The structure of bovine F$_1$–ATPase in complex with its regulatory protein IF$_1$

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In mitochondria, the hydrolytic activity of ATP synthase is prevented by an inhibitor protein, IF$_1$. The active bovine protein (84 amino acids) is an α-helical dimer with monomers associated via an antiparallel α-helical coiled coil composed of residues 49–81. The N-terminal inhibitory sequences in the active dimer bind to two F$_1$–ATPases in the presence of ATP. In the crystal structure of the F$_1$–IF$_1$ complex at 2.8 Å resolution, residues 1–37 of IF$_1$ bind in the α$_{DP}$β$_{DP}$ interface of F$_1$–ATPase, and also contact the central γ subunit. The inhibitor opens the catalytic interface between the α$_{DP}$ and β$_{DP}$ subunits relative to previous structures. The presence of ATP in the catalytic site of the β$_{DP}$ subunit implies that the inhibited state represents a pre-hydrolysis step on the catalytic pathway of the enzyme.

ATP synthase (also called F$_1$F$_o$–ATPase) is a multisubunit, membrane-bound assembly central to biological energy conversion. It is composed of a globular F$_1$ catalytic domain (subunit composition α$_3$β$_3$γδε) and an F$_o$ domain, linked together by central and peripheral stalks. Energy from the transmembrane proton-motive force (PMF) generates the rotation of a ring of hydrophobic c subunits in the Fo domain against another from the transmembrane proton-motive force (PMF) generates the rotation of the central stalk, in 120° steps, takes each catalytic site through three states, and three ATP molecules are synthesized.

When a cell is deprived of oxygen, for example by ischemia, the PMF across the inner mitochondrial membrane collapses and the ATP synthase switches from synthesis to hydrolysis. This hydrolytic activity is prevented by a natural inhibitor protein, IF$_1$ (refs. 10,11). Its action depends on the presence of ATP, and in the inhibited complex an ATP molecule becomes trapped in the enzyme. Residues 14–47 of the 84-residue bovine IF$_1$ have been defined by deletion analysis as the minimal inhibitory sequence. This region is 87% identical in the bovine and human proteins. In vitro, the active form of bovine IF$_1$ is a dimer associated by formation of an antiparallel α-helical coiled coil between the C-terminal regions of monomers. The dimeric IF$_1$ binds simultaneously to two F$_1$ domains via its inhibitory regions. The dimeric state of the inhibitor is favored by pH values <6.5 (ref. 16). Under ischemic conditions in vivo, glycolysis becomes the only source of cellular ATP. The pH values of the cytosol and the mitochondrial matrix decrease, promoting inhibition of ATP hydrolysis by IF$_1$ to preserve ATP. Upon re-energization of mitochondria, the pH increases, dimeric IF$_1$ dissociates from F$_1$ and, in vitro, it forms inactive tetramers and higher oligomers via coiled-coil interactions in the N-terminal inhibitory regions.

This paper describes the structure of the inhibited complex between F$_1$–ATPase and IF$_1$, formed in the presence of ATP. The structure confirms that the N-terminal region of the dimeric inhibitor is bound to F$_1$. It exerts its effect by binding selectively to the interface between the α$_{DP}$ and β$_{DP}$ subunits of F$_1$, thereby blocking rotary catalysis. In the inhibited state, the enzyme is locked in a conformation in which ATP (or AMP-PNP) is entrapped in subunit β$_{DP}$. The structure of the complex seems to represent a pre-hydrolysis state in the catalytic cycle.

RESULTS

Disorder in the crystals of the F$_1$–IF$_1$ complex

The structure of the bovine F$_1$–IF$_1$ complex was solved by molecular replacement and refined against X-ray data to a resolution of 2.8 Å.
In regions that deviate significantly from three-fold pseudo symmetry, such as parts of the nucleotide binding domain and the C-terminal domain of the β subunit, the density is weaker, but still generally unambiguous. The quality of the electron density for the inhibitor and the βDP catalytic site is apparent in Figure 2.

