The structure and nucleotide occupancy of bovine mitochondrial F_1-ATPase are not influenced by crystallisation at high concentrations of nucleotide

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Abstract Analysis of tryptophan mutants of F_1-ATPase from *Escherichia coli* [Löbau et al. (1997) FEBS Lett. 404, 15–18] suggested that nucleotide concentrations used to grow crystals for the determination of the structure of bovine F_1-ATPase [Abrahams et al. (1994) Nature 370, 621–628] would be sufficient to occupy only two catalytic sites, and that higher concentrations of nucleotide would result in all three sites being occupied. We have determined the structure of bovine F_1-ATPase at 2.9 Å resolution with crystals grown in the presence of 5 mM AMPPNP and 5 μM ADP. Similar to previous structures of bovine F_1-ATPase determined with crystals grown in the presence of lower nucleotide concentrations, only two β-subunits have bound nucleotide and the third subunit remains empty. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Mitochondrion; ATP synthase; Catalytic site; Nucleotide; Crystal structure

1. Introduction

The catalytic domain of ATP synthase is an assembly of five different subunits, α, β, γ, δ and ε, in the stoichiometry 3:3:1:1:1. The α- and β-subunits each contain a nucleotide binding site, but catalysis occurs at the nucleotide binding sites in the β-subunits. The nucleotide binding sites in the α-subunits are not catalytically competent, but they may have a regulatory role. The three catalytic sites have different affinities for substrate and products, and models of catalysis have been proposed in which each catalytic site undergoes a series of concerted conformational changes (the binding change mechanism) [1,2]. In these models, at least one catalytic site has no bound nucleotide at any point in the cycle. The structure of bovine F_1-ATPase [3] is consistent with these models: one catalytic site is occupied by ADP (subunit β_{DP}), a second by the ATP analogue 5′-adenylyl-imidodiphosphate (AMPPNP) (subunit β_{TP}) and the third β-subunit (subunit β_E) has no bound nucleotide. The structure also suggested that the different affinities of the catalytic sites were imposed by their interaction with the asymmetric γ-subunit, and that rotation of this subunit steps each catalytic site through the three different conformational states. Rotation of the γ-subunit dependent on ATP hydrolysis was demonstrated subsequently [4–6].

The occupancy of nucleotide binding sites in F_1-ATPase from *Escherichia coli* has been monitored by the fluorescence of tryptophan residues introduced nearby [7]. By this technique, it was confirmed that the three catalytic sites have different affinities for ATP [8,9], and that at nucleotide concentrations greater than 1 mM, all three catalytic sites appeared to be occupied with nucleotide [8]. Most importantly, it was demonstrated that to maintain physiological levels of activity, all sites apparently need to be occupied [8,10]. It was also shown that under conditions similar to those used for crystallisation of bovine F_1-ATPase (250 μM AMPPNP and 250 μM ADP, but in the absence of polyethylene glycol (PEG) 6000), only two catalytic sites were occupied [11]. However, addition of 5 mM ATP under the same conditions led to the occupation of all three catalytic sites [11].

None of the reported structures of bovine F_1-ATPase has all three catalytic sites filled. A structural model of rat F_1-ATPase [12] has all three catalytic sites occupied by nucleotide, but this occupancy arises from the averaging of three α–β pairs in the crystal lattice, and so it is questionable that the model represents a true catalytic state. In this paper, we describe the structure of bovine F_1-ATPase crystallised in the presence of 5 mM AMPPNP, which according to the fluorescence studies [10] should lead to crystals in which all three catalytic sites of the F_1-ATPase complexes are occupied with nucleotides.

2. Materials and methods

2.1. Crystal growth

Crystals of bovine F_1-ATPase were grown by microdialysis as described previously [13]. The protein solution (11 mg/ml) was mixed with an equal volume of buffer containing 100 mM Tris–HCl pH 7.2, 400 mM sodium chloride, 2 mM EDTA, 4 mM magnesium chloride, 0.04% (w/v) sodium azide, 0.002% (w/v) phenylmethylsulphonyl fluoride, 14% (w/v) PEG 6000, 10 mM dithiothreitol, 2 mM AMPPNP and 40 μM ADP and dialysed against buffer containing 50 mM Tris–HCl pH 8.2, 200 mM sodium chloride, 1 mM EDTA, 20 mM magnesium sulphate, 0.02% (w/v) sodium azide, 0.001% (w/v) phenylmethylsulphonyl fluoride, 5 mM dithiothreitol, 5 mM AMPPNP, 5 μM

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Abbreviations: AMPPNP, 5′-adenylyl-imidodiphosphate; rms, root mean square

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Table 1
Data processing and refinement statistics

