Binding of TNP-ATP and TNP-ADP to the non-catalytic sites of *Escherichia coli* F_1-ATPase

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Abstract Using site-directed-tryptophan fluorescence, parameters for equilibrium binding of (Mg)TNP-ATP and (Mg)TNP-ADP to non-catalytic sites of *Escherichia coli* F_1-ATPase were determined. All three non-catalytic sites showed the same affinity for MgTNP-ATP (K_d = 0.2 μM) or MgTNP-ADP (K_d = 6.5 μM) whereas even at concentrations of 100 μM no binding of uncomplexed TNP-ATP or TNP-ADP was observed. The results demonstrate that the three non-catalytic sites bind TNP-nucleotides non-cooperatively, and emphasize the importance of Mg^{2+} for non-catalytic-site nucleotide binding. Parameters for binding of (Mg)TNP-ADP to the three catalytic sites were also determined, and showed marked cooperativity. This work completes the set of thermodynamic parameters for equilibrium binding of (Mg)TNP-ATP and (Mg)TNP-ADP to all six nucleotide sites of F_1, providing essential information to fully exploit the potential of these nucleotide analogs in studies of F_1-ATPase.

Key words: Oxidative phosphorylation; F_1-ATPase; Nucleotide binding site

1. Introduction

F_1 is the catalytic sector of F_1F_o-ATP synthase, the enzyme that catalyzes ATP synthesis by oxidative phosphorylation. F_1 has six nucleotide binding sites, and in isolated form acts as an ATPase (F_1-ATPase). Only three of the nucleotide binding sites, those located mainly on the β-subunits, participate in catalysis ('catalytic sites'). The remaining three sites, which are located on the α-subunits, are not directly involved in the catalytic process; the function of these 'non-catalytic sites' is not yet established (for reviews, see [1,2]).

Attempts to assess the possible role(s) of the non-catalytic sites were seriously hampered in the past by lack of a reliable technique to determine their degree of occupancy under given experimental conditions. This obstacle was overcome by introducing a tryptophan residue specifically into the adenine-binding subdomain of the non-catalytic sites, at position αR365[(1)] of *Escherichia coli* F_1-ATPase [3]. In αR365W mutant F_1, the fluorescence of the introduced tryptophan is substantial and is virtually completely quenched upon binding of nucleotide; this response allowed us to determine kinetic and thermodynamic parameters for binding of nucleotide specifically to the non-catalytic sites [3–5]. The mutant enzyme was found to be functionally-normal [3]. The X-ray structure of mitochondrial F_1-ATPase [6] shows that the aliphatic portion of the residue αR365 side-chain makes Van der Waals contact with the adenine moiety of non-catalytic-site-bound nucleotide (see fig. 11 of [2]).

TNP-ATP and TNP-ADP [7] are frequently used as analogs of ATP or ADP to investigate nucleotide binding sites in proteins, because they have useful fluorescence properties and generally bind with high affinity. For example, we recently used TNP-ATP to elucidate properties of the three catalytic sites of F1-ATPase, showing that MgTNP-ATP exhibited the same strong binding cooperativity as MgATP, but with the binding affinities of MgTNP-ATP being higher by a factor of 20–30 than those for MgATP. The data obtained were very valuable in correlating catalytic activity with nucleotide binding, and we were able to determine rates of catalysis in enzyme molecules with one, two or all three catalytic sites occupied [8].

So far, little is known as to whether TNP-ATP or TNP-ADP bind to the non-catalytic sites of F_1. Binding of both TNP-ATP and TNP-ADP to isolated α-subunit has been demonstrated [9,10], and characteristic features of the absorption difference spectrum obtained upon binding of TNP-ADP to isolated α [10] were noted upon binding of the nucleotide analog to an $\alpha_3\beta_3\gamma$ sub-complex of F_1-ATPase from *Bacillus* PS3 [11]. Also, under suitable experimental conditions a photoaffinity label, 2-azido-TNP-ATP, was found to bind to a non-catalytic site of F_1 [12].

