The structure of bovine F₁-ATPase inhibited by ADP and beryllium fluoride

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This paper is dedicated to Professor Yasuo Kagawa

The structure of bovine F₁-ATPase inhibited with ADP and beryllium fluoride at 2.0 Å resolution contains two ADP·BeF₃[−] complexes mimicking ATP, bound in the catalytic sites of the β_{TP} and β_{DP} subunits. Except for a 1 Å shift in the guanidinium of αArg373, the conformations of catalytic side chains are very similar in both sites. However, the ordered water molecule that carries out nucleophilic attack on the γ-phosphate of ATP during hydrolysis is 2.6 Å from the beryllium in the β_{DP} subunit and 3.8 Å away in the β_{TP} subunit, strongly indicating that the β_{DP} subunit is the catalytically active conformation. In the structure of F₁-ATPase with five bound ADP molecules (three in α-subunits, one each in the β_{TP} and β_{DP} subunits), which has also been determined, the conformation of αArg373 suggests that it senses the presence (or absence) of the γ-phosphate of ATP. Two catalytic schemes are discussed concerning the various structures of bovine F₁-ATPase.

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Introduction

The ATP synthase (F₁F₀-ATPase) in the inner membranes of mitochondria is a multisubunit enzyme that uses energy from the proton motive force (PMF) generated by electron transfer to catalyse the synthesis of ATP from ADP and inorganic phosphate (Boyer, 1997; Walker, 1998; Senior *et al*, 2002). It has a membrane domain F₀, attached by central and peripheral stalks to the catalytic domain, F₁, which lies outside the membrane in the matrix of the organelle. The central stalk consisting of subunits γ, δ and ε penetrates into the core of the F₁ catalytic domain, and the three α-subunits and the three β-subunits that together with subunits γ, δ and ε make

up the F₁ domain are arranged in alternation around a six-fold axis of pseudo-symmetry passing through the central stalk (Abrahams *et al*, 1994). The foot of the central stalk (Gibbons *et al*, 2000) is attached firmly to a ring of hydrophobic c-subunits in the membrane domain (Stock *et al*, 1999). The central stalk and the c-ring rotate as an ensemble, and the rotation is driven by energy from the PMF (Yoshida *et al*, 2001). This rotation modulates the nucleotide-binding properties and catalytic activities of each catalytic β-subunit by bringing about a series of structural transitions that takes each of them through states with high, medium and low affinities for substrates and products.

The F₁ domain can be released intact from the membrane and retains the ability to hydrolyse ATP. Over the past 10 years, bovine F₁-ATPase has been studied intensively by X-ray crystallography. The first crystal structure, called ‘the reference structure’ below, was determined with crystals grown in the presence of ADP and the nonhydrolysable ATP analogue AMP-PNP (Abrahams *et al*, 1994). In this structure, AMP-PNP was found in one β-subunit (β_{TP}), ADP in the second (β_{DP}) and the third (β_E) had adopted an open conformation that was incapable of binding a nucleotide. On the basis of the reduced solvent accessibility to the catalytic site, and the arrangement of the catalytic residues, the β_{DP} subunit was designated as the catalytic (tight) binding site. As MgADP, but not phosphate, was bound to this subunit, it was suggested that the structure represents the ‘ADP-inhibited state’. Subsequently, the structures of the enzyme inhibited by the antibiotics efrapeptin (Abrahams *et al*, 1996) and aurovertin B (van Raaij *et al*, 1996), and of the enzyme covalently inhibited with 4-chloro-7-nitrobenzofurazan (Orriss *et al*, 1998) and dicyclohexyl-carbodiimide (DCCD) (Gibbons *et al*, 2000) were determined. All of them were very similar to the reference structure. In each of them, MgADP was bound to the β_{DP} subunit, and the β_E subunit remained empty. When crystals were grown in the presence of a high concentration (5 mM) of AMP-PNP and the usual concentration of ADP, the β_E subunit still remained empty (Menz *et al*, 2001a). However, when they were grown from enzyme inhibited with ADP and aluminium fluoride (and aluminium fluoride was not maintained in the mother liquor), the resulting structure (Braig *et al*, 2000) had an AlF₃ group bound close to ADP in the β_{DP} subunit, mimicking a catalytic intermediate. The presence of aluminium fluoride resulted in small movements of the catalytic residues (αArg373, βGlu188, βArg189) in the β_{DP} catalytic site, but the overall conformation of the enzyme was still very similar to that of the reference structure. These experiments provided overwhelming evidence that the reference structure (and the related structures) represents a conformation on the catalytic pathway of ATP hydrolysis by F₁-ATPase. Other crystals of F₁-ATPase inhibited by ADP and aluminium fluoride, where excess aluminium fluoride and ADP were maintained in the mother liquor, led to a structure where the ADP·AlF₄[−] complex was bound to both β_{TP} and β_{DP} subunits, and in addition ADP

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and a sulphate moiety (mimicking phosphate) were found in the β_E subunit (Menz *et al*, 2001b). This structure, referred to below as the (ADP.AlF₄)₂F₁ structure, displayed a novel 'half-closed' conformation of the β_E subunit in which the formerly open or empty site had closed partially forming both an adenine-binding pocket that allowed ADP to bind and a distinct phosphate-binding site that was occupied by sulphate. In this structure, the central stalk had rotated significantly in a clockwise direction as viewed from the foot of the central stalk relative to the reference structure. The coordination distances of the AlF₄ group bound to the β_{DP} subunit suggested that this structure was mimicking a transition state in the catalytic pathway. Another structure of F₁-ATPase inhibited by the regulatory protein, IF₁, appears to represent a prehydrolysis state where ATP is poised for hydrolysis (Cabezón *et al*, 2003).