The disorder observed in the crystals results from the dimerization of F1-ATPase by the inhibitor protein15. The distance between two F1-ATPase complexes related by a unit cell translation along the crystallographic b-axis is such that they can be cross-linked by a dimeric inhibitor molecule, but only if the second F1-ATPase complex is rotated by 120° relative to the first. If cross-linked F1-ATPase dimers were arranged perfectly in the crystal, this arrangement would give rise to a doubling in length of the b-axis. In reality, the packing of F1-ATPase dimers in the lattice is imperfect, resulting in a statistically disordered structure that gives rise to the continuous diffraction observed in the diffraction images.

Overall protein structure

The two F1-ATPase complexes adopt similar but not identical conformations. The overall r.m.s. deviation in Ca coordinates is 0.82 Å. Significant differences are restricted to the αE and αDP subunits, which show small rigid-body rotations. Because these subunits are involved in intermolecular contacts, it is likely that the rotations are the result of crystal packing forces.

The structure of dimeric F1–IF1 (Fig. 1a,b) contains 5,980 amino acids. The region defined as complex A consists of residues αE24–510, αTP24–401 and αTP410–510, αDP19–404 and αDP409–510, β9–474, βDP9–47, γ1–30, γ77–88 and γ221–272 and residues 4–40 of IF1. Complex B includes the same residues as complex A, except that residues αDP9–474 are absent and residues 41–47 of IF1 were resolved. The δ and ε subunits could not be modeled in either complex. The ordered region of the inhibitor protein adopts a helix-turn-helix structure, with at least residues 4–37 involved directly in contact with F1. There was no interpretable density for the C-terminal regions of IF1 (residues 48–84), which form an antiparallel coiled coil in isolated dimeric IF1. In the dimeric F1–IF1 complex (Fig. 1c, green) superimposed on the structure of dimeric IF1 alone14 (blue and red). The r.m.s. deviations in Ca coordinates in the overlapping segments (residues 19–40 in complex A and residues 20–47 in complex B) are 0.99 Å and 0.92 Å, respectively. In the F1–IF1 complex, residues 4–18 of IF1 were resolved, whereas in the structure of the IF1 dimer alone they were disordered. The distance between the two inhibitory regions in the isolated IF1 dimer in the fully extended form is 62 Å (Fig. 1c), whereas in the crystal structure of the F1–IF1 complex, it is reduced to 31 Å. Crystals of the F1–IF1 complex

Figure 1 The dimeric bovine F1-ATPase–IF1 complex. (a) View of the dimeric complex along the central axes of F1 particles viewed from the protruding region of the central stalk toward the catalytic sites. Complexes A and B (left and right, respectively) are shown in ribbon representation. The α, β and γ subunits are red, yellow and blue, respectively, and residues 4–40 and 4–47 of the two IF1 molecules are green. The green arrows indicate that the N-terminal regions of IF1 are linked via an antiparallel coiled coil in isolated dimeric IF1. IF1 in the F1–IF1 complex, residues 4–18 of IF1 were resolved, whereas in the isolated IF1 dimer they were disordered. The distance between the two inhibitory regions in the isolated IF1 dimer is 62 Å (Fig. 1c), whereas in the crystal structure of the F1–IF1 complex, it is reduced to 31 Å. Crystals of the F1–IF1 complex

(Table 1). The crystals of the complex are statistically disordered so that, on average, each site in the crystal is occupied by two complexes of half occupancy. These two complexes, A and B, are in different orientations and are related by a rotation of 120° around the axis of pseudo symmetry of the (αβ)3 assembly. This arrangement superimposes the βDP, αDP, βTP, αTP, βE and αE subunits of F1-ATPase complex A onto the βTP, αTP, βE, αE, βDP and αDP subunits, respectively, of complex B (Fig. 1). Because a significant proportion of F1-ATPase follows three-fold pseudo symmetry, the resulting electron density for the superimposed structures is of reasonable quality, much like that obtained for a fully ordered structure at a resolution of 3.5 Å.
were redissolved and examined by SDS-PAGE. The IF1 was intact, and there was no evidence of its degradation in the crystals. Therefore, the unresolved region must have a greater curvature than in the crystals of the IF1 dimer and also be disordered. In the crystals of IF1 alone, two types of dimers were observed with markedly different curvatures in this region, suggesting that the coiled-coil region is flexible.