However, none of the previous reports has reported equilibrium binding of TNP-nucleotides to the non-catalytic sites in intact F_1, and it is important therefore to establish whether this occurs and to determine values of binding parameters. In the study described here, we use the fluorescence of residue αW365 in αR365W mutant F_1 to measure binding of (Mg)TNP-ATP and (Mg)TNP-ADP to the non-catalytic sites of *E. coli* F_1-ATPase. We also report parameters for binding of (Mg)TNP-ADP to the catalytic sites, thus completing the set of binding parameters for (Mg)TNP-ATP and (Mg)TNP-ADP at all six nucleotide sites in *E. coli* F_1.

2. Materials and methods

Wild-type F_1, αR365W mutant F_1, and βY331W mutant F_1 were isolated from strains SWM1 [13], AW7 [3], and pSWM4/JP17 [14], respectively, as described in [15]. Nucleotide-depleted αR365W mutant F_1 was prepared as described in [16]. TNP-ATP and TNP-ADP were purchased from Molecular Probes (Eugene, OR). MgTNP-ATP hydrolysis was measured as in [8].

Throughout this study, the signal used to measure TNP- nucleotide binding to F_1 non-catalytic sites was the tryptophan fluorescence of

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Abbreviations: TNP-ATP or TNP-ADP, 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate or -diphosphate, respectively

(1) *E. coli* residue numbers are used throughout. For mutations, αR365W indicates that the arginine residue in position α365 has been replaced by tryptophan.

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residue αW365 [3]; for the catalytic sites, we used the fluorescence of residue βW331 [14]. All fluorescence experiments were carried out at 23°C in spectrofluorometers type SPEX Fluorolog 2 or SLM AMINCO Bowman 2. The excitation wavelength was 295 nm. Before use, the enzyme was pre-equilibrated with 50 mM Tris-H_2SO_4, pH 8.0, by passage through a 1 ml Sephadex G-50 centrifuge column; in the case of βY331W mutant F_1, two consecutive column passages were used, to ensure complete removal of catalytic-site-bound nucleotides [17]. Titrations with MgTNP-ATP as the ligand were performed by adding $MgSO_4$/TNP-ATP at a ratio of 4:10 to the cuvette containing 50–100 nM enzyme in 50 mM Tris-H_2SO_4, pH 8.0. Each data point was generated in an independent experiment, to avoid interference by the hydrolysis product TNP-ADP. Titrations with MgTNP-ADP were performed by adding TNP-ADP to 100–200 nM F_1 in 50 mM Tris-H_2SO_4, 2.5 mM $MgSO_4$, pH 8.0. Concentrations of MgTNP-ATP and MgTNP-ADP were calculated according to [18], assuming no influence of the TNP moiety on Mg-nucleotide complexation. Binding of free (uncomplexed) TNP-ATP and TNP-ADP was measured in a buffer containing 50 mM Tris-H_2SO_4, 0.5 mM EDTA, pH 8.0.

Due to significant absorbance by TNP-nucleotides in the experimentally-relevant wavelength range, two effects had to be corrected for: (1) To compensate for inner filter effects, parallel titrations were performed with wild-type and mutant enzymes. Nevertheless, the titration curves could not be extended significantly above 100 μM TNP-ATP or TNP-ADP as the correction factors became too large. (2) Because there is overlap of tryptophan emission and TNP nucleotide excitation, fluorescence resonance energy transfer between residue αW365 and catalytic-site-bound TNP-nucleotide had to be taken into account. The distance between adjacent catalytic and non-catalytic sites is 27 Å (from β-phosphate to β-phosphate [6]) and the characteristic energy transfer distance (with a transfer efficiency of 0.5) for the donor/acceptor pair tryptophan/TNP nucleotide is 23 Å [19]. From these values the energy transfer efficiency was calculated to be 0.3, and the experimental results were corrected accordingly, using K_d values for binding of TNP nucleotides to the catalytic sites given in [8] for TNP-ATP or as reported here for TNP-ADP. As discussed in [8], an analogous correction for energy transfer between residue βW331 and non-catalytic-site-bound TNP nucleotide is not necessary as the non-catalytic sites of βY331W mutant F_1 are filled with endogenous ATP and ADP.