This series of structures has helped to define the catalytic pathway of ATP hydrolysis by F₁-ATPase, but structural information about an enzyme-substrate complex with ATP bound to the catalytically active β_{DP} subunit has been lacking, and all attempts to obtain crystals with ATP or an ATP analogue bound to the β_{DP} subunit have been unsuccessful. Beryllium fluoride is well established as a phosphate analogue (Petsko, 2000), and it has been used to study a variety of NTPases including G-proteins (Bigay *et al*, 1987), F-actin, microtubules (Combeau and Carlier, 1988, 1989) and rabbit muscle kinases (Garin and Vignais, 1993). Its use has resulted in a number of protein structures in which the beryllium fluoride is mimicking phosphate. In others, it is associated with ADP. In the structures of the myosin motor domain from *Dictyostelium discoideum* (Fisher *et al*, 1995; Bauer *et al*, 1997; Gulick *et al*, 2000), of scallop myosin S1 (Himmel *et al*, 2002) and of nucleotide diphosphate kinase (Xu *et al*, 1997) the ADP.BeF complex was interpreted as mimicking bound ATP, whereas in a UMP/CMP kinase a BeF₂ group formed a strong bridge between the terminal oxygens of the bound nucleotides (ADP and UDP) (Schlichting and Reinstein, 1997). ADP and beryllium fluoride together inhibit bovine F₁-ATPase (Dupuis *et al*, 1989).

This paper describes the crystal structure of bovine F₁-ATPase inhibited by ADP and beryllium fluoride at 2.2 Å resolution. In this structure, an ADP.BeF complex appears to mimic ATP bound to both β_{DP} and β_{TP} subunits. Two possible reaction schemes for ATP hydrolysis are discussed in the context of the known crystal structures of the bovine enzyme.

Results

Structure determination of BeF₃⁻-inhibited bovine F₁-ATPase

The crystals of bovine F₁-ATPase inhibited with BeF₃⁻ and ADP (BeF₃⁻-F₁) were grown by microdialysis in the presence of beryllium chloride and sodium fluoride. The crystallization buffer did not contain AMP-PNP. The structure was solved by molecular replacement (see Materials and methods) using data to 2.2 Å resolution. Data processing and refinement statistics are summarized in Table I.

The refined model consists of residues α_E 24–510, α_{TP} 23–401, 410–510, α_{DP} 13–510, β_{DP} 9–475, β_E 9–474, β_{TP} 9–474, γ 1–47, 67–90, 105–116, 127–148, 159–173 and 201–272. The electron density for the δ - and ϵ -subunits was too weak to allow reliable modelling. The mobility of these subunits

appears to be correlated to the *a* dimension of the unit cell, as they are well defined in both the DCCD-inhibited structure (Gibbons *et al*, 2000) (*a* = 267 Å) and the (ADP.AlF₄)₂F₁ structure (Menz *et al*, 2001b) (*a* = 268 Å), but not in other structures with *a* in the range 282–286 Å. This is probably because the smaller cell edge is associated with additional lattice contacts between the γ - and δ -subunits of one complex and the N-terminal region of the α_E subunit of an adjacent molecule.

Structure determination of the ADP bound form of bovine F₁-ATPase

Since the BeF₃⁻-F₁ crystals were grown with ADP as the only nucleotide present, the structure of F₁-ATPase with ADP as the only bound nucleotide (ADP-F₁) was determined to allow comparison with the BeF₃⁻-inhibited structure. The ADP-F₁ crystals were grown under the same conditions as the reference crystals (Lutter *et al*, 1993) except that AMP-PNP was omitted from both the inside and outside buffers, and the ADP concentration was increased from 5 μ M to 2 mM in the outside buffer. The structure was solved by molecular replacement (see Materials and methods), but the resolution was limited by crystal quality to 2.85 Å. Data processing and refinement statistics are summarized in Table I.

The refined structure consists of α_E 24–510, α_{TP} 24–401, 410–510, α_{DP} 19–510, β_{DP} 9–475, β_E 9–474, β_{TP} 9–474, γ 1–44, 77–90 and 209–272. One residue (Asp411) in the α_{TP} subunit is in a disallowed region of the Ramachandran plot, but this region of the protein is poorly ordered, with *B*-factors above 100 Å².

Overall structure of BeF₃⁻-F₁

When the beryllium fluoride-inhibited structure is compared to the reference structure (Braig *et al*, 2000), the root mean square deviation (r.m.s.d.) in C α positions for the entire

Table I Data processing and refinement statistics

	BeF ₃ ⁻ -F ₁	ADP-F ₁
Resolution (Å)	20.0–2.2	20.0–2.85
Number of reflections	185 113	93 001
Multiplicity ^a	2.0 (1.4)	2.6 (2.3)
Completeness (%) ^{a,b}	87.1 (51.1)	93.3 (96.2)
R _{merge} (%) ^c	8.0 (35.4)	6.3 (18.9)
<I/σ(I)> ^a	8.1 (1.7)	14.3 (6.1)
B-factor from Wilson plot (Å ²)	37.7	51.8
<i>Refinement</i>		
Number of reflections	175 588	88 256
Total number of atoms	24 479	23 092
Number of waters	1019	266
R-factor (%)	18.4	22.2
R _{free} (%) ^d	23.6	27.8
R.m.s.d. in bond lengths (Å)	0.012	0.007
R.m.s.d. in bond angles (deg)	1.3	1.0

^aThe values for the highest resolution bins (2.32–2.20 Å for BeF₃⁻-F₁ data and 3.00–2.85 Å for ADP-F₁ data) are given in parentheses.

^bFor BeF₃⁻-F₁ data, the completeness is 97.4% at 2.46 Å. The completeness at 2.20 Å is low because these data were only recorded in the corners of the detector.

^cR_{merge} = $\sum_h \sum_i |I(h)_i - \bar{I}(h)| / \sum_h \sum_i I(h)_i$, where $I(h)_i$ is the mean weighted intensity after rejection of outliers.

^dR_{free} values were calculated using randomly selected subsets of reflections (5.0% for BeF₃⁻-F₁ and 4.8% for ADP-F₁) that were excluded from the refinement.

complex is 0.41 Å, ranging from 0.30 Å (β_{TP}) to 0.62 Å (γ) for individual subunits. The relatively large r.m.s.d. for the γ -subunit probably arises because it is more mobile: the average *B*-factors for individual subunits range from 38.7 Å² (α_{DP}) to 82.9 Å² (γ).

