Nucleotide occupancy of F_1-ATPase catalytic sites under crystallization conditions

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Abstract **Using site-directed tryptophan fluorescence we studied nucleotide occupancy of the catalytic sites of *Escherichia coli* F_1-ATPase, under conditions used previously for crystallization and X-ray structure analysis of the bovine mitochondrial enzyme [Abrahams et al. (1994) Nature 370, 621–628]. We found that only two of the three catalytic sites were filled in the *E. coli* enzyme under these conditions (250 μM MgAMPPNP plus 5 μM MgADP), consistent with what was reported in the bovine F_1 X-ray structure. However, subsequent addition of a physiological concentration of MgATP readily filled the third catalytic site. Therefore the enzyme form seen in the X-ray structure results from the fact that it is obtained under sub-saturating nucleotide conditions. The data show that the X-ray structure is compatible with a catalytic mechanism in which all three F_1-ATPase catalytic sites must fill with MgATP to initiate steady-state hydrolysis [e.g. Weber and Senior (1996) Biochim. Biophys. Acta 1275, 101–104]. The data further demonstrate that the site-directed tryptophan fluorescence technique can provide valuable support for F_1 crystallography studies.**

Key words: Oxidative phosphorylation; F_1-ATPase; Catalytic site; Nucleotide occupancy

1. Introduction

ATP synthase is the membrane enzyme responsible for ATP synthesis in mitochondria and chloroplasts. In bacteria it performs both ATP synthesis and ATP-driven proton transport. The F_1 catalytic sector may be purified in soluble form as an active ATPase [1–5]. Since 1981 the model of Cross [6] has provided a working hypothesis for the enzyme mechanism. Briefly it proposes that the three catalytic sites of F_1 have widely different affinities for substrate. Under substrate saturation conditions, one of the three sites is always empty, and immediately after the product release step, two sites are empty. A structure of bovine heart mitochondrial F_1 determined by X-ray crystallography [7] showed two catalytic sites occupied by nucleotide and one site empty. This was interpreted as consistent with the Cross model [7].

Direct equilibrium measurement of nucleotide binding parameters for the three catalytic sites of F_1 became possible only recently, when we introduced the technique of site-directed tryptophan fluorescence to provide specific optical probes [8]. We demonstrated that substrate MgATP, at physiological, saturating (V_{max}) concentration (≥1 mM), binds to all three catalytic sites, and that the sites do indeed show widely different binding affinity (K_{d1}, 10^{-10} M; K_{d2}, 10^{-6} M; K_{d3}, 10^{-4} M) [9,10]. We further demonstrated that all three catalytic sites must bind MgATP for physiological activity to be achieved, and that molecules with only two sites filled show negligible activity [9]. Under steady-state MgATP hydrolysis conditions, one site is occupied by MgATP and two by MgADP [11]. Independent corroboration of the site-directed tryptophan fluorescence technique came from 2-azido-ATP photolabelling experiments [12].

These new data point strongly to a model for the catalytic mechanism of F_1-ATPase in which all three catalytic sites are filled at saturating substrate concentration, and immediately after product release only one catalytic site is empty, as proposed in [8]. This model therefore supersedes the Cross model, while incorporating several of its important features.

The X-ray structure [7] is not incompatible with the new model, because the F_1 form seen in the crystals could well correspond to the species that occurs immediately after product release during steady-state turnover. However, we felt it important to examine why F_1 in the crystals has only two catalytic sites occupied. One plausible explanation was that, under the conditions of crystallization, the concentrations of nucleotide (250 μM MgAMPPNP, 5 μM MgADP) are insufficient to fill the third catalytic site. Using *Escherichia coli* F_1, we measured nucleotide occupancy of catalytic sites under the crystallization conditions, and we tested what happened when saturating concentrations of MgATP were subsequently added.

2. Materials and methods

2.1. Enzyme preparations

E. coli F_1 was purified as described [13]. Wild-type F_1 was from strain SWM1 [14], βY331W F_1 from strain SWM4 [9], βF148W F_1 from strain CB1 [11], and αR365W F_1 from strain AW7 [15]. Preparation of nucleotide-depleted F_1 was as in [16]. Before use nucleotide-depleted enzyme was passed once through a 1 ml centrifuge column of Sephadex G-50 [17] equilibrated in 50 mM Tris-SO_4, pH 8.0. 'Native' F_1 was enzyme that was passed twice through centrifuge columns as above. Previous work has established that this procedure effectively removes catalytic but not noncatalytic site-bound nucleotide [10,18].