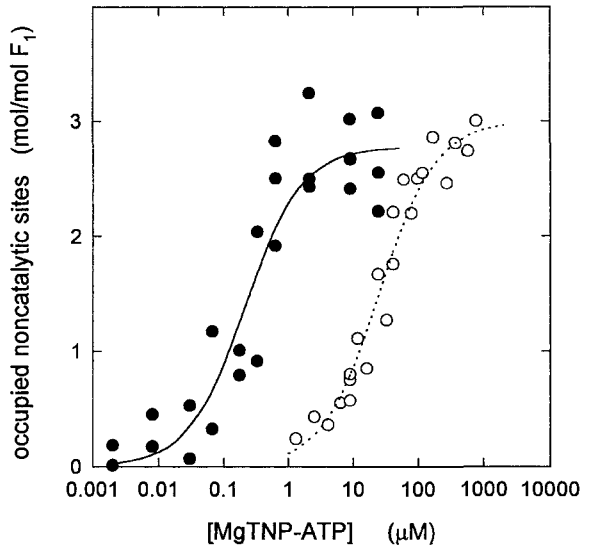

Fig. 1. Binding of MgTNP-ATP to non-catalytic sites of *E. coli* F_1. Nucleotide binding was measured as described in Materials and Methods, using the αW365 fluorescence as signal. Each point represents a single independent experiment. ●, MgTNP-ATP; ○, MgATP (taken from [3]). The lines are theoretical curves based on a model with N identical, independent binding sites. Calculated binding parameters are given in Table 1.

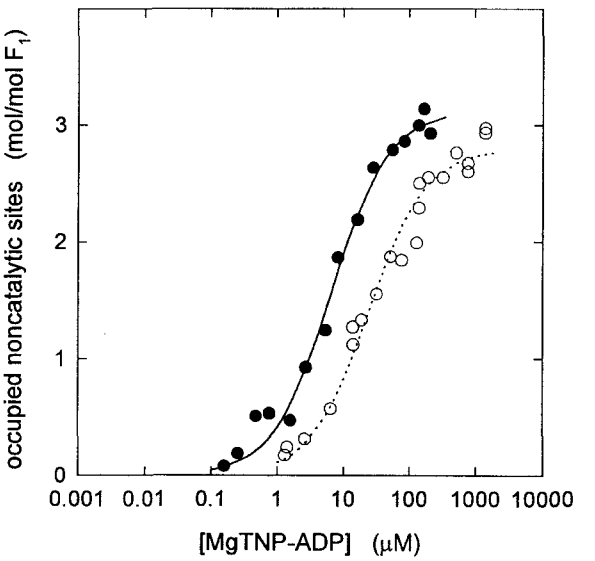

Fig. 2. Binding of MgTNP-ADP to non-catalytic sites of *E. coli* F_1. Nucleotide binding was measured as described in Section 2, using the αW365 fluorescence as signal. ●, MgTNP-ADP; ○, MgADP (taken from [3]). The lines are theoretical curves based on a model with N identical, independent binding sites. Calculated binding parameters are given in Table 1.

3. Results

3.1. Binding of MgTNP-ATP and MgTNP-ADP to non-catalytic sites

As can be seen from Fig. 1 (filled circles), MgTNP-ATP binds to all three non-catalytic binding sites, and saturation is achieved at relatively low ligand concentrations. Binding parameters were determined by fitting theoretical curves to the data points. A reasonable fit was obtained when $N = 2.8$ identical, independent binding sites, with a K_d of 0.2 μM at each site. The fit could not be improved by assuming binding models with multiple classes of sites, or cooperative models. This binding behavior is similar to that found with MgATP (Fig. 1, open circles), except that the affinity for MgTNP-ATP is around 100-fold higher (K_d(MgATP) = 25 μM [3]).