Interactions between F1-ATPase and IF1
Bovine IF1 binds to F1 at the interface between the \( \alpha_{\text{DP}} \) and \( \beta_{\text{DP}} \) subunits, and it also makes contacts with the \( \gamma \) subunit and with residues \( \beta_{\text{TP}} 386 \) and \( \alpha_{\text{E}} 355 \) (Fig. 3). The extensive binding surface has an area of 1,500 \( \text{Å}^2 \) and is a mixture of 60\% hydrophobic and 40\% hydrophilic interactions. Residues \( \alpha_{\text{DP}} 405-408 \) may also contribute to binding IF1, but the electron density did not allow this region to be modeled. Earlier, the interactions between yeast F1Fo–ATPase and its inhibitor protein were shown by cross-linking and peptide mapping experiments to involve residues at an interface between \( \alpha \) and \( \beta \) subunits. On the basis of similar experiments with the bovine F1-ATPase, it was proposed that a region within residues \( \beta_{\text{DP}} 394-459 \) interacts with IF1. Both observations are consistent with the X-ray structure.

By deletion analysis, residues 14–47 of bovine IF1 were defined as the smallest segment to retain full inhibitory activity, except that, unlike the situation with intact IF1, the inhibition was lost with time, presumably because the peptide was dissociating from the enzyme.

Conformational changes in F1-ATPase
The reference state structure of F1-ATPase was determined with crystals grown in the presence of AMP-PNP and ADP, whereas the present structure was determined with crystals of the F1–IF1 complex preformed in the presence of ATP, and then grown in the presence of AMP-PNP. In the structure of the complex, the catalytic sites in subunits \( \beta_{\text{TP}} \) and \( \beta_{\text{DP}} \) are occupied by ATP or AMP-PNP (which cannot be distinguished on the basis of the electron density), whereas subunit \( \beta_{\text{E}} \) is unoccupied. Either ATP or AMP-PNP is bound to each of the three \( \alpha \) subunits. The presence of ATP (or AMP-PNP) rather than ADP in the \( \beta_{\text{DP}} \) subunit is very clear (Fig. 2b) and is presumably a consequence of IF1 binding, because AMP-PNP was present at high concentration (250 \( \mu \text{M} \)) in the crystallization of the reference state structure. The conformational changes in F1 due to inhibitor binding were analyzed by comparison of structures of the reference state of F1 with the F1–IF1 complex. For this purpose, a common point of reference was defined as the six N-terminal domains of the \( (\alpha\beta)_{6} \) subassembly, which form a stable crown to the structure. After superimposing all six corresponding N-terminal domains, changes in quaternary and tertiary structure were demonstrated by examining the r.m.s. deviations in C\( \alpha \) positions for each domain. As expected, the N-terminal domains are very similar (r.m.s. deviations up to 0.5 \( \text{Å} \), and the largest r.m.s. deviations are in the C-terminal domains of the \( \alpha_{\text{DP}} \) and \( \beta_{\text{DP}} \) subunits (4.5 \( \text{Å} \) and 3.0 \( \text{Å} \), respectively). They arise from rotations of the coiled-coil structure.
8.7° and 5.8°, respectively (Fig. 4) about axes that lie close to the peptides linking the nucleotide binding and C-terminal domains in the respective subunits, in an orientation almost orthogonal to the axis of the (αβ)₆ assembly. In consequence, the interface between the two subunits is sufficiently open to accommodate the binding of IF₁ (Fig. 5), resulting in an arrangement closely resembling the α₂β₃-β₁P interface in the reference state structure. In the other subunits, the r.m.s. deviations in this domain are comparatively small (~1.5 Å), with the exception of the α₅ subunit (r.m.s. deviation 2.1 Å), which rotates by 3.4°, probably as a consequence of the changes in the adjacent subunits (β₁P and β₁). Residue α₂G355 is also involved in contacts with residues 4 and 5 of IF₁ (Fig. 3), and this interaction could explain the higher r.m.s. deviation found in that subunit (note that although residues 1–3 in IF₁ are not present in the model, they are probably involved in additional contacts with subunit α₅). The rotation of the C-terminal domain of the α₁P subunit results in a slight opening of the nucleotide binding pocket, which is probably responsible for the disorder observed in the adenine ring of the nucleotide bound to that subunit.

