cient to drive the actin filament to one end of the myosin filament where it becomes temporarily arrested by rigor heads. Motility was much more reproducible when filaments were loaded at high density so that they overlapped or aggregated side-to-side. On the few occasions actin sliding was observed on isolated native-like filaments, there was no detectable burst of Cy3-nucleotide displacement, indicating that most of the labelled heads did not participate in the sliding activity. This is perhaps not surprising given the poor signal-to-noise ratio and that at least two-thirds of the heads in the myosin filament point away from the actin filament. Improvements using dual-view imaging [9] should allow a better characterization of this reaction by separating the signals from actin and myosin into two channels.

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The structure of bovine mitochondrial F$_1$-ATPase: an example of rotary catalysis

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Introduction

F$_1$F$_0$ ATP synthases are found in the membranes of bacteria, the thylakoid membranes of chloroplasts and the inner membranes of mitochondria. They utilize energy derived from proton movement down an electrochemical gradient to synthesize ATP from ADP and inorganic phosphate. In bacteria the enzyme is reversible, and under anaerobic conditions it can use ATP hydrolysis to generate a transmembrane proton gradient. The ATP synthase is a complex oligomeric assembly, composed of 16 different subunits in the case of the mitochondrial enzyme, and eight or nine for the simpler bacterial homologues, with some subunits present in multiple copies. Morphologically the enzyme has three components; a membrane-bound sector, F$_0$, that contains the proton channel, which is linked by a narrow stalk to an approximately spherical assembly, F$_1$, that contains the catalytic sites.

The F$_1$ component can be separated from the membrane, and isolated F$_1$ is active as an ATPase, hence the designation F$_1$-ATPase. F$_1$-ATPase from all characterized sources consists of five different subunits, with a stoichiometry $\alpha_3\beta_2\gamma_3\delta_1\epsilon_1$. The composition and amino acid sequences of the bovine mitochondrial F$_1$-ATPase were determined by Walker and co-workers [1,2].

Structure determination

Crystals of F$_1$-ATPase suitable for structure determination were obtained only after many years of effort [3]. An essential step in obtaining high quality crystals was to ensure a homogeneous nucleotide content. This was achieved by first stripping the enzyme of all bound nucleotides, and then adding back the non-hydrolysable ATP analogue AMPPNP. Even then, the crystals showed a significant degree of non-isomorphism,
The overall structure of the enzyme \[5\] is shown basis of a single heavy atom derivative (methyl mercury), albeit at two different concentrations. The use of novel density modification techniques \[4\] resulted in a final electron density map of excellent quality, which was readily interpreted in terms of an atomic model.

**Structure description**

The overall structure of the enzyme \[5\] is shown schematically in Figure 1(a). The three \(\alpha\) and three \(\beta\)-subunits associate like the segments of an orange to form a roughly spherical assembly about 100 Å in diameter. The \(\gamma\)-subunit forms a left-handed coiled coil which runs approximately along the axis of the \(\alpha/\beta_1\) complex, protruding from it by about 30 Å. There was no interpretable electron density for approximately one half of the \(\gamma\)-subunit or the entire \(\delta\)- and \(\epsilon\)-subunits, even though the latter were known to be present in the crystal. These subunits, and the remainder of the \(\gamma\)-subunit, must therefore be disordered in the crystal lattice, although it cannot be ruled out that the \(\delta\)- and \(\epsilon\)-subunits were present in sub-stoichiometric amounts. The overall shape and dimensions of the structure are very similar to those determined by cryo electron-microscopy of the enzyme from *Escherichia coli* \[6\]. In particular, the protruding \(\gamma\)-subunit probably corresponds to part of the central stalk that links \(F_1\) to the membrane-bound component \(F_0\).