MgTNP-ADP also bound to all three non-catalytic sites (Fig. 2, filled circles), and again the affinity was the same at each site. Calculated binding parameters were: $N = 3.1$, $K_d = 6.5$ μM. Comparison with MgADP (Fig. 2, open circles) showed the same general binding behavior, i.e a single type of site, with a four-fold increased affinity for MgTNP-ADP ($K_d = 6.5$ μM) versus MgADP ($K_d = 24$ μM [3]).

3.2. Activity of F_1 with MgTNP-ATP-filled non-catalytic sites

MgTNP-ATP hydrolysis by nucleotide-depleted and native αR365W F_1 was measured at 23°C, pH 8.0, with 9 μM MgTNP-ATP. Pi production was linear in both enzymes for several minutes. Initial rates were: nucleotide-depleted, 0.083 U/mg; native, 0.078 U/mg. It was confirmed that under the conditions used, all three non-catalytic sites in the nucleotide-depleted enzyme became occupied by MgTNP-ATP. In native enzyme, the three non-catalytic sites were already filled by endogenous adenine nucleotide [3,8]. Therefore there is no effect on nucleoside triphosphatase activity of F_1 whether the three non-catalytic sites are filled by adenine nucleotide, TNP-nucleotide, or are empty ([3], this work).

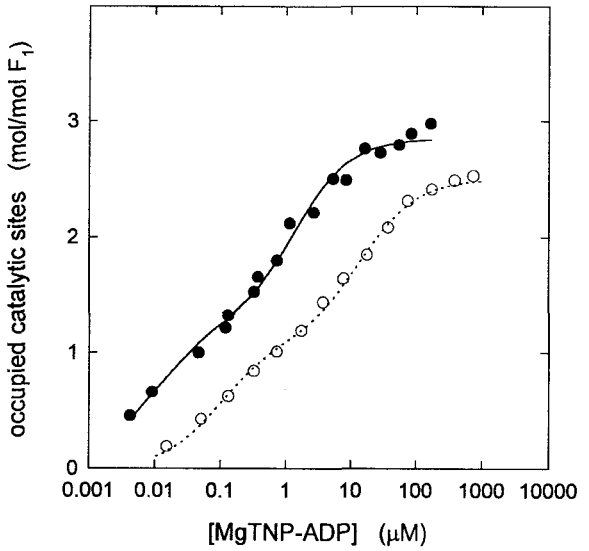

Fig. 3. Binding of MgTNP-ADP to catalytic sites of *E. coli* F_1. Nucleotide binding was measured as described in Materials and Methods, using the βW331 fluorescence as signal. ●, MgTNP-ADP; ○, MgADP (taken from [14,20]). The lines are theoretical curves based on a model with two types of sites. Calculated binding parameters are given in Table 1.

3.3. Interaction of TNP-ATP and TNP-ADP with non-catalytic sites in absence of Mg^{2+}

Titration of nucleotide-depleted αR365W mutant F_1 with TNP-ATP or TNP-ADP in absence of Mg^{2+} (presence of 0.5 mM EDTA) resulted in a decrease of the technical fluorescence signal. However, after application of corrections (see Section 2) the response turned out to be due to inner filter effects and energy transfer between residue αW365 and catalytic-site-bound TNP-ATP or TNP-ADP. Therefore at concentrations up to 100 μM, no significant binding of free TNP-ATP or TNP-ADP occurred.

3.4. Binding of MgTNP-ADP and free TNP-ADP to catalytic sites

It was necessary to know the parameters for binding of TNP-nucleotides to catalytic sites in order to correct for decrease of the αW365 fluorescence due to energy transfer between the αW365 residue and catalytic-site-bound TNP-nucleotide. For MgTNP-ATP and free TNP-ATP the relevant parameters were taken from [8]; for MgTNP-ADP and free TNP-ADP they were determined in this study, using the fluorescence of residue βW331 as signal [14]. Fig. 3 shows occupancy of catalytic sites as a function of MgTNP-ADP concentration (filled circles). As with MgADP (Fig. 3, open circles), two classes of binding site were found, one site of higher affinity and two sites of lower affinity. For MgTNP-ADP, the calculated binding parameters were $N_1 = 1.2$, $K_{d1} = 0.008$ μM, $N_2 = 1.6$, $K_{d2} = 1.3$ μM, indicating an approximately 10-fold increase in affinity as compared to MgADP (for MgADP: $K_{d1} = 0.1$ μM, $K_{d2} = 14$ μM [3,20]).