**DISCUSSION**

The binding of IF₁ to F₁–ATPase requires an active enzyme and the presence of ATP²². In the inhibited complex, the ratio of ATP to ADP is higher than in the active enzyme²³,²⁴, and ATP appears to be trapped in a nucleotide binding site. It has been proposed that IF₁ binds initially to F₁–ATPase in an unproductive mode and upon hydrolysis of Mg-ATP, this changes to a productive inhibitory complex, preventing product release¹¹,²⁵. In the structure of the complex, the inhibited state formed in the presence of ATP and crystallized in the presence of AMP-PNP traps ATP or its analog in the β₁P active site, which is occupied by ADP in the reference state structure. Therefore, the structure seems to represent a pre-hydrolysis state in the catalytic cycle. A number of studies have indicated that ultimately ADP and IF₁ are associated with a final ‘dead-end’ product, not in the catalytic cycle of F₁–ATPase²⁴,²⁵. It remains possible that if the crystals of the preformed complex were grown in the presence of ATP (and not a nonhydrolyzable analog), then ATP subsequently becomes hydrolyzed and a ‘dead-end’ complex with ADP in the inhibitory site might be observed.

In principle, inhibition of hydrolysis could be achieved either by preventing the conformational changes in the α and β subunits that are required for rotary catalysis (and are responsible for generating the rotation of the γ subunit), or alternatively by directly blocking the rotation of the γ subunit itself. The extent of the interaction between IF₁ and the α₁P and β₁P subunits compared with the rather limited interactions with the γ subunit strongly suggests that the former have the dominant function in inhibition. A possible scheme for inhibition of hydrolysis by IF₁ based on the binding change mechanism²⁶ is presented (Fig. 6). The ground state (Fig. 6, step 1) binds ATP and IF₁ at an open site (O), resulting in the committed hydrolysis of ATP at an adjacent tight site, T' (Fig. 6, step 2). It is probable that ATP binds first, inducing a conformational change (O → L'), which creates the binding site for IF₁, ADP and phosphate are released, and a cyclic interconversion of states occurs (Fig. 6, step 3). A second molecule of ATP binds to the newly formed open site (Fig. 6, step 4), resulting in another round of hydrolysis and product release (Fig. 6, step 5). The presence of IF₁ bound to the L site, L (Fig. 6, step 4) prevents binding of a second inhibitor molecule. Also, the inhibitor blocks the conversion of the L site to a catalytically competent tight site, even if ATP binds to the vacant open site (Fig. 6, step 6). Further catalysis, which depends on a cyclic interconversion of states, cannot occur. The crystal structure corresponds to the state shown in step 5 in this scheme (Fig. 6). An alternative scheme is possible if IF₁ binds to an L site rather than a closing (O → L') site. If IF₁ binds to site L in step 2 (Fig. 6), then after hydrolysis and product release from the T' site, it will block the conversion of the L site to a T site, effectively locking the complex in the state shown in step 3 (Fig. 6). Binding of IF₁ prevents the closure of the α₁P–β₁P catalytic interface, so that it resembles the α₁P–β₁P interface of the reference state structure. By analogy with that structure, this may prevent the close approach of the guanidino group of α₂₃5,Arg373 to the β- and
γ-phosphate groups of the nucleotide bound to the βDP subunit, although the electron density for this side chain is too weak to confirm this fully. This interpretation is consistent with the presence of ATP bound to the βDP subunit, because αArg373 is known to be essential for catalysis3,27.

