \mu M}$). 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 Mg⁡2⁺ 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 subunit of this enzyme contains three catalytic nucleotide binding sites, located on the three $\beta$-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 $V_{\text{max}}$ 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 $10^3$–$10^6$ on going from “unisite” to “bisite” catalysis, not only for the mitochondrial enzyme (Cross et al., 1982) but also for F₃ 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 $V_{\text{max}}$ 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 yj331W 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 $V_{\text{max}}$ 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 $V_{\text{max}}$ 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 $\beta$-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 yj331W 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 $K_o$ values for binding of MgTNP-ATP and free TNP-ATP to F₁ catalytic sites.

²This 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.

¹The abbreviations used are: TNP, trinitrophenyl; TNP-ATP or TNP-ADP, 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate or -diphosphate, respectively.
Wild-type and βY331W mutant F₁ were prepared from strains SW1 (Rao et al., 1988) and pSW44/P17 (Weber et al., 1993), respectively, as described (Weber et al., 1992). Before use, F₁ was equilibrated in 50 mM Tris/H₂SO₄, pH 8.0, by passing 100-μl 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 F₁, as judged from the fluorescence signal of βY331W F₁. Protein concentration of F₁ solutions was determined using the Bio-Rad protein assay (Bradford, 1976). The molecular mass of F₁ was taken as 382,000 Da (Senior and Wise, 1983). The F₁ 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 °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 H₂SO₄ and gave values of 3.13 and 3.02 mol P per mol of TNP, respectively. Before hydrolysis, the Pₐ 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 °C in buffer containing 50 mM Tris/H₂SO₄, pH 8.0, with further additions as indicated. For measurements of hydrolytic activity, TNP-ATP (or ATP) and MgSO₄ were added in a concentration ratio of 2.5:1 to 1. MgTNP-ATP (and MgATP) concentrations were calculated according to Fabiato and Fabiato (1979), assuming that the TNP moiety did not affect Mg²⁺-complexation. Hydrolysis activities were calculated from the amount of Pₐ liberated; Pₐ 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/H₂SO₄, pH 8.0, with nucleotide and MgSO₄ present in a ratio of 2.5:1. For measurements of binding of TNP-ATP in the absence of Mg²⁺, the buffer contained 0.5 mM EDTA instead of MgSO₄. It should be emphasized that the signal used to measure catalytic site MgTNP-ATP binding throughout this study was the tryptophan fluorescence of residue βW331. 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 βW331. Energy transfer from βW331 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. In previous work with native E. coli F₁, we showed that under the conditions used here for fluorescence measurements in the presence of Mg²⁺, 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 F₁ was incubated in 50 mM Tris-H₂SO₄, pH 8.0, 5 mM EDTA at 23 °C, and nucleotide release was followed as described by Weber and Senior (1995) (the presence of EDTA prevents rebinding of released nucleotide, which is Mg²⁺-dependent). The calculated τ₀ for release of 1 mol of noncatalytic site nucleotide per mol of F₁ 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 βW331 and noncatalytic site-bound TNP-nucleotide would not occur. 2 J. Weber and A. E. Senior, unpublished data.

**RESULTS**

Fig. 1 shows hydrolysis of MgTNP-ATP by wild-type E. coli F₁ as a function of substrate concentration. At saturation, the MgTNP-ATPase activity was 0.16 units/mg, which is 1.4% of the MgATPase activity under the same conditions (23 °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 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.

In previous work we have used the βY331W mutant E. coli F₁ 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 βY331W mutant F₁ 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. 1 represents a model with a single K_m. The fit could be improved considerably by 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 F₁ hydrolyzes MgTNP-ATP relatively better than does mitochondrial F₁. E.g., the ratio V_max(MgTNP-ATP)/V_max(MgATP) is about 10-fold higher in E. coli F₁ as compared to mitochondrial F₁.

![Image](http://www.jbc.org/)
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 F1 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 βY331W mutant F1 with the same affinity ($K_d = 76 \mu M$) (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 identical, independent binding sites gave a reasonable fit (Fig. 4, solid line) with calculated $K_d = 4.1 \mu M$ at 2.8 sites. However, a better fit was obtained using a model with three different independent binding sites, with $K_{d1} = 1.3 \mu M$, $K_{d2} = 4.1 \mu M$, and $K_{d3} = 32 \mu M$ (Fig. 4, dashed line).