Both \(\alpha\)- and \(\beta\)-subunits are known to contain nucleotide binding sites, but chemical modification experiments using nucleotide analogues have identified the \(\beta\)-subunit as containing the catalytic nucleotide-binding site (reviewed in \[7\]). In the crystal structure, all the \(\alpha\)-subunits bind AMPPNP. However, one \(\beta\)-subunit (\(\beta_{1\tau}\)) binds AMPPNP, the second (\(\beta_{2\nu}\)) binds ADP, and the third (\(\beta_2\)) has no nucleotide bound. The nucleotide binding sites are located at the interfaces of the \(\alpha\)- and \(\beta\)-subunits; the \(\alpha\)-subunit that contributes to the \(\beta_{1\tau}\) nucleotide-binding site is therefore denoted \(\alpha_{1\tau}\), and the other two \(\alpha\)-subunits are similarly labelled \(\alpha_{2\nu}\) and \(\alpha_2\) (see Figure 1). A cross-section through the complex (Figure 1b), showing the \(\alpha_{1\tau}\), \(\beta_{2\nu}\) and \(\gamma\)-subunits, allows the fold of the individual subunits to be seen more clearly. The fold of the \(\alpha\)- and \(\beta\)-subunits is very similar (they have approximately 20% sequence identity). The N-terminal domain is a six-stranded \(\beta\)-barrel. This is followed by a central nucleotide-binding domain, containing a nine-stranded predominantly parallel \(\beta\)-sheet with nine associated \(\alpha\)-helices, live on one side of the sheet and four on the other. The final C-terminal domain is an \(\alpha\)-helical bundle, made up of seven helices in the \(\alpha\)-subunit and six in the slightly smaller \(\beta\)-subunit.

**Conformational asymmetry of the \(\alpha\)- and \(\beta\)-subunits**

The three \(\alpha\)-subunits adopt similar conformations, although in the \(\alpha_{1\tau}\)-subunit there is a small rigid body rotation of the N-terminal domain relative to the other two domains. The two \(\beta\)-subunits \(\beta_{1\tau}\) and \(\beta_{2\nu}\) also adopt similar conformations, but the nucleotide-free \(\beta_2\)-subunit shows a dramatic conformational change, which is most simply described as a rigid body rotation of the lower part of the nucleotide binding domain and the C-terminal domain. This rotation, almost 30° in magnitude, moves the C-terminal domain outwards from the axis of the \(\alpha/\beta\), assembly. It is accompanied by a significant disruption in the \(\beta\)-sheet of the nucleotide binding domain (illustrated by an arrow in Figure 1d) with the loss of several hydrogen bonds. The novel conformation of the \(\beta_2\)-subunit is clearly associated with the asymmetric location of the \(\gamma\)-subunit relative to the axis of the \(\alpha/\beta\), complex. The coiled-coil structure of the \(\gamma\)-subunit does not lie along this axis, but is displaced to one side towards the \(\beta_2\)-subunit. This displacement is particularly marked in the lower region of the coiled-coil, and prevents the \(\beta_2\)-subunit adopting the conformation observed in the \(\beta_{1\tau}\) and \(\beta_{2\nu}\)-subunits.

**The active site**

The nucleotide binding site in the \(\beta_{1\tau}\)-subunit is shown schematically in Figure 2. There is a water molecule (labelled HO1) positioned 4 Å from the \(\gamma\)-phosphate group which hydrogen bonds to the carboxylate group of Glu-188. This residue is appropriately positioned to activate the water molecule for an in-line nucleophilic attack on the terminal phosphate in the hydrolysis reaction. Lys-162, Arg-189 and \(\alpha\)-Arg-373 (from the adjacent \(\alpha_{1\tau}\)-subunit) may stabilize the negative
charge that develops in a penta-coordinated transition state. All four residues have been implicated as playing an important role in catalysis by site-directed mutagenesis of the *E. coli* and *Bacillus* PS3 enzymes [8–11].