Nucleotide-binding sites of the BeF₃⁻-inhibited structure

The difference electron density map calculated after the first round of refinement (using a model with ADP as the bound nucleotide) showed positive density in both the β_{DP} and the β_{TP} nucleotide-binding sites adjacent to the β -phosphate of the bound ADP. Beryllium fluoride (BeF₃⁻) gave an excellent fit to the electron density in both subunits (Figure 1). There was no evidence for bound nucleotide in the third β -subunit, β_E . The presence of two BeF₃⁻ molecules in the structure is consistent with the stoichiometry estimated in biochemical studies (Dupuis *et al*, 1989).

The P-loop residues of the β_{DP} and β_{TP} subunits were superimposed, and the positions of the catalytic residues were compared (Figure 2A). The only significant difference is in the position of the guanidinium group of α Arg373, which is ca. 1 Å further away from the BeF₃⁻ group in the β_{TP} subunit. Other side-chain positions differ by less than 0.3 Å. However, comparison of the water structure in the catalytic sites revealed a significant shift in the position of the water molecule lying between the beryllium and the carboxylate group of β Glu188. This water molecule is 2.6 Å from the beryllium in the β_{DP} subunit, but 3.8 Å distant in the β_{TP} subunit. The β_{DP} catalytic site of BeF₃⁻-F₁ is also very similar to the β_{DP} catalytic site of the (ADP·AlF₄⁻)₂F₁ structure (which is believed to represent the pentacovalent intermediate). Following superposition of the P-loop residues, the side-chain positions differ by less than 0.5 Å (Figure 2B).

By superimposing the catalytic site of the β_{TP} subunit of BeF₃⁻-F₁ onto the β_{TP} subunit of the reference structure, it became apparent that the beryllium fluoride group overlaps the γ -phosphate of AMP-PNP, demonstrating that in terms of the geometry of binding ADP·BeF₃⁻ is a good mimic of AMP-PNP (and ATP). The noncatalytic nucleotide-binding sites in

the three α -subunits all contain ADP, with no evidence for bound sulphate or beryllium fluoride.

Structure of bovine ADP-F₁

The structure of ADP-F₁ is also very similar to the reference structure, with an r.m.s.d. in C α positions for the entire complex of 0.54 Å. The r.m.s.d. values for individual subunits range from 0.32 Å (α_{DP}) to 0.70 Å (γ). When compared with the BeF₃⁻-F₁ structure, the overall r.m.s.d. is 0.55 Å, with the values for individual subunits ranging from 0.28 Å (β_{DP}) to 0.55 Å (γ).

The nucleotide-binding sites of the β_{DP} and β_{TP} subunits and all three α -subunits have ADP bound, whereas the β_E subunit has no bound nucleotide. The conformations of the catalytic residues of the β_{DP} catalytic site are very similar (within 0.6 Å when superimposed by the P-loop residues) in the ADP-F₁ and BeF₃⁻-F₁ structures. However, in the β_{TP} catalytic site, α_{TP} Arg373 adopts a very different conformation in ADP-F₁ (Figure 3), which has previously only been observed in the DCCD-inhibited structure (Gibbons *et al*, 2000). The re-arrangement of the Arg373 side chain is accompanied by a shift in its α -carbon position of 2 Å (adjacent residues shift less than 0.5 Å), and somewhat larger shifts (up to 3 Å) in residues 421–427 in the β_{TP} subunit, including Phe424, which forms part of the nucleotide-binding pocket (Figure 3). In its new conformation, the N ϵ of Arg373 can form a hydrogen bond to the 2' hydroxyl of the ADP bound to the β_{TP} subunit.

Discussion

Comparison with other structures of bovine mitochondrial F₁-ATPase

The overall structure of BeF₃⁻-F₁ is very similar to that of the reference structure, and also to the majority of the previously determined structures of bovine F₁-ATPase in a variety of inhibited states (Abrahams *et al*, 1994, 1996; van Raaij *et al*, 1996; Orriss *et al*, 1998; Braig *et al*, 2000; Gibbons *et al*, 2000; Menz *et al*, 2001a). The two exceptions are the (ADP·AlF₄⁻)₂F₁ structure (Menz *et al*, 2001b) and the complex with the

Figure 1 The nucleotide-binding site in the β_{DP} subunit of bovine F₁-ATPase inhibited with ADP and beryllium fluoride (BeF₃⁻-F₁). The view is in stereo. The difference electron density for beryllium fluoride is shown as are catalytic residues in the vicinity of the site. The attacking nucleophilic water molecule (Wat) is shown as an orange sphere and a magnesium ion (Mg) is yellow. In this and subsequent figures, carbon atoms are green in β -subunits and white in α -subunits. Nitrogen, oxygen and phosphorus atoms are blue, orange and pink, respectively. The BeF₃⁻ group is also coloured pink.

Figure 2 Comparison of the catalytic sites in bovine F₁-ATPase. (A) Superimposition of the β_{TP} and β_{DP} subunits from the structure of bovine $\text{BeF}_3\text{-F}_1$. The view is shown in stereo and the β_{TP} and β_{DP} subunits are coloured and grey, respectively. The attacking water (Wat) is also shown. Except for αArg373 , the catalytic side chains have similar conformations in the two subunits (as observed in previous structures of bovine F₁-ATPase), but the positions of the nucleophilic water molecule differ significantly. (B) Superimposition of the β_{DP} subunits of the $(\text{ADP}.\text{AlF}_4)_2\text{F}_1$ structure (coloured) and $\text{BeF}_3\text{-F}_1$ (grey).

Figure 3 Superimposition of nucleotide-binding site residues in the β_{TP} subunits in the structures of bovine ADP-F_1 and $\text{BeF}_3\text{-F}_1$. The view is shown in stereo and the ADP-F_1 and $\text{BeF}_3\text{-F}_1$ structures are coloured and grey, respectively. The $\alpha_{\text{TP}}\text{Arg373}$ side chain adopts dramatically different conformations in the two structures as does $\beta_{\text{TP}}\text{Phe424}$.

natural inhibitor protein (Cabezón *et al*, 2003). The structural similarities include the catalytic residues in the β_{DP} and β_{TP} subunits. The r.m.s.d. values for all atoms in the P-loop and

the side chains of βLys162 , βGlu188 , βArg189 and αArg373 between the $\text{BeF}_3\text{-inhibited}$ and the reference structures are 0.29 and 0.45 Å for the β_{DP} and β_{TP} subunits, respectively. In

the BeF_3^- - F_1 structure, the BeF_3^- group mimics the γ -phosphate of ATP, and this structure provides the first picture of how ATP binds to the β_{DP} subunit.