In absence of Mg^{2+}, again all three catalytic sites could be filled with TNP-ADP (Fig. 4, filled circles). A reasonable fit was obtained assuming a single type of binding site (Fig. 4, solid line) yielding values of $N = 2.8$, $K_d = 3.0$ μM. However, as was previously observed for TNP-ATP [8], in the case of TNP-ADP the fit could be improved by assuming a model with three different independent binding sites, with $K_{d1} = 0.4$ μM, $K_{d2} = 5.5$ μM, $K_{d3} = 23$ μM (Fig. 4, dashed line). The binding affinity for TNP-ADP was about 30-fold higher than that for ADP ($N = 2.9$, $K_d = 82$ μM [20]).

Table 1
Binding affinity of F_1-ATPase non-catalytic and catalytic sites for TNP-nucleotides

	Non-catalytic sites	Catalytic sites
MgTNP-ATP	K_{d1}, K_{d2}, $K_{d3} = 0.2$	$K_{d1} < 0.001$[a] $K_{d2} = 0.023$[a] $K_{d3} = 1.39$[a]
MgTNP-ADP	K_{d1}, K_{d2}, $K_{d3} = 6.5$	$K_{d1} = 0.008$ K_{d2}, $K_{d3} = 1.3$
TNP-ATP	$K_{d1} > 100$	$K_{d1} = 1.3$[a,b] $K_{d2} = 4.1$[a,b] $K_{d3} = 32$[a,b]
TNP-ADP	$K_{d1} > 100$	$K_{d1} = 0.4$[c] $K_{d2} = 5.5$[c] $K_{d3} = 23$[c]

K_d values are given in μM.
[a]Values from ref. [8].
[b]Note that a reasonable fit was also obtained where K_{d1}, K_{d2}, $K_{d3} = 4.1$ μM [8] in which case the catalytic sites appear symmetrical.
[c]Note that a reasonable fit was also obtained where K_{d1}, K_{d2}, $K_{d3} = 3.0$ μM, see text, in which case the catalytic sites appear symmetrical.

4. Discussion

The goal of the present study was to characterize equilibrium binding of (Mg)TNP-ATP and (Mg)TNP-ADP to the non-catalytic sites of F_1-ATPase. In addition, we also analyzed (Mg)TNP-ADP binding to the catalytic sites. Taken together with the data on (Mg)TNP-ATP interaction with the catalytic sites which we published previously [8], the affinities of all six nucleotide binding sites for (Mg)TNP-ATP and (Mg)TNP-ADP are now known. Table 1 summarizes the values.

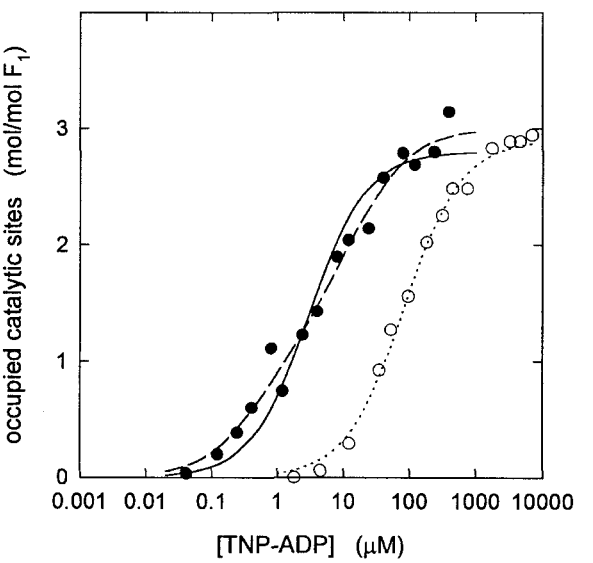

Fig. 4. Binding of uncomplexed TNP-ADP to catalytic sites of *E. coli* F_1. ●, TNP-ADP; ○, ADP (taken from [20]). The dotted and solid lines are theoretical curves based on a model with N identical, independent binding sites; the dashed line is for a model with three different sites. Calculated binding parameters are given in Table 1.