Diffraction data	
Space group	$P2_12_12_1$
Cell parameters (Å)	$a = 280.0$; $b = 107.0$; $c = 139.1$
Resolution (Å)	2.87
Measured intensities	217 558
Unique reflections	79 330
Completeness[a] (%)	95.8 (90.4)
Multiplicity[a]	2.5 (2.3)
R^a_{merge} (%)	6.3 (16.6)
Refinement	
Resolution (Å)	20–2.9
R_{cryst} (%)	23.6
R_{free} (%)	29.2
Reflections	110 254
Protein atoms	22 663
Solvent atoms	117
Nucleotide atoms	161
Geometry	
Rmsd in bond lengths (Å)	0.010
Rmsd in bond angles (°)	1.5

$R_{merge} = \Sigma_h\Sigma_i |I(h) - I(h)_i| / \Sigma_h\Sigma_i I(h)_i$, where $I(h)$ is the mean weighted intensity after rejection of outliers.
$R_{cryst} = \Sigma_h |F_o - F_c| / \Sigma_h F_o$ where F_o and F_c are the observed and calculated structure factor amplitudes, respectively, and were determined using 95% of the data.
R_{free} was determined from the residual 5% of the data.
Hydrogen atoms were excluded.
[a]Values in parentheses are for the highest resolution shell.

ADP and 11–12% (w/v) PEG 6000. Crystals appeared after 2–4 weeks and reached their maximum size in 6–8 weeks.

Before data collection, the crystals were stabilised by increasing the glycerol concentration of the crystallisation buffer gradually from 0 to 20% (v/v) in 5% increments, and then freezing them in liquid nitrogen.

2.2. Data collection and processing

Data were collected to 2.87 Å resolution on beam-line BM14 at the ESRF (Grenoble, France), at a wavelength of 0.95 Å. A data set was collected at 100 K from a single frozen crystal, using a Mar Research image plate detector. The crystals belonged to space group $P2_12_12_1$ with unit cell dimensions $a = 280.0$ Å, $b = 107.0$ Å, $c = 139.1$ Å. The diffraction images were processed with MOSFLM [14] and further processing was performed with programs from the Collaborative Computational Project Number 4 Suite [15]. The structure was solved by molecular replacement using the coordinates of the frozen-native bovine F_1-ATPase crystal structure (Protein Data Bank (PDB) code 1E1Q) [16] as a search model. For calculation of the free R value [17], 5% of the observed diffraction data were set aside and excluded from the entire refinement including the initial rigid body refinement. To avoid bias, the same reflections were used for calculating the free R value as in the original structure determination. The structure was refined by a combination of rigid body, positional and temperature factor refinement using REFMAC [18]. Several rounds of rigid body refinement were performed with both the number of rigid domains and the resolution increasing with each round until all data between 25 and 2.9 Å were included. Positional refinement using the maximum likelihood target was then carried out with data between 20 and 2.9 Å. Where appropriate, the structure was adjusted manually and solvent molecules were added with the programme O [19].

The quality of the refined model was assessed with PROCHECK [20] and superpositions were carried out with LSQMAN [21].

The coordinates of the high AMPPNP structure have been deposited in the PDB with the accession code 1H8H.

3. Results

3.1. Crystallisation

Crystallisation trials were conducted with 5 μM ADP and either 1 mM, 1.5 mM or 5 mM AMPPNP. Crystals formed at all three concentrations. However, in all cases the diffraction quality was inferior to crystals grown under standard conditions (250 μM AMPPNP and 5 μM ADP) [13]. Crystals grown with 1 mM and 1.5 mM AMPPNP diffracted to high resolution, but the diffraction patterns indicated twinned or multiple crystals, and none was suitable for data collection. The crystals grown in the presence of 5 mM AMPPNP also had a tendency to twin or give multiple diffraction patterns, but single crystals that diffracted to high resolution were also present and enabled data to be collected to 2.87 Å resolution.

3.2. Structure determination

The refinement statistics (Table 1) are very similar to those of other bovine F_1-ATPase structures at this resolution. During the rigid body refinement the R_{cryst} and R_{free} values decreased from 37.3% and 38.4% to 26.7% and 29.3%, respectively. Following final adjustment and refinement of the model, the values of R_{cryst} and R_{free} were 23.6% and 29.2%, respectively. The final model contained residues α_E 24–510, α_{TP} 24–401, 410–510, α_{DP} 19–510, β_{DP} 9–475, β_E and β_{TP} 9–474, and γ 1–44, 77–90 and 209–272, very similar to the frozen-native structure [16] that was used as a search model. As in the frozen-native structure, several regions of the structure (α_{DP} 405–409, β_E 385–400, β_E 423–426 and β_E 470–474) had high main chain B-factors (> 100 Å^2). The Ramachandran plot statistics for this structure are very similar to those for published structures of bovine F_1-ATPase with 87.9% of residues in most favoured regions, 11.8% in additionally allowed regions, 0.4% in generously allowed regions, and none in disallowed regions.