Bovine IF1 is an equally effective inhibitor of both the bovine and yeast enzymes28. Of the 32 residues in the α, β and γ subunits of bovine F1-ATPase that are involved in binding to IF1, 25 of them are identical in yeast F1-ATPase. A total of 21 of the 32 IF1 binding residues are also identical in the enzyme from Escherichia coli, but neither the bovine nor the yeast IF1 inhibits the bacterial enzyme (E.C. and J.E.W., unpublished observations). Bacteria have no IF1 homologs to regulate the activity of their ATP synthases, and there is no physiological requirement to inhibit the ATP hydrolytic activity of ATP synthase under anaerobic conditions. Instead, as the PMF diminishes during the onset of anaerobiosis, ATP made by substrate-level phosphorylation is hydrolyzed and provides energy to generate the PMF by pumping protons out of the bacterial cytoplasm.

Nonetheless, the ε subunit of E. coli F1-ATPase does inhibit ATP hydrolysis in vitro29, and a electron density map of the bacterial enzyme30 at a resolution of 4.4 Å has been interpreted with the aid of a structure of a complex of the ε subunit and residues 11–258 of the γ subunit31 as showing a direct interaction between the C-terminal α-helices of the ε subunit and the αβγ hexamer32, supporting crosslinking experiments33,34. Such an interaction might impede the inhibitory effect of IF1 on the bacterial enzyme. These interpretations of the structure of the E. coli F1-ATPase have led to the suggestion that by interacting with the α and β subunits via its C-terminal helix, the ε subunit might act in the intact ATP synthase as a ratchet to restrict or prevent the conformational changes required for ATP hydrolysis, with little effect on ATP synthesis33. There is no evidence that the mitochondrial δ subunit (the equivalent of the bacterial ε subunit) forms similar interactions3,8, and, given the extensive interactions of this subunit with the ring of c subunits in the membrane domain7, augmented by the bovine ε subunit8, it is unlikely to do so. It has been suggested that in the mitochondrial enzyme IF1 fulfils a similar role to that proposed for the bacterial ε subunit33. However, the mode of binding of IF1 to bovine F1-ATPase does not support the notion that it acts as a ratchet that could inhibit rotation in one direction (corresponding to hydrolysis) in preference to the opposite direction (corresponding to synthesis). Moreover, the interface (1,500 Å2) between F1 and IF1 is

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Figure 5 The catalytic interface between subunits αDP and βDP in the reference state structure of F1−ATPase and in the F1−IF1 complex. (a) Space-filling representations of the αDP and βDP subunits in the reference state structure. (b) Space-filling representations of the αDP and βDP subunits in the F1−IF1 complex with IF1 removed. (c) Space-filling representations of the αDP and βDP subunits in the F1−IF1 complex with IF1 present. (d−f) Ribbon representations of the C-terminal domains of α and β subunits and part of the central coiled coil made from residues γ3–30 and γ221–272 viewed from underneath (90° rotation) relative to the views in a−c. For details of colors, see the legend to Figure 1.

Figure 6 The inhibition of the ATP hydrolytic activity of ATP synthase by IF1. The scheme is based on the binding change mechanism of ATP hydrolysis26. See text for further details.
Structure determination and refinement. The diffraction images were indexed on an orthorhombic cell (space group $P2_12_12_1$) with unit cell dimensions $a = 272.3 \, \text{Å}$, $b = 107.2 \, \text{Å}$, $c = 152.4 \, \text{Å}$, which are similar to those of native $F_1$-ATPase ($a = 280.8 \, \text{Å}$, $b = 107.4 \, \text{Å}$, $c = 139.6 \, \text{Å}$). The diffraction data were integrated with MOSFLM and processed further with programs from the CCP4 suite. A close examination of the diffraction images revealed the presence of semi-continuous diffraction, lying between layer lines predicted for the orthorhombic unit cell, suggesting the presence of statistical disorder in the crystals.