In view of the similarities in the crystallization conditions of the $(\text{ADP}.\text{AlF}_4^-)_2F_1$ and the BeF_3^- - F_1 structures, which differ primarily in the replacement of aluminium fluoride by beryllium fluoride, it is not apparent why the BeF_3^- - F_1 structure did not bind ADP in the β_{E} subunit. One difference between the two structures is the length of the crystallographic a axis ($a = 283.5 \text{ \AA}$ for BeF_3^- - F_1 , $a = 268 \text{ \AA}$ for $(\text{ADP}.\text{AlF}_4^-)_2F_1$). This difference suggests that the shorter a axis favours the nucleotide-bound half-closed conformation of the β_{E} subunit. However, the β_{E} subunit is not involved in lattice contacts in either structure, and therefore the β_{E} conformation must be influenced indirectly via intermolecular interactions involving the γ - and δ -subunits. While lattice contacts may influence the conformation and nucleotide occupancy of the β_{E} subunit, they cannot be the sole factor because the DCCD-inhibited F_1 structure, which also has a short a axis ($a = 267 \text{ \AA}$; Gibbons *et al*, 2000), has the 'open' (nucleotide free) conformation of the β_{E} subunit. In recent experiments, both the open and half-closed conformations of the β_{E} subunit have been observed within the same crystal (S Bartoschek, S Contessi, MG Montgomery, AGW Leslie and JE Walker, unpublished work), suggesting that the energetic barrier between the two conformations is rather small, and could be influenced by crystal packing forces. However, there is no simple explanation that accounts for all the observations.

Which β -subunit is catalytically active?

The β_{DP} subunit rather than the β_{TP} subunit was identified as being closest to the catalytically active conformation in the original structure of the bovine enzyme because the nucleotide is more buried at the $\beta_{\text{DP}}/\alpha_{\text{DP}}$ interface than at the $\beta_{\text{TP}}/\alpha_{\text{TP}}$ interface. The presence of MgADP in the β_{DP} subunit is also consistent with the enzyme being in the MgADP-inhibited form, in which MgADP (but no phosphate) is bound to the catalytic site with the highest affinity. The assignment of β_{DP} as the catalytic subunit was supported by the structure of AlF_3 -inhibited F_1 (Braig *et al*, 2000), where $\text{MgADP}.\text{AlF}_3$ was bound to the β_{DP} subunit but not to the β_{TP} subunit.

In the structure of $(\text{ADP}.\text{AlF}_4^-)_2F_1$, both the β_{TP} and the β_{DP} catalytic sites bind $\text{MgADP}.\text{AlF}_4^-$, which is believed to mimic a pentacovalent intermediate in the hydrolysis reaction. As discussed previously (Menz *et al*, 2001b), the arrangement of catalytic residues in the two sites is very similar. The principal difference is in the position of the guanidinium group of $\alpha\text{Arg}373$, which is ca. $1 \text{ \AA}$ further from the γ -phosphate site in the β_{TP} subunit (the γ -phosphate site is the location of the γ -phosphate of AMP-PNP bound to the β_{TP} subunit in the reference structure). Comparison of the β_{TP} and β_{DP} catalytic sites in BeF_3^- - F_1 (Figure 2A) also shows that the two sites are almost identical, except that $\alpha\text{Arg}373$ has shifted, as in the $(\text{ADP}.\text{AlF}_4^-)_2F_1$ structure. The spatial arrangement of the catalytic residues in the β_{DP} subunit of the $(\text{ADP}.\text{AlF}_4^-)_2F_1$ structure is also very similar to that in the β_{DP} subunit of the BeF_3^- - F_1 structure (representing the catalytic enzyme-ATP complex), with differences in side-chain positions of less than $0.5 \text{ \AA}$ (Figure 2B). This is consistent with the proposed catalytic states of these two sites: a significant conformational change occurs only after ATP hydrolysis, to

a state represented by the 'half-closed' $\beta_{\text{ADP}+\text{P}_i}$ subunit of the $(\text{ADP}.\text{AlF}_4^-)_2F_1$ structure.

All available structural and biochemical data support a catalytic mechanism in which the carboxylate group of $\beta\text{Glu}188$ polarizes or extracts a proton from a water molecule, promoting an in-line attack on the γ -phosphate of ATP. Other side chains ($\beta\text{Lys}162$, $\beta\text{Arg}189$, $\alpha\text{Arg}373$) and main-chain atoms in the P-loop play roles in orienting the γ -phosphate and stabilizing negative charges that develop on the γ -phosphate during catalysis. Either ADP or $\text{ADP}.\text{AlF}_x$ was bound to the β_{DP} subunit in all previously determined structures of the bovine enzyme, and the solvent structure in the region of the γ -phosphate must differ when ATP is bound. Only in the present structure, with $\text{ADP}.\text{BeF}_3^-$ mimicking bound ATP in both the β_{TP} and the β_{DP} catalytic sites, has it been possible to examine which site is more likely to represent the catalytically active conformation on the basis of the location of the attacking water. The relatively high resolution of the BeF_3^- - F_1 structure ($2.2 \text{ \AA}$) also allows greater confidence in the observed positions of well-ordered water molecules. The distinction between the two sites is very clear (Figure 4). In the β_{DP} subunit, the water molecule (B -factor $33 \text{ \AA}^2$) is $2.6 \text{ \AA}$ from the beryllium, and it also interacts with both carboxylate oxygens of $\beta\text{Glu}188$ (2.8 and $3.5 \text{ \AA}$ away). In the β_{TP} subunit, the corresponding water molecule (B -factor $33 \text{ \AA}^2$) is $3.8 \text{ \AA}$ from the beryllium, and it is not favourably placed for in-line attack on the γ -phosphate of an ATP molecule. This observation strongly supports the view that the β_{DP} subunit represents the catalytically active conformation. The overall similarity of the two catalytic sites (Figure 2A) suggests that the shift in the position of the attacking water is an indirect result of a small shift of the side chain of $\alpha\text{Arg}373$. In the β_{TP} subunit, the guanidinium group of $\alpha\text{Arg}373$ is positioned about $1 \text{ \AA}$ further away from the γ -phosphate site than in the β_{DP} subunit. This change is presumably responsible for the observed shift in the BeF_3^- group, which effectively pivots around the fluorine atom bound to the magnesium ion, producing a shift of $0.7 \text{ \AA}$ in the beryllium and $0.8 \text{ \AA}/1.0 \text{ \AA}$ in the other two fluorine atoms (Figure 5). The combined shifts of $\alpha\text{Arg}373$ and the BeF_3^- group result in a shift of the attacking water by $0.6 \text{ \AA}$ in the opposite direction, towards the carboxylate group of $\beta\text{Glu}188$, so that the distance to the beryllium changes from 2.6 to $3.8 \text{ \AA}$. There are two further water molecules in the vicinity of the BeF_3^- group, but their positions change by less than $0.3 \text{ \AA}$ between the two sites. This assignment of catalytic activity to the β_{DP} subunit agrees with a similar suggestion based on molecular dynamics simulations (Gao *et al*, 2003).