3.3. Structure comparison

The high AMPPNP structure and the frozen-native structure [16] are essentially identical. The main chain atoms of the two complete structures superimpose with an root mean square deviation (rmsd) of 0.19 Å. This value is typical of coordinate error for structures determined at this resolution. Superposition of all the protein atoms gives an rmsd of 0.27 Å, highlighting the similarity in both tertiary and quaternary structure.

The high AMPPNP structure has the same nucleotide occupancy as the frozen-native and native structures [3,16] (Table 2). All the non-catalytic nucleotide binding sites in α-subunits contain AMPPNP. The β_{DP}-subunit contains ADP, the β_{TP}-subunit contains AMPPNP and the β_E-subunit adopts the same open conformation as in the frozen-native structure. In this latter subunit, there is no evidence in the electron density map for a nucleotide molecule associated with P-loop residues which are involved in binding the phosphate groups of nucleotide in the two other β-subunits (Fig. 1). As in other bovine

Table 2
Comparison of occupancies of nucleotide binding sites in bovine F_1-ATPase

Subunit	α_{DP}	α_E	α_{TP}	β_{DP}	β_E	β_{TP}
Frozen-native	AMPPNP	AMPPNP	AMPPNP	ADP	sulphate	AMPPNP
High AMPPNP	AMPPNP	AMPPNP	AMPPNP	ADP	sulphate	AMPPNP

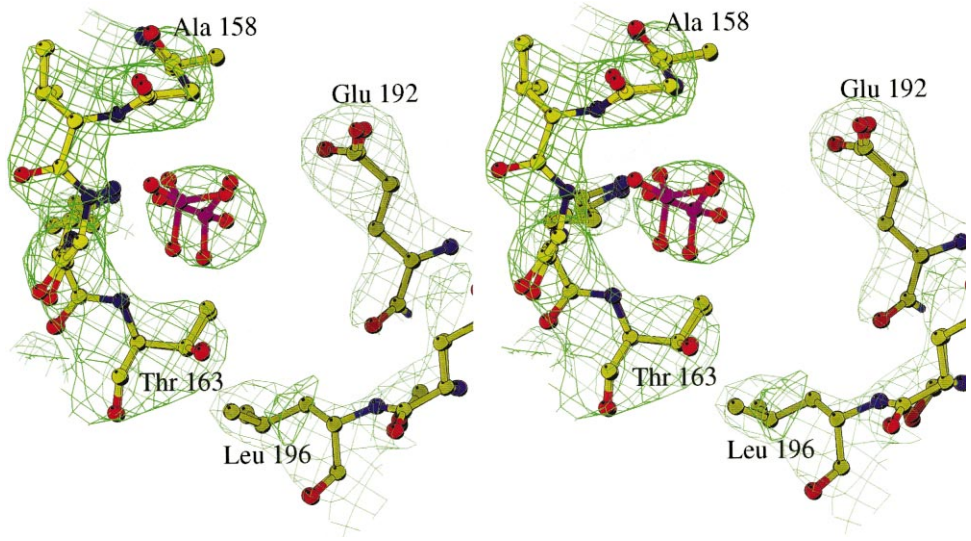

Fig. 1. Stereo view of a superposition of the β_E catalytic sites of the high AMPPNP and frozen-native [16] structures. All α-carbon atoms were used to superimpose the β_E-subunits with rmsd of 0.15 Å. There is a slightly larger difference in the position of the bound phosphate (or sulphate) group, but this group has a very high temperature factor (80 Å^2). The carbon, nitrogen, oxygen and phosphorous atoms are coloured yellow, blue, red and pink, respectively. The $2F_o$–F_c electron density map for the high AMPPNP structure is shown contoured at 1.3 σ.

F_1-ATPase structures there is density in the region of the P-loop residues which is consistent with a bound phosphate (or more likely sulphate, Fig. 1). Therefore, although the crystals were grown at high nucleotide concentration (5 mM AMPPNP), the overall conformation of the enzyme and the occupancy of the nucleotide binding site remain the same as when the crystals were grown at a lower nucleotide concentration (250 μM).