**The binding change mechanism of catalysis**

Both F₁-ATPase and ATP synthase display negative co-operativity in binding substrates but positive co-operativity in catalysis. Boyer and
colleagues have shown that, in the case of ATP synthase, the release of ATP (the product) is greatly enhanced by the binding of ADP+Pi (the substrates) to an adjacent catalytic site (reviewed in [12]). Similarly, the release of ADP+Pi is promoted by ATP binding to an adjacent catalytic site in F₁-ATPase. To account for these observations and other biochemical evidence, Boyer proposed that the three catalytic sites alternate sequentially between three different states — open, loose and tight — which have differing affinities for nucleotides (Figure 3). Energy from the proton gradient is used to convert a tight site into an open site, with the release of ATP. Simultaneously, the loose site, with bound ADP+Pi, is converted into a tight site, leading to ATP synthesis, while the open site, which has low affinity for nucleotides, is converted into a loose site ready to bind the substrates. This model is known as the binding change mechanism for catalysis [12,13].

The crystal structure provides support for Boyer’s hypothesis. The nature of the three catalytic sites is indeed rather different, as demonstrated by the buried surface areas at the catalytic interfaces. The \( \alpha_\gamma/\beta_\gamma \) interface has a buried surface area of 3030 Å², the \( \alpha_\gamma/\beta_\delta \) interface 2200 Å² and the \( \alpha_\delta/\beta_\delta \) interface 1760 Å². Thus the catalytic site on the \( \beta_\gamma \)-subunit would represent the open conformation, with low affinity for nucleotides, \( \beta_\delta \) corresponds to the loose conformation and \( \beta_\delta \) to the tight conformation. At first sight, the binding of ATP to the tight site appears to be inconsistent with Boyer’s scheme. However, there is a well-characterized Mg–ADP–inhibited form of the enzyme which has ADP and Mg, but no phosphate, bound to the tight catalytic site, and the crystal structure may well represent this inhibited state.

**Rotational catalysis**

The stoichiometry of \( F_1 \) led Boyer to suggest that the conformational changes required for interconversion between the three types of catalytic site might be achieved by rotation of the three catalytic \( \beta \)-subunits relative to the single copy subunits \( \gamma, \delta \), and \( \epsilon \). Cross-linking studies provided some support for this suggestion. It was shown that cross-linking the \( \gamma-\) and \( \alpha-/\beta-\)subunits inhibited \( F_1 \)-ATPase activity, but on reducing the S–S bond in the middle of the cross-linker, thereby breaking the link, activity was restored [14].

A number of features of the crystal structure suggest that the \( \gamma \)-subunit can indeed rotate relative to the \( \alpha-/\beta- \)complex. The first of these is that the interaction between the C-terminal region of the \( \gamma \)-subunit and the \( \alpha-\) and \( \beta-\)subunits (uppermost in Figure 1) has the characteristics of a molecular bearing. Six loops, one from each \( \alpha- \) and \( \beta-\)subunit (Figure 1), form a circular hydrophobic collar with a diameter of 15 Å and a depth of 17 Å. This ‘collar’ perfectly accommodates the final residues (261–271) of the C-terminal helix of the \( \gamma \)-subunit. These residues all have small hydrophobic side-chains, so that the whole structure resembles a shaft (formed by the \( \gamma \)-subunit) passing through a bearing (formed by the six loops). The second feature of interest is the presence of a large, solvent-filled cavity in the centre of the \( \alpha_\gamma/\beta_\gamma \) complex (Figure 1), which is traversed by the \( \gamma \)-subunit. As a result there are very few specific interactions between the central segment of the \( \gamma \)-subunit and the \( \alpha-\) and \( \beta-\)subunits that would impede their relative rotation. Finally, the interactions between the lower segment of the \( \gamma \)-sub-
The binding change mechanism of catalysis