Structural basis for the difference between the β_{DP} and β_{TP} active sites

Because the catalytic sites are located at the interfaces between the α - and β -subunits, the conformation of the active site depends on both the conformation of the individual α - and β -subunits (their tertiary structures) and on the relative positioning of the subunits (the quaternary structure). The β_{DP} and β_{TP} subunits have very similar tertiary structures, particularly in the region of the nucleotide-binding site, as have the α_{DP} and α_{TP} subunits. Therefore, the observed differences between the β_{DP} and β_{TP} catalytic sites are primarily the result of differences in the quaternary structure. This conclusion is apparent when the $\beta_{\text{DP}}/\alpha_{\text{DP}}$ subunits are

β_{DP}

 β_{TP}

Figure 4 Schematic representations of the nucleotide-binding sites in the β_{DP} and β_{TP} subunits in the structure of bovine BeF_3^- -F₁. The β_{DP} and β_{TP} subunits are on the left and right, respectively. Distances between atoms are given in Ångstroms, and possible hydrogen bonds and interactions involving the magnesium ion are shown as dotted lines.

Figure 5 Comparison in stereo of the positions of ADP, beryllium fluoride and the attacking water (Wat) after superimposition of the β_{DP} and β_{TP} subunits of the bovine BeF_3^- -F₁ structure. The pivoting motion of the BeF_3^- group around the fluorine that coordinates the magnesium ion is more apparent in this view than in Figure 2. The α -carbons of the P-loops were used for the superimposition. The β_{TP} subunit is in colour and the β_{DP} subunit is in grey.

superimposed (as a pair) onto the β_{TP}/α_{TP} subunits using only atoms from the β -subunits to define the superposition. As expected, the β -subunits superimpose well (r.m.s.d. 0.66 Å for all C α atoms, 0.47 Å for C α atoms in the nucleotide-binding domain), but the α -subunits do not (r.m.s.d. 3.56 Å for all C α atoms, 2.09 Å for C α atoms in the nucleotide-binding domain). A rigid-body rotation of 6.7° is required to superimpose the nucleotide-binding domains of the α -subunits (r.m.s.d. 0.63 Å after superposition). The rotation axis runs through the upper part of the nucleotide-binding domain and is oriented approximately at right angles to the axis of the F₁ assembly and at an angle of 45° to the α_{TP}/β_{TP} interface (the axis lies very close to the α -carbons of αGly135 and αIle206). This rigid-body rotation is primarily responsible for the shift in the position of αArg373 relative to the catalytic residues of the β -subunits. Additional small rotations are required to superimpose the N- or C-terminal domains, as the relative orientation of these domains differs in the α_{DP} and α_{TP} subunits. The positioning of the α_{TP} subunit in the F₁ complex is determined by its interactions with the neighbouring β -subunits (β_{TP} and β_E) and with the centrally located γ -subunit. These interactions are quite extensive, involving numerous segments of polypeptide, suggesting

that no single interaction is responsible for the observed rotation of the nucleotide-binding domain of the α_{TP} subunit. However, it is clear that the asymmetry of the $(\alpha\beta)_3$ sub-assembly is fundamentally due to the inherent lack of three-fold symmetry in the γ -subunit. Each α - and β -subunit interacts with the γ -subunit in a unique manner that results in the asymmetric quaternary structure of the complex.

The arginine finger $\alpha_{TP}\text{Arg373}$

The ADP-F₁ structure is very similar to the reference structure, but there is a significant difference in the conformation of the side chain of $\alpha_{TP}\text{Arg373}$. This side chain is believed to play a critical role in catalysis (Futai *et al*, 1989; Abrahams *et al*, 1994; Nadanaciva *et al*, 1999), but in the ADP-F₁ structure it points away from the active site rather than towards it, resulting in a shift of almost 10 Å in the position of the guanidinium group. This conformation has been observed previously in the α_{TP} subunit of the DCCD-inhibited enzyme (Gibbons *et al*, 2000), and the common feature of the two structures is the absence of a γ -phosphate (or an analogue such as BeF_x or AlF_x) in the β_{TP} site. There are several basic residues in the vicinity of the γ -phosphate site, including βLys162 , βArg189 and αArg373 . It is possible that in the

absence of favourable interactions between the γ -phosphate (or analogue) and the guanidinium group of α Arg373, this arrangement of basic residues becomes energetically unfavourable. Of these basic side chains, α Arg373 would seem to have the greatest conformational freedom, as it is not involved with as many packing interactions as the other residues, and it has the opportunity for a radical change in conformation.

Comparison of the β_{TP}/α_{TP} interface in the ADP-F₁ structure and the reference structure (after superposing the β_{TP} subunits) shows that the change in the conformation of α Arg373 is accompanied by a rigid-body rotation (1.5°) of the entire α_{TP} subunit relative to the β_{TP} subunit. The rotation results in a slight opening of the α_{TP}/β_{TP} catalytic interface, and in shifts of up to $1.5 \text{ \AA}$ for residues in the C-terminal domain of the α_{TP} subunit. A rotation of a similar magnitude (1.3°) is observed in the β_E subunit, which forms the other interface with the α_{TP} subunit, whereas the remaining α - and β -subunits show much smaller changes ($<0.5^\circ$ rotation). These changes in the relative positions of the α - and β -subunits (quaternary structure) are found in both ADP-F₁ and DCCD-F₁ (both have ADP bound to the β_{TP} subunit) when compared with either the reference structure or BeF₃-F₁ (both contain a γ -phosphate or analogue in the β_{TP} subunit). The position of the segment of the γ -subunit that interacts with the β_{TP} and α_{TP} subunits is very similar in all these structures, which suggests that the change in the quaternary structure is a direct result of the presence or absence of a γ -phosphate group in the catalytic site of the β_{TP} subunit.