2.2. Nucleotide binding assays by fluorescence measurement

Fluorescence measurements were made at 22°C and enzyme concentration of 100 nM, in 2 ml buffer consisting of 50 mM Tris-HCl, pH 8.2, 1 mM EDTA, 200 mM NaCl, 5 mM 2-mercaptoethanol, 5 mM dithiothreitol, 20 mM $MgSO_4$, 3 mM NaN_3, 0.001% (w/v) phenylmethylsulfonyl fluoride, with other additions or subtractions as indicated in the text. Excitation was at 295 nm, fluorescence was monitored in a SPEX Fluorolog 2 Spectro-fluorimeter equipped with DataMax Software. Background signals (buffer, Raman scatter) were subtracted; inner filter and volume effects were corrected for by control experiments with wild-type enzyme. Wild-type enzyme showed no significant fluorescence changes on addition of nucleotides.

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3. Results and discussion

βY331W mutant *E. coli* F_1, which shows normal function [9], contains a Tyr→Trp substitution in each of the three catalytic sites. In wild-type, Tyr-331 makes Van der Waals contact with the adenine ring of bound nucleotide [7]. On binding of nucleotide to βY331W enzyme, the large fluorescence signal of the three Trp-331 residues is virtually fully quenched [9,10]. Fig. 1 shows fluorescence spectra of βY331W and wild-type F_1 obtained in buffer which mimics the crystallization buffer used by Walker and colleagues for X-ray crystallography [19]. The fluorescence of both enzymes was reduced by about 50% as compared to previous measurements made in 50 mM Tris-SO_4, pH 8.0, buffer [9,10], but the signals were nevertheless easily sufficient for nucleotide binding measurements. Curve a in Fig. 1 represents the spectrum of nucleotide-depleted βY331W enzyme alone. On addition of 250 μM AMPPNP simultaneously with 5 μM ADP, the fluorescence was partly quenched (curve b). The degree of quenching of fluorescence at λ = 360 nm indicates an average occupation of two catalytic sites per enzyme molecule. On addition of 5 mM ATP, further quenching occurred (curve c) close to the level of fluorescence seen in wild-type (curve d), indicating that all three catalytic sites were now occupied. Multiple replicate experiments of this kind were carried out, with highly consistent results, and the data are summarized in Table 1. The conclusion is that under these conditions, either 250 μM AMPPNP alone, or the combination of 250 μM AMPPNP with 5 μM ADP, are not sufficient to fill the third catalytic site, but subsequent addition of physiological concentration of ATP readily does so, and the resultant enzyme has all three sites filled. It should be noted that since the buffer contains 20 mM $MgSO_4$, each nucleotide is present as Mg-complex.

The conditions used in the above experiments were intended to mimic as closely as possible those used by Lutter et al. [19] for crystallization of bovine heart mitochondrial F_1. It is important to discuss how well this goal was met. The major

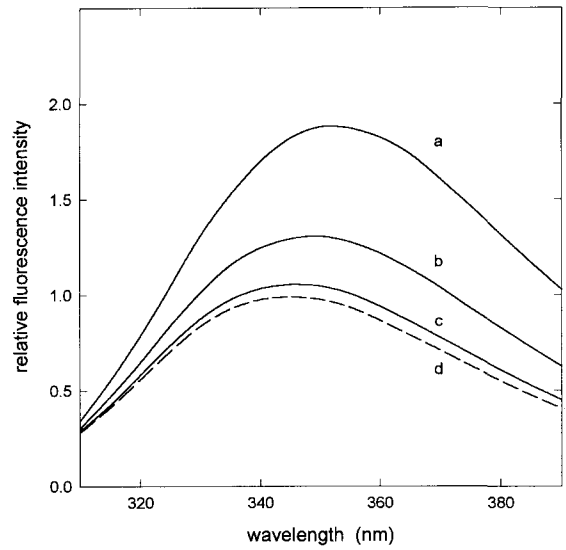

Fig. 1. Fluorescence spectra of βY331W mutant *E. coli* F_1 in 'crystallization buffer'. Fluorescence emission spectra (λ_{exc} = 295 nm) were obtained as described in Section 2. Curve a, βY331W mutant F_1; curve b, βY331W F_1 with 250 μM AMPPNP plus 5 μM ADP; curve c, subsequent addition of 5 mM ATP; curve d, wild-type *E. coli* F_1, with or without the same nucleotide additions.