Inhibitor studies

The structures of a number of inhibited forms of F$_1$-ATPase provide further support for rotary catalysis. The efrapeptins are a family of peptide antibiotics produced by the fungus Tolypocladium niveum, and are potent inhibitors of F$_1$-ATPase. The structure of the F$_1$-ATPase–enfrapeptin complex [15] shows that the antibiotic binds to the 'open' nucleotide-free β-subunit ($f_\beta$) in a manner that would prevent this subunit adopting the conformation of the β-subunits which bind nucleotide ($f_{\beta\gamma}$ and $f_{\beta\delta}$). This provides an attractive mechanism for inhibition, as the binding change mechanism requires that during catalysis each subunit sequentially adopts a different conformation (Figure 3). By preventing the conversion of the $f_\beta$-subunit to a conformation with a high affinity for nucleotide, efrapeptin effectively locks the enzyme in an inactive state.

Essentially the same conclusions can be drawn from the structures of F$_1$-ATPase complexed with aurovertin [16] (an antibiotic produced by the fungus Calcarisporium arbuscula) and 4-chloro-7-nitrobenzofurazan [17]. In both cases the inhibitors bind at the interface between β- and α-subunits in a manner that would block the interconversion of the catalytic sites that is required by the binding change mechanism.

Additional evidence for rotation

Subsequent to the publication of the structure, further evidence has been presented that supports the model of rotational catalysis. This includes additional cross-linking studies [18], an analysis based on polarized absorption relaxation measurements [19,20], and most recently the direct observation of rotation [21]. In these last experiments, F$_1$-ATPase was tethered to a Ni-coated glass substrate via a His-tag at the N-terminus of the β-subunits so that the complex was oriented 'upside-down' relative to the orientation shown in Figure 1. Fluorescently labelled actin filaments were then coupled to the γ-subunit using a biotin/streptavidin linker. When the coated slide was immersed in a microscope flow cell containing ATP, the actin filaments were seen to rotate at about 4 Hz. This rotation is slower than expected from the turnover rate of the free enzyme in solution, but the viscous drag of the actin filaments could easily account for the discrepancy.

Summary

There is now compelling evidence in support of a rotary catalytic mechanism in F$_1$-ATPase, and, by extension, in the intact ATP synthase. Although models have been proposed to explain how protein translocation in F$_0$ results in rotation of the γ-subunit relative to the $z_\gamma$/$z_\beta$ assembly in F$_1$ [22], these are still speculative. It seems likely that a satisfactory explanation of this mechanism will ultimately depend on structural information on the intact ATP synthase.

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Visualizing enzyme intermediates using fast diffraction and reaction trapping methods: isocitrate dehydrogenase

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Introduction

In order to collect complete diffraction data sets from crystals of macromolecules, the standard technique employed has been monochromatic oscillation photography, with the crystal irradiated using a single incident wavelength and simultaneously oscillated through a large rotational range in order to satisfy Bragg's law for the majority of reflections. Such data sets may be collected in as little as several minutes at a synchrotron with strongly diffracting crystals, and it was shown in the late 1980s that such data can be used to observe the accumulation of intermediate states with extremely long chemical lifetimes [1,2].

Time-resolved crystallography uses a group of related techniques for reaction trapping and data collection. The experiments are designed to accumulate a specific catalytic intermediate throughout the crystal for a short period of time, during which diffraction data is collected [3-6]. In this context, three parameters can be defined: the method used to isolate the intermediate, the longest exposure time allowed for data collection (which is limited either by the lifetime of the intermediate or by the lifetime of the crystal during the experiment), and the method of data collection. At one end of the spectrum is a single-turnover experiment, triggered by a photolytic event, in which the lifetime of the rate-limited species is short, necessitating rapid data collection. At the other extreme is a reaction intermediate trapped by chemical and physical techniques, usually visualized by the use of a slower method of data collection.

The techniques used to extend the lifetime of related techniques for reaction trapping and data collection. The experiments are designed to accumulate a specific catalytic intermediate throughout the crystal for a short period of time, during which diffraction data is collected [3-6]....

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