On the other hand, α Arg373 in the α_{DP} subunit of ADP-F₁ (or the reference structure) does not have the same conformation as in the α_{TP} subunit of ADP-F₁, even though ADP is bound to the β -subunit in both cases. A possible explanation is that differences in the quaternary structure between the β_{TP}/α_{TP} and the β_{DP}/α_{DP} catalytic interfaces (for example, the more extensive interface between β_{DP} and α_{DP} subunits, or interactions with the γ -subunit) make it energetically unfavourable for the β_{DP}/α_{DP} interface to open to accommodate the alternative conformation of α Arg373 in the same way as observed in the β_{TP}/α_{TP} catalytic interface of the F₁-ADP or F₁-DCCD structures.

If the conformational flexibility of α Arg373 is indeed associated with the type of nucleotide (ADP or ATP) bound to the catalytic subunit, and if this flexibility is coupled to the changes in the quaternary structure (as suggested by the ADP-F₁ and F₁-DCCD structures), then this residue may play an active role in catalysis by providing discrimination between ADP and ATP binding to the β_E site (which transforms into the β_{TP} site) during ATP hydrolysis.

Stoichiometry of beryllium fluoride binding

In BeF₃-F₁, all three α -subunits bind ADP rather than ADP.BeF₃⁻. This observation probably reflects the rate of formation of an ADP.BeF₃⁻ complex from free ADP and BeF₃⁻ being likely to be very slow in solution, and not being enhanced significantly when the nucleotide is bound to the α -subunits. In contrast, the catalytic residues in the β -subunits that promote ATP hydrolysis/synthesis are also very effective in catalysing the formation of ADP.BeF₃⁻. Once formed, ADP.BeF₃⁻ binds very tightly to the β -subunits, and so the concentration of free ADP.BeF₃⁻ in solution (which could then bind to the α -subunits) will always be very low.

The presence of ADP.BeF₃⁻ bound to both the β_{DP} and β_{TP} subunits does not necessarily imply that there is a state in the catalytic cycle where both subunits bind ATP. The presence of ADP.BeF₃⁻ in these two sites could arise from a step that is not part of the normal catalytic cycle, for example a 120° rotation of the γ -subunit without ATP hydrolysis. Similar considerations apply to the aluminium fluoride-inhibited form of the enzyme, where ADP.AlF₄⁻, mimicking the pentacovalent intermediate, is found in both the β_{DP} and β_{TP} subunits, although all current models suggest that only one of the three β -subunits is catalytically active at any given time.

Two possible reaction schemes for ATP hydrolysis

The crystal structures of bovine F₁-ATPase complexed with aluminium and beryllium fluoride, together with the reference structure, provide detailed information on distinct conformational states of the enzyme. A remaining challenge is to relate these structures to an overall catalytic scheme, which also takes account of the results of both single-molecule studies (Nishizaka *et al*, 2004) and kinetic data including tryptophan fluorescence experiments (Weber and Senior, 2000).

Two possible schemes that relate the rotation of the γ -subunit to ATP binding and hydrolysis events in F₁-ATPase are shown in Figure 6 (adapted from Leslie and Walker, 2000). It is assumed that the nucleotide occupancy of the catalytic sites alternates between two and three, corresponding to tri-site catalysis. This proposal is favoured by the results of tryptophan fluorescence experiments that show that on average all three catalytic sites are occupied during ATP hydrolysis at physiological rates (Weber and Senior, 2000) and by single-molecule studies (Nishizaka *et al*, 2004), although the original bi-site model (with catalytic site occupancies alternating between one and two) has also been defended recently (Boyer, 2002). The two schemes differ in the assignment of the β -subunit where ATP hydrolysis occurs. In scheme (A), ATP binding to the β_E subunit (state I) induces a large conformational change in that subunit, generating a rotation of the γ -subunit to an intermediate position as shown in state II. More subtle conformational changes are simultaneously transmitted to the active site of the β_{TP} subunit, promoting the hydrolysis of bound ATP to ADP + P_i. The combination of the conformational changes due to ATP binding to the β_E subunit and ATP hydrolysis on the β_{TP} subunit generates a 120° rotation of the γ -subunit, converting the β_{DP} subunit in state I to an open conformation (β_E) in state III, releasing ADP and P_i. State III is equivalent to state I, but there has been a cyclic interconversion of the three catalytic sites. In the next step (not shown), ATP binds to the newly formed β_E subunit of state III, resulting in hydrolysis of the ATP that had bound to the β_E subunit in the previous step (which is transformed into a β_{TP} subunit in state III). This produces a further rotation of the γ -subunit by 120° and release of ADP + P_i from the newly created β_{DP} subunit (repeating states I–III). A third round of ATP binding, ATP hydrolysis and product release brings the enzyme back to the original position.

In the alternative scheme (B), ATP binding to the β_E subunit promotes hydrolysis in the β_{DP} subunit (rather than β_{TP}). This corresponds to hydrolysis of the nucleotide that bound two steps earlier in the sequence (where a step corresponds to a 120° rotation of the γ -subunit). As in scheme

Figure 6 Two possible reaction schemes for ATP hydrolysis by F₁-ATPase. In parts (A) and (B) F₁-ATPase is depicted as viewed from the membrane proximal aspect of the intact ATP synthase. For simplicity, only the catalytic β-subunits and the centrally located γ-subunit are shown. ATP* represents an ATP molecule that is committed to hydrolysis. See text for further details.

(A), the combination of ATP binding and ATP hydrolysis (on different subunits) results in a 120° rotation of the γ-subunit, and ADP + P_i release from the β_{DP} subunit. Two further steps (each consisting of substeps of ATP binding followed by ATP hydrolysis and product release) bring the system back to its original position.