4. Discussion

By studying the fluorescence of tryptophan residues close to catalytic sites in F_1-ATPase from *E. coli*, all three catalytic sites were found to be occupied in the presence of 5 mM ATP, 250 μM AMPPNP and 5 μM ADP under conditions similar, but not identical, to those used in crystallisation experiments [11]. The same technique has also been used to measure the binding affinities of the three catalytic sites in the presence of magnesium and either ATP ($K_{d1} < 50$ nM, $K_{d2} = 0.5$ μM $K_{d3} = 25$ μM [9]), ADP ($K_{d1} = 0.14$ μM, $K_{d2} = 20$ μM, $K_{d3} = 20$ μM [10]) or AMPPNP ($K_{d1} = 0.14$ μM, $K_{d2} = 47$ μM, $K_{d3} = 47$ μM [10]). Because the affinity constants for the three nucleotides are all within the same range, it is likely that a mixture of these nucleotides (5 mM ATP, 250 μM AMPPNP and 5 μM ADP) will result in non-homogeneous occupancy of catalytic sites between individual F_1-ATPase complexes. The non-homogeneity of nucleotide occupancy is likely to be exacerbated further by 5 mM ATP ($K_{d1} < 50$ nM) which is likely to out-compete inhibitory AMPPNP ($K_{d1} = 0.14$ μM) and ADP ($K_{d1} = 0.14$ μM) and in turn be hydrolysed. For crystallisation, usually a homogeneous protein population is required. Therefore, we chose to perform the crystallisation in the presence of only AMPPNP and ADP. Assuming that the empty site observed in the original crystal structure [3] had an affinity constant for AMPPNP similar to that observed for the low affinity sites in the *E. coli* mutants (47 μM) [10], then it would be expected at AMPPNP concentrations of 250 μM, 1 mM and 5 mM that 84.2%, 95.5% and 99.1% of the sites, respectively, would be occupied. However, in the structure determined from crystals grown with 5 mM AMPPNP and 5 μM ADP, bound nucleotide was resolved at only two of the catalytic sites.

Bovine and *E. coli* F_1-ATPases both exhibit three distinct affinity constants for the binding of MgATP and their values (10^{-12}, 10^{-5} and 10^{-4} and 10^{-10}, 10^{-6} and 10^{-4}, respectively) are similar [11,22]. Hence, it is unlikely that the difference in occupancy observed between the solution and crystalline states arises from the different sources of enzyme. However, several possibilities can be advanced to explain the difference. First, the occupancy of the *E. coli* enzyme has not been investigated in the presence of both ADP and AMPPNP at concentrations greater than 250 μM AMPPNP. However, it is unlikely that affinity for AMPPNP would fall dramatically due to the presence of 5 μM ADP compared with AMPPNP alone, especially as binding of ADP can occur at all three sites with similar affinity constants to those observed with AMPPNP. Second, the tryptophan fluorescence experiments [11] were performed under the same conditions as used for crystallisation [3], except for the absence of 14% (w/v) PEG 6000, which was excluded because it quenched the tryptophan fluorescence. Therefore, PEG 6000 may affect (reduce) the binding of AMPPNP at the third catalytic site. Third, if an affinity constant of 47 μM is assumed for the binding of AMPPNP to the third catalytic site, then under the crystallisation conditions used here (5 mM AMPPNP) only 0.9% of the F_1-ATPase complexes would have two catalytic sites occupied. If there is a difference in solubility of the form with two sites occupied, compared with that with three sites occupied, then it is possible, although very unlikely, that the minor form may be selectively crystallised. Recently the structure of bovine F_1-ATPase inhibited with aluminium fluoride has been solved with crystals grown under the same salt and precipitant conditions as used here, and all three catalytic sites are occupied (I.R. Menz, J.E. Walker and A.G.W. Leslie, submitted for publication). However, this aluminium fluoride-inhibited enzyme and F_1-ATPase with all three catalytic sites occupied

by AMPPNP may adopt different conformations. Finally, in mutants with tryptophan substitutions at different positions around the catalytic site, quenching of their fluorescence may be observed when bound and unbound nucleotides are in rapid equilibrium, or if there is positional disorder in nucleotide binding resulting in no detectable electron density for the bound substrate. The catalytic site of the β_E-subunit is much more open than that in the β_{TP}-subunit. For example, Phe 424 and Tyr 345, which define the adenine binding pocket, are 8.1 and 12.2 Å apart in the β_{TP} and β_E sites, respectively, and in the same sites, respectively, Tyr 345 lies 12.6 and 13.3 Å from the P-loop residue Thr 163. This more open conformation would result in fewer hydrogen bonds being formed between the substrate and enzyme, and, given the symmetry of the terminal phosphate groups of AMPPNP, it may be possible to bind the substrate in more than one conformation. This situation could lead to the tryptophan fluorescence being quenched, and the substrate unresolved by X-ray crystallography.

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