Table 1
Nucleotide occupancy of catalytic sites of βY331W mutant *E. coli* F_1 under conditions mimicking those used for crystallization of beef heart mitochondrial F_1

	Nucleotide occupancy of catalytic sites			
	Nucleotide-depleted F_1		Native F_1	
	+ NaN_3	− NaN_3	+ NaN_3	− NaN_3
AMPPNP	1.9	1.9	1.8	1.95
AMPPNP then ATP	2.9	2.8	2.75	2.8
AMPPNP/ADP	2.05	1.9	1.95	2.05
AMPPNP/ADP then ATP	2.8	2.75	2.8	2.8
ATP alone	2.8	–	2.8	2.9

Catalytic site nucleotide occupancy was determined by fluorescence spectroscopy as described in Section 2 and is presented as mol/mol F_1. The enzyme concentration was 100 nM, and NaN_3 was 3 mM when present. Additions are described in column 1. 'AMPPNP' indicates addition of AMPPNP alone (final concentration = 250 μM); 'AMPPNP/ADP' indicates addition of 250 μM AMPPNP and 5 μM ADP (final concentrations) simultaneously; 'then ATP' indicates the subsequent addition of 5 mM ATP after either AMPPNP or AMPPNP/ADP; 'ATP alone' indicates addition of 5 mM ATP without AMPPNP or ADP. The buffer contained 20 mM $MgSO_4$, hence all nucleotides are present as Mg-complexes. Data shown are means of 3 or 4 experiments which showed excellent agreement in every case.

difference between our experiments and those of Lutter et al. is that we used *E. coli* F_1 and they used bovine heart mitochondrial F_1, so the question arises as to whether the former provides a valid experimental model for the latter. As to binding of MgAMPPNP and/or MgADP at the first, highest affinity catalytic site, it is clear from data in the literature [9,20–23] that in both enzymes the first catalytic site would be expected to be completely filled under the conditions used, and obviously this was the case. There is currently no equilibrium technique available for specifically determining nucleotide binding parameters of the second and third catalytic sites in bovine mitochondrial F_1, this can as yet only be done in *E. coli* enzyme. However, there are strong indications that the two enzymes are very similar. First, K_m(MgATP) for ATPase activity at V_{max} rate in bovine heart mitochondrial F_1, as reported from numerous laboratories, is 100–250 μM [24–29]. For wild-type *E. coli* enzyme, the same value was reported, also from several laboratories [9,30,31], in both ε-depleted and ε-replete enzyme [32]. k_{cat}/K_m(MgATP) for bovine mitochondrial F_1 is close to 2×10^6 M^{-1} s^{-1} in all the reports cited above, and the value for *E. coli* F_1 is the same. These two parameters reflect the affinity and rate of MgATP binding at the loosest catalytic site, and it is clear that both enzymes behave very similarly. βY331W mutant *E. coli* enzyme has K_m(MgATP) lower by 2-fold than wild-type, while retaining the same k_{cat}/K_m(MgATP) [9]. Second, the structure of the catalytic sites in the two enzymes appears to be very similar indeed. The sequences of the β-subunits are strongly conserved, especially in the nucleotide binding domain, and all the residues identified at the catalytic sites in the X-ray structure of the bovine mitochondrial enzyme are present in *E. coli* enzyme [1–5,7]. Certainly the results obtained here substantiate the approach – under the same conditions the same fractional nucleotide occupancy was found.

We investigated other factors that might have affected nucleotide binding to catalytic sites under the crystallization

conditions. As Table 1 shows, sodium azide could be omitted without any effect. Lutter et al. [19] used D_2O-containing buffers, which stabilize subunit interactions in bovine heart mitochondrial F_1 at low or high temperature [33]. We did not think this necessary, since it was shown that at 20°C as used here, D_2O had no effect on V_{max}(ATPase) or K_m(MgATP) [33]. Polyethylene glycol 6000 was used to encourage crystal formation [19]. We found that, at 7% (w/v) concentration, it caused large and rapid decrease in enzyme fluorescence signal, and could not therefore be included in the buffer.