We found that in absence of Mg$^{2+}$, TNP-ATP was not a hydrolysis substrate. After incubation of 400 nM βY331W mutant F1 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 Pi 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 F1-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

---

**TABLE I**

Binding and hydrolysis of MgTNP-ATP by E. coli F1-ATPase.

| Model          | $K_{M1}$ | $V_{max1}$ | $K_{M2}$ | $V_{max2}$ |
|----------------|----------|------------|----------|------------|
| Single $K_m$   | 0.24     | 0.153      | 1.33     | 0.092      |
| Two $K_m$      | 0.023    | 0.067      | 1.33     | 0.056      |

* ND, not determined.
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 $V_{\text{max}}$, if it occurred at all (Weber et al., 1993). The previous data allow us to conclude that bisite hydrolysis by MgTNP-ATP amounted to a substantial fraction (38%) of $V_{\text{max}}$. It may be noted that in absolute terms this rate of bisite hydrolysis by MgTNP-ATP may well imply that the rate of binding of substrate to site 2 was 1 order of magnitude 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 MgTNP-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 Mg2+ was seen to cause a huge increase in affinity for MgTNP-ATP at catalytic sites, just as it has previously been observed for MgATP (Weber et al., 1994a). The $K_{d}$ for MgTNP-ATP at catalytic site 3 was of similar magnitude to that for MgTNP-ATP binding to isolated $\beta$-subunit (Rao et al., 1988) and also for binding of free TNP-ATP at each of the catalytic sites in absence of Mg2+. 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 Mg2+ 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 Mg2+), 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 Mg2+, the catalytic sites of F1-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.

REFERENCES

Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Nature 370, 626–628

Allision, W. S., Jault, J. M., Zhuo, S., and Paik, S. R. (1992) Bioenerg. Biomembr. 24, 469–477

Bradford, M. M. (1976) Anal. Biochem. 72, 248–254

Capaldi, R. A., Aggeler, R., Turina, P., and Wilkens, S. (1994) Trends Biochem. Sci. 19, 284–289

Cross, R. L., Grubmeyer, C., and Penefsky, H. S. (1982) J. Biol. Chem. 257, 12101–12105

Fabiano, A., and Fabiato, F. (1979) J. Physiol. (Paris) 75, 463–505

Fillinger, R. H. (1990) in The Bacteria (Krulwich, T. A., ed) Vol. 12, pp. 345–391, Academic Press, New York

Garboczi, D. N., Shenbagamurthi, P., Kirk, W., Hullihen, J., and Pedersen, P. L. (1988) J. Biol. Chem. 263, 812–816

Grubmeyer, C., and Penefsky, H. S. (1988a) J. Biol. Chem. 263, 3728–3734

Grubmeyer, C., and Penefsky, H. S. (1988b) J. Biol. Chem. 257, 3718–3727

Gryczynski, I., Wilczek, W., Inesi, G., Squier, T., and Lakowicz, J. R. (1989) Biochemistry 28, 3490–3498

Haughton M. A., and Capaldi, R. A. (1995) J. Biol. Chem. 270, 20568–20574

Hiratsuka, T., and Uchida, K. (1973) Biochim. Biophys. Acta 320, 635–647

Penefsky, H. S., and Cross, R. L. (1991) Adv. Enzymol. 64, 173–214

Rao, R., Al-Shawi, M. K., and Senior, A. E. (1988) J. Biol. Chem. 263, 5569–5573

Senior, A. E. (1988) Physiol. Rev. 68, 177–231

Senior, A. E., and Wise, J. G. (1988) J. Membr. Biol. 105–124

Tausky, H. S., and Shorr, E. (1953) J. Biol. Chem. 202, 675–685

Tiedge, H., and Schäfer, G. (1986) Biol. Bull. 172, 699–704

van Veldhoven, P. P., and Mannerts, G. P. (1987) Anal. Biochem. 163, 45–48

Wagner, J. R., and Senior, A. E. (1995) J. Biol. Chem. 270, 12653–12658

Weber, J., Lee, R. S.-F., Grell, E., Wise, J. G., and Senior, A. E. (1992) J. Biol. Chem. 267, 1712–1718

Weber, J., Wilke-Mounts, S., Lee, R. S.-F., Grell, E., and Senior, A. E. (1993) J. Biol. Chem. 268, 20126–20133

Weber, J., Wilke-Mounts, S., and Senior, A. E. (1994a) J. Biol. Chem. 269, 20462–20467

Weber, J., Wilke-Mounts, S., Grell, E., and Senior, A. E. (1994b) J. Biol. Chem. 269, 11261–11268

FIG. 4. Binding of TNP-ATP to $\beta$Y331W mutant E. coli F1-ATPase in the absence of Mg2+. Free TNP-ATP binding in absence of Mg2+ 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).