In respect to non-catalytic-site-binding it is clear that the affinity of all three sites for MgTNP-ATP or MgTNP-ADP is the same, as was seen previously for MgATP or MgADP [3], demonstrating that the three non-catalytic sites bind Mg-nucleotide non-cooperatively. However, whereas the non-catalytic sites bind both MgATP and MgADP with similar affinity [3], MgTNP-ATP binding is approximately 30-fold tighter than that of MgTNP-ADP. A preference of non-catalytic sites for nucleoside triphosphate over diphosphate has previously been observed with guanine and inosine nucleotides [3]. Filling of non-catalytic sites with MgTNP-ATP had no effect on catalytic activity.

The results obtained with free (uncomplexed) TNP-ATP and TNP-ADP emphasize the importance of Mg^{2+} ions for nucleotide binding to the non-catalytic sites. In absence of Mg^{2+}, no binding of free TNP-ATP or TNP-ADP was observed even at concentrations up to 100 μM. At this concentration, free ATP fills 0.1 non-catalytic sites per F_1, free ADP fills 0.2 sites (calculated from N(ATP) = 3.0, K_d(ATP) = 3500 μM; N(ADP) = 2.6, K_d(ADP) = 1300 μM [5]). Thus, the TNP moiety does not increase the affinity of non-catalytic sites for free nucleotide to any significant extent.

At the three catalytic sites, MgTNP-ATP [8] and MgTNP-ADP (this work) are bound with pronounced cooperativity, and therefore the catalytic sites appear strongly asymmetric when binding TNP-nucleotide in presence of Mg^{2+}, just as they do when binding adenine nucleotide in presence of Mg^{2+} [2]. At catalytic sites, K_d values for MgTNP-ATP and MgTNP-ADP are lower by factors of 10–30 as compared to K_d(MgATP) and K_d(MgADP). Thus, the TNP moiety increases binding energy by 1.4–2.0 kcal/mol. In absence of Mg^{2+}, the average affinity (and binding energy) for free TNP-ATP and TNP-ADP were also seen to be enhanced, by similar amounts, when compared to the corresponding adenine nucleotides.

Catalytic-site-binding of free (uncomplexed) ATP and ADP was seen previously to occur with the same affinity at all three sites, and so the catalytic sites appear symmetrical with adenine nucleotide in absence of Mg^{2+} [2]. With free (uncomplexed) TNP-ATP and TNP-ADP the best fits to the data (Fig. 4, Table 1, ref. [8]) showed that the affinities differed somewhat among the three sites, although to not nearly the same extent as was seen in presence of Mg^{2+}. Thus, even in absence of Mg^{2+}, the catalytic sites may not be perfectly symmetrical in presence of TNP-nucleotide.

Knowledge of the binding affinities of TNP-nucleotides will be very helpful in the design and analysis of future experiments. Information in Table 1 allows calculation of the occupancy of each of the six F_1 nucleotide binding sites by TNP-nucleotides under given experimental conditions (different concentrations, absence or presence of Mg^{2+}). Such information is essential before statements about filling of a specific site or sites can be made. Based on our results, it is clear that when nucleotide-depleted *E. coli* F_1 is titrated with increasing concentration of MgTNP-ATP, the site with the highest affinity, which fills first, is catalytic site 1. The affinity of all three non-catalytic sites is 9-fold lower than that of catalytic site 2, and 7-fold higher than that of catalytic site 3. Computer simulations show that the differences are not sufficiently large to allow specific filling of any one of these five sites by MgTNP-ATP. In native F_1, where the three non-catalytic sites are filled with endogenous adenine nucleotide, specific sequential filling of catalytic sites 1, 2, and 3 by MgTNP-ATP is feasible. With MgTNP-ADP as ligand, similar considerations apply. With free TNP-ATP or TNP-ADP, in absence of Mg^{2+}, the catalytic sites will clearly fill preferentially.

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