Nucleotide-depleted and 'native' βY331W enzymes behaved very similarly (Table 1), showing that the initial state of occupation of the noncatalytic sites, or the ligands occupying the noncatalytic sites, had no effect. We examined the occupancy of the three noncatalytic sites under the 'crystallization buffer' conditions using αR365W mutant *E. coli* F_1. In this enzyme the fluorescence of the αW365 residues is virtually completely quenched upon binding of nucleotide to noncatalytic nucleotide sites [15]. Nucleotide-depleted αR365W F_1 was incubated under the conditions of Table 1, and it was seen that upon addition of 250 μM AMPPNP alone, or 250 μM AMPPNP simultaneously with 5 μM ADP, the αW365 fluorescence was maximally quenched, and subsequent addition of 5 mM ATP had no further effect. Therefore under the 'crystallization' conditions all three noncatalytic sites were occupied by nucleotide, consistent with the fact that bovine heart mitochondrial F_1 in the X-ray structure has all three noncatalytic sites occupied by AMPPNP [7].

The crystallization buffer contains a high (20 mM) concentration of Mg^{2+} ion. In separate experiments with βY331W mutant *E. coli* F_1 we have studied MgATP binding to catalytic sites at concentrations of 2.5, 20 and 25 mM $MgSO_4$. With MgATP, we found maximal binding stoichiometries of 3.0, 2.8 and 2.8 mol/mol F_1, respectively. Thus high Mg^{2+} concentrations caused a slight reduction in apparent stoichiometry of ATP binding to catalytic sites. High Mg^{2+} concentrations are also inhibitory to hydrolysis [22]. Lower concentrations may favor full occupancy of sites in future crystallization trials.

It was demonstrated previously that the fluorescence signal of βF148W mutant *E. coli* F_1 allows one to discriminate between nucleoside di- and tri-phosphate bound in catalytic sites [11]. Unfortunately we found here that, under the 'crystallization buffer' conditions, the fluorescence signal of βF148W enzyme was reduced, such that useful results could not be obtained. Instead we performed experiments in 50 mM Tris-SO_4, pH 8.0, 2.5 mM $MgSO_4$ buffer as reported previously in [11]. We compared the spectra obtained on addition of 250 μM AMPPNP alone versus addition of 250 μM AMPPNP plus 5 μM ADP. The total catalytic site nucleotide binding stoichiometry was the same in both cases, but in the former all the bound nucleotide was in the form of nucleoside triphosphate, as expected, whereas in the latter the ratio of bound AMPPNP/bound ADP was 2/1. Thus up to 67% of the molecules would be in the form seen in the crystal structure, i.e. with one bound AMPPNP, one bound ADP, and one empty catalytic site.

Our conclusions are as follows. (i) The reason that F_1 molecules in the X-ray structure of Abrahams et al. [7] have only two out of three catalytic sites occupied is that the nucleotide concentrations in the crystallization buffer are insufficient to saturate the third site. Further addition of MgATP at physiological concentration under these conditions readily saturates the third site. The open structure of the empty catalytic site seen in the 'β_E' subunit in [7] is the native, unoccupied form; it clearly undergoes profound conformational change when it binds and closes around a nucleotide molecule. (ii) The work provides support for a 'three-site' mechanism of F_1 catalysis (e.g. as in [8]), by showing that the X-ray structure is compatible with such a mechanism. The F_1 species seen in the X-ray structure may well correspond to the form of the enzyme that occurs immediately after release of product from one of the three catalytic sites in the catalytic cycle during steady-state turnover (state D of [8]). Interestingly, recent work on the X-ray structure of the efrapeptin-F_1 complex [34] suggests that efrapeptin inhibits F_1 by specifically binding to the enzyme at this step of the catalytic cycle, when one catalytic site is empty, and preventing the third site from filling with nucleotide. (iii) Quantitative estimation of nucleotide binding parameters obtained from site-directed tryptophan fluorescence experiments can be used in future work to predict crystallization conditions favoring enzyme molecules with specific stoichiometry of bound nucleotide; conversely, conditions found by trial and error to favor crystal formation can be readily checked to determine the nucleotide content of the F_1 species present.

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