Both schemes are consistent with the direction of rotation of the γ-subunit observed in single-molecule studies (Noji *et al*, 1997). However, they differ in the average nucleotide occupancy of the catalytic sites, which is two ADP molecules and one ATP for scheme (A), and one ADP and two ATP molecules for scheme (B). On the basis of free energy simulations, Gao *et al* (2003) have described a similar scheme to (A). Tryptophan fluorescence measurements that distinguish between bound ADP and ATP support scheme (A) (Weber *et al*, 1996).

Recent single-molecule experiments in which the rotation of the γ-subunit and the binding of a fluorescent ATP analogue 2'-O-Cy3-EDA-ATP were monitored simultaneously have provided new insights into the catalytic cycle (Nishizaka *et al*, 2004). These results have been interpreted as showing that ATP binding to a nucleotide free β-subunit generates an 80° rotation of the γ-subunit, which triggers ATP hydrolysis and/or P_i release in the β-subunit that bound ATP one step earlier in the sequence, resulting in a further rotation of 40° in the γ-subunit. With the significant assumption that the use of a fluorescent ATP analogue while greatly reducing the rate of hydrolysis does not alter the basic reaction sequence, these results also support scheme (A).

Can the two proposed schemes be distinguished on the basis of the available crystal structures? Previously, the β_{DP} subunit has been assigned as being closest to a catalytically active conformation on the basis of the arrangement of the catalytic residues in the structures of the aluminium fluoride-

inhibited forms of the enzyme (Braig *et al*, 2000; Menz *et al*, 2001b). On this basis, a detailed catalytic sequence based on scheme (B) was proposed (Menz *et al*, 2001b). Although the BeF₃⁻-F₁ structure supports the assignment of β_{DP} as being closer to the catalytic conformation, it is now apparent that this does not necessarily imply support for scheme (B). The recently reported single-molecule experiments (Nishizaka *et al*, 2004) have been interpreted as suggesting that ATP hydrolysis probably occurs only after the 80–90° rotation of the γ-subunit induced by ATP binding to an empty site (i.e. state II in Figure 6). There are no structural data available for an intermediate position of the γ-subunit such as that shown in state II, but such a large rotation is likely to have a significant effect on the conformations at the catalytic sites of the β_{DP} and β_{TP} subunits. In particular, the β_{DP} subunit will change towards the β_E conformation, while the β_{TP} subunit will change towards the β_{DP} conformation. If ATP hydrolysis does indeed occur in state II, at this point the β_{TP} subunit may adopt a conformation that is actually closest to that of the β_{DP} subunit in the crystal structures (the catalytically active conformation), in which case the structural data also support scheme (A) in preference to scheme (B).

Because none of the crystal structures correspond exactly to the states depicted in Figure 6 in terms of the occupancy of the catalytic sites, they do not allow a clear distinction to be made between the alternative schemes. State I is best represented by the (ADP.AIF₃)F₁ structure (Braig *et al*, 2000), where AMPPNP is bound to the β_{TP} subunit and ADP.AIF₃ to the β_{DP} subunit, but with relatively long distances between the aluminium and both the oxygen of the β-phosphate (2.4 Å) and the nucleophilic water (3.1 Å). In terms of the overall structure of the enzyme, this arrangement is very similar to both the reference structure and BeF₃⁻-F₁. The (ADP.AIF₄⁻)₂F₁ structure, which, by analogy with other

NTPases complexed with aluminium fluoride, is assumed to mimic the pentacovalent intermediate in the hydrolysis reaction, to some extent represents state II. However, the position of the γ -subunit in this structure is closer to that in state III and the presence of ADP + P_i bound to the half-closed β_E subunit suggests that this structure represents a posthydrolysis, preproduct release state. Thus, it is best interpreted as representing an intermediate between states II and III in scheme (A).

The limited range of movement of the γ -subunit seen in the crystal structures is to be expected if the larger rotational substep (80 – 90°) is associated with nucleotide binding rather than a catalytic event. All of the current structures represent conformations of the enzyme between states II and III in scheme (A) (note that states I and III are equivalent), which corresponds to a maximum rotation of 30 – 40° in the γ -subunit. The possibility that lattice contacts influence the position of the γ -subunit also cannot be ruled out, particularly since a significant fraction of the γ -subunit (corresponding to the foot of the central stalk) was sufficiently mobile that it could not be modelled in the reference structure. However, it is unlikely that lattice contacts have a significant effect on the catalytic sites in the β -subunits because the most flexible and structurally variable region of the γ -subunit is below the point at which it interacts with the $(\alpha\beta)_3$ sub-assembly.

The crystal structures demonstrate that the β_{DP} and β_{TP} catalytic sites are remarkably similar in their arrangements of catalytic residues. The most significant difference between them is the position of the guanidinium of α Arg373, which is determined by the interactions between the relevant α - and β -subunits, and the interactions of both α - and β -subunits with the γ -subunit. Furthermore, the BeF_3^- - F_1 structure illustrates how a small (ca. $1\text{ \AA}$) shift in the position of the guanidinium can produce a more radical difference in the position of the attacking water molecule, clearly differentiating the two sites in terms of their potential for catalysing ATP hydrolysis.

Materials and methods

Enzyme purification and inhibition

Bovine F_1 -ATPase was purified from heart mitochondria as described previously (Lutter *et al*, 1993) with minor changes: a Sephacryl S-300 column was replaced by an XK26/60 Superdex 200pg column, amastatin has been added to prevent proteolysis of the δ -subunit and ATP in buffers has been replaced by ADP, 5 mM 2-mercaptoethanol was replaced with 5 mM dithiothreitol. Glycerol treatment to remove endogenous bound nucleotides was not carried out. The purified F_1 -ATPase was stored as an ammonium sulphate precipitate. It was collected by centrifugation ($30\,600g$, 20 min). The enzyme was exchanged on a spin column (Bio-Gel P-6 Medium, Bio-Rad) into a buffer containing 100 mM Tris- SO_4 , pH 7.8, 4 mM EDTA, 1 mM ADP and 10 mM MgSO_4 . To prepare the beryllium fluoride-inhibited enzyme, the protein sample (1.3 ml, protein concentration 18.5 mg/ml) was mixed with a solution (440 μ l) containing 40 mM MgSO_4 and 10 mM ADP. After 20 min, 0.5 M NaF (100 μ l) was added to the mixture, and 20 min later 250 mM BeCl_2 (50 μ l). At this point, the ATP hydrolase activity was inhibited by 98.5% (Pullman *et al*, 1960).