The structure was solved by molecular replacement with AmoRe using the frozen native structure (PDB entry 1E1Q) as a starting model. After molecular replacement, the final $R$-factor and correlation coefficient were 43.7% and 58.0%, respectively, for data between resolutions 20 Å and 4 Å. Rigid-body refinement and restrained refinement against a maximum-likelihood target was carried out with CNS. Electron density maps calculated at this stage of the refinement ($R = 32.9\%$, $R_{free} = 37.1\%$) were difficult to interpret. The electron density for the $\beta_{\text{TP}}$ and $\beta_{\text{TP}}$ and the three $\epsilon$ subunits was reasonably well defined, with clear side chain densities, but the density for the $\beta_{\text{g}}$ and $\gamma$ subunits was poor, with frequent breaks. Additional uninterpreted density was also present. A close examination of the electron density for the C-terminal domain of the $\beta_{\text{g}}$ subunit revealed additional weak density, suggesting an alternative conformation for this domain. Fitting a Cm model to this density resulted in a conformation very similar to that of the $\beta_{\text{DP}}$ or $\beta_{\text{TP}}$. This suggested that the electron density represents an average of two structures of $F_1$-ATPase, related by a rotation of 120° such that, to a first approximation, the $\epsilon$ subunits of one $F_1$-ATPase molecule would superimpose on different $\alpha$ subunits of the second and similarly for the $\beta$ subunits. This interpretation is consistent with the poor density for the $\beta_{\text{g}}$ and $\gamma$ subunits, which deviate most from the pseudo-threefold symmetry of the molecule, and also the diffuse scattering on the diffraction images.

Two models of the disorder were considered, corresponding to rotations of +120° and −120°, respectively. After rigid-body refinement using a maximum-likelihood target, these models gave $R_{free}$ values of 36.7% and 33.3%, respectively, for data to a resolution of 2.8 Å, compared with 38.2% for a single $F_1$-ATPase. This provided strong support for the proposed disordered model with a rotation of −120°. All subsequent refinement was carried out with two molecules per asymmetric unit. Each molecule was assigned an occupancy of 0.5, and the second complete molecule was treated as an alternative conformation of the first in CNS. The presence of two molecules in the asymmetric unit doubles the number of refined parameters, resulting in a poor ratio of data to parameters (0.6:1). To help stabilize the refinement, non-crystallographic symmetry (NCS) restraints were applied to make the two molecules as similar as possible, using the $R_{free}$ as the criterion to set the NCS weighting. Further refinement and modeling of Mg-ATP in the $\beta_{\text{DP}}$ and $\beta_{\text{TP}}$ residues 4–40 at the interface between the $\alpha_{\text{DP}}$ and $\beta_{\text{DP}}$ subunits, reduced the $R_{free}$ and working $R$ values to 27.7% and 23.0%, respectively. Averaging the density of the two copies of the inhibitor made it much easier to fit the sequence. No solvent molecules were included in the model. The final $R_{free}$ value is typical for a structure determined at this resolution, suggesting that the refined model is reliable. However, it is certainly the case that the accuracy of the model, particularly the side chains, will be somewhat lower than a typical structure determined at a resolution of 2.8 Å. The stereochemistry was assessed with PROCHECK, which assigned 83.8% of the residues to the most favored region of the Ramachandran plot, 15.4% to additional allowed regions and 0.8% to generously allowed regions. Structure comparisons and the determination of rotation axis parameters were carried out with the CCP4 program SUPERPOSE. Interactions between $F_1$-ATPase and IF1 were analyzed using the program AREAIMOL, from the CCP4 suite. Figures 1–5 were generated with BobScript.

Coordinates. Coordinates and structure factors have been deposited in the Protein Data Bank (accession code 1OHH).

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The ATP synthase: a splendid molecular machine.