Bovine F_1 -ATPase used for growing crystals of ADP- F_1 was purified as described above except that amastatin was replaced by bestatin and diprotin A. The purified enzyme was then subjected to nucleotide stripping as described previously (Lutter *et al*, 1993).

Crystallization and data collection

Crystals of BeF_3^- - F_1 were grown in microdialysis buttons (50 μ l) with SpectraPor dialysis membranes (3500 MW cutoff). An equal volume of inside buffer (100 mM Tris-HCl, pH 7.2, 400 mM sodium chloride, 4 mM magnesium chloride, 0.04% (w/v) sodium azide, 2 mM EDTA, 0.004% (w/v) phenylmethylsulphonyl fluoride, 14% (w/v) polyethylene glycol (PEG) 6000, 660 μ M ADP, 10 mM dithiothreitol, 1 mM beryllium chloride, 5 mM sodium fluoride) was added to the protein (final concentration 6 mg/ml). The samples were dialysed for 3 days against outside buffer (50 mM Tris-HCl, pH 8.2, 200 mM sodium chloride, 20 mM magnesium sulphate, 0.02% (w/v) sodium azide, 1 mM EDTA, 0.004% (w/v) phenylmethylsulphonyl fluoride, 9% (w/v) PEG 6000, 660 μ M ADP, 5 mM dithiothreitol, 1 mM beryllium chloride, 5 mM sodium fluoride). Then, the outside buffer was replaced and the PEG 6000 concentration was increased to between 9.5 and 12.0% (in 0.25% steps). After a week, crystals began to appear and they were fully grown 2 weeks later. Typical maximum dimensions were 0.2–0.3 mm. In order to cryoprotect the crystals, the outside buffer was exchanged to one containing 13.75% PEG 6000 and 5% glycerol. Then, the glycerol concentration was increased stepwise to 10, 20 and 22% (30 min at each concentration). The crystals were harvested with a cryoloop (0.2–0.3 mm, Hampton Research, Laguna Niguel, CA), plunged into liquid nitrogen and stored at 100 K. ADP- F_1 crystals were grown at the final protein concentration of 5 mg/ml under the same conditions as the native F_1 crystals (Lutter *et al*, 1993) except that AMP-PNP was omitted from both the inside and outside buffers, and in the outside buffer the concentration of ADP was increased from 5 μ M as in the native F_1 crystallization conditions to 2 mM. The crystals were grown at a PEG 6000 concentration of 11.5%. They were cryoprotected with 25% glycerol (overnight soak at 10%, then 2 h each at 15, 20 and 25%). The crystals were frozen in a cryostream just before data collection.

Diffraction data for the BeF_3^- - F_1 crystal were collected to $2.2\text{ \AA}$ resolution using an ADSC Q4R CCD detector on beamline ID14-4 ($\lambda = 0.94\text{ \AA}$) at the ESRF (Grenoble, France). The ADP- F_1 diffraction data were collected using a Mar Research image plate detector on beamline ID2 ($\lambda = 0.91\text{ \AA}$) also at the ESRF. Both the BeF_3^- - F_1 and ADP- F_1 crystals belonged to the space group $P2_12_12_1$ with unit cell dimensions $a = 283.5\text{ \AA}$, $b = 107.4\text{ \AA}$, $c = 137.9\text{ \AA}$ and $a = 283.0\text{ \AA}$, $b = 107.6\text{ \AA}$, $c = 139.6\text{ \AA}$, respectively. There is one F_1 molecule in the crystallographic asymmetric unit. The diffraction data were processed with MOSFLM (Leslie, 1992) and with programs from the Collaborative Computational Project Number 4 (CCP4) suite (CCP4, 1994).

Structure solution and refinement

The structure of BeF_3^- - F_1 was solved by molecular replacement with AMoRe (Navaza, 1994). The starting model was the reference structure refined against data collected from a single cryocooled crystal (PDB accession code 1E1Q) (Braig *et al*, 2000) but with the γ -phosphate deleted from the nucleotide bound to the β_{TP} subunit. After rigid-body refinement with AMoRe, the R -factor and correlation coefficient were 30.2 and 78.8%, respectively, for all data from 20.0 – $4.0\text{ \AA}$ resolution. Further refinement was carried out using REFMAC5 (CCP4, 1994; Murshudov *et al*, 1997) alternating with manual rebuilding using O (Jones *et al*, 1991). The ADP- F_1 structure was solved by molecular replacement with AMoRe (Navaza, 1994) using the reference structure (PDB accession code 1BMF) as a starting model. The model was refined initially with X-PLOR (Brünger, 1996) and then with REFMAC5 (CCP4, 1994; Murshudov *et al*, 1997) alternating rounds of refinement with manual rebuilding using O (Jones *et al*, 1991).

For the calculations of the R_{free} value of the BeF_3^- - F_1 and ADP- F_1 structures, respectively 5 and 4.8% of the observed diffraction data were set aside and excluded from the refinement including the initial rigid-body refinement. The final model for the BeF_3^- - F_1 structure includes four glycerol molecules and 1019 water molecules, and that of the ADP- F_1 structure includes three glycerol molecules and 266 water molecules.

The stereochemistry was assessed with PROCHECK (Laskowski *et al*, 1993). For the BeF_3^- - F_1 structure, 91.0% of the residues were

assigned to the most favoured region of the Ramachandran plot, 8.6% to allowed regions and 0.3% to generously allowed regions. There are no residues in disallowed regions. For the ADP-F₁ structure, 90.0% of the residues are in the most favoured region, 9.6% in the allowed regions and 0.3% are in generously allowed regions.

Figures 1, 2, 3 and 5 were produced using PyMOL (De Lano, 2002).

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Coordinates

The structures reported in this paper have been deposited in the Protein Data Bank with accession codes 1W0J (BeF₃⁻-F₁) and 1W0K (ADP-F₁).

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