The structure of bovine F1-ATPase complexed with the antibiotic inhibitor aurovertin B

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ABSTRACT In the structure of bovine mitochondrial F1-ATPase that was previously determined with crystals grown in the presence of adenylyl-imidodiphosphate (AMP–PNP) and ADP, the three catalytic β-subunits have different conformations and nucleotide occupancies. Adenylyl-imidodiphosphate is bound to one β-subunit (βTP), ADP is bound to the second (βDP), and no nucleotide is bound to the third (βE). Here we show that the uncompetitive inhibitor aurovertin B binds to bovine F1 at two equivalent sites in βTP and βE, in a cleft between the nucleotide binding and C-terminal domains. In βDP, the aurovertin B pocket is incomplete and is inaccessible to the inhibitor. The aurovertin B bound to βTP interacts with α-Glu399 in the adjacent αE subunit, whereas the aurovertin B bound to βE is too distant from αE to make an equivalent interaction. Both sites encompass βArg412, which was shown by mutational studies to be involved in binding aurovertin. Except for minor changes around the aurovertin pockets, the structure of bovine F1-ATPase is the same as determined previously. Aurovertin B appears to act by preventing closure of the catalytic interfaces, which is essential for a catalytic mechanism involving cyclic interconversion of catalytic sites.

The aurovertins are a family of related antibiotics from the fungus Calcarisporium arbuscula (1). They inhibit oxidative phosphorylation in mitochondria (2) and in many bacterial species (3). Aurovertins B (Fig. 1) and D have identical biological properties and are more potent than aurovertin A. Citreoviridin (4, 5) (from Penicillium citreoviride and Aspergillus terreus) and asteltoxin (6, 7) (produced by Aspergillus stellatus) are metabolites with related structures and similar properties to aurovertins. The aurovertins inhibit the proton-pumping F1-F0-ATP synthase by binding to β-subunits in its β-catalytic sector (8). This is a globular domain that can be released as a water-soluble ATP hydrolase by disruption of a slender stalk that joins it to the membrane domain of ATP synthase. In the membrane-bound enzyme, both ATP hydrolysis and synthesis are inhibited, as is ATP hydrolysis by isolated F1-ATPase (1). The inhibition by aurovertins is uncompetitive with nucleotides (9). Three β-subunits are present in the ATP synthase complex; each β-subunit contains a nucleotide binding site that is directly involved in catalysis (10). The number of aurovertin binding sites per F1 assembly is uncertain, but there is at least one high affinity binding site (KD ∼1 μM), and either one or two additional sites with lower affinities (KD ∼4–6 μM) (8, 11). One site has low affinity in the presence of ADP but high affinity in the presence of ATP (11). Through the analysis of aurovertin-resistant mutants in Escherichia coli, β-Arg398 (equivalent to bovine β-Arg412) has been implicated in aurovertin binding (12, 13). In some other microorganisms that are naturally insensitive to aurovertins, this residue is also substituted by other amino acids (3, 14, 15).

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In the atomic structure of F1-ATPase from bovine mitochondria (17), determined with crystals grown in the presence of adenylyl-imidodiphosphate (AMP–PNP) and ADP, the three chemically identical β-subunits have different conformations and different occupancies by nucleotides. AMP–PNP is bound to one, ADP to the second, and no nucleotide at all is bound to the third β-subunit, despite the presence of excess nucleotides in the mother liquor. These three conformations are referred to as βTP, βDP, and βE, respectively. In the structure, the three β-subunits and the three noncatalytic α-subunits are arranged alternately like six segments of an orange around the central anti-parallel coiled-coil of α-helices in the γ-subunit. During the catalytic cycle, the three conformations of β-subunits could be interconverted by the relative rotation of this central coiled-coil. Therefore, this structure supports a binding change mechanism that proposed a cyclic interconversion of three different catalytic sites during the enzyme’s catalytic cycle (18).

The sites at which aurovertin B binds to bovine F1-ATPase have now been determined by x-ray analysis of crystals soaked in the inhibitor.

MATERIALS AND METHODS

Crystalization and Data Collection. Crystals of bovine heart F1-ATPase were grown by microdialysis in the presence of AMP–PNP and ADP (19). An ethanolic solution of aurovertin B (2 mM; Sigma) was then added to the solution outside the dialysis membrane to give a final concentration of 20 μM and 1% (vol/vol) ethanol. Immediately before data collection

Abbreviations: AMP–PNP, adenylyl-imidodiphosphate; rms, root-mean-square.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (reference R1COWSF).

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at the Synchrotron Radiation Source (Daresbury, U. K.), the glycerol concentration was increased by dialysis from 0 to 20% (wt/vol) in steps of 5%, with at least 2 h of dialysis between steps. One crystal, transferred to a similar solution but containing 25% (wt/vol) glycerol, was frozen at 100 K, and data were collected to 3.1 Å resolution (1 Å = 0.1 nm). Reflections were integrated with MOSFLM (20) and processed further with programs from the Collaborative Computational Project Number 4 suite (21).

**Structure Solution and Refinement.** The structure of the bovine F_{1}-ATPase–aurovertin B complex was solved by difference Fourier analysis using the calculated amplitudes and phases from the bovine F_{1}-ATPase coordinates (17). The starting model had an R factor of 17.2% and a free R factor (22) of 25.4% with respect to the F_{1}-ATPase data, and an R factor of 34.9% and free R factor of 34.8% with respect to the data obtained from the aurovertin–F_{1} complex. It was subjected to rigid body refinement using the computer program TNT (23) and all data from 20 to 3.1 Å. In this and in all subsequent refinement steps, 2% of the data were set aside for calculation of the free R factor, and refinement procedures were chosen so that the R factor decreased and the free R factor either decreased or remained constant. Two aurovertin molecules, one in b_{TP} and the other in b_{E}, were built into the structural model with the graphics program O (24), using bond lengths and angles from astetoxin (6) and 3,6-anhydro-α-D-galactose(25). The pyrone ring and the spacer of conjugated double bonds were kept planar (6, 26). The shape of the positive difference density in both sites agreed with the absolute stereochemistry of aurovertin (27). A few regions, particularly around the aurovertin binding sites, were rebuilt with O. The positions of 603 water molecules in the starting model were refined by real-space refinement using TNT. Those water molecules that were too close either to the aurovertins or to other parts of the model were removed, as were those incapable of making potential hydrogen bonds. Where appropriate, additional water molecules were introduced into the model. The temperature factors of all water molecules were refined, and those with temperature factors greater than 100 Å² were removed. The positions of individual atoms were refined with TNT, restraining the coordinates of the model to those of F_{1}-ATPase (for details, see ref. 28). The individual temperature factors of the atoms and the occupancies of the aurovertins were refined in a separate run, in which temperature factors were restricted to less than 100 Å² were removed. The positions of individual atoms were refined with TNT, restraining the coordinates of the model to those of F_{1}-ATPase (for details, see ref. 28). The individual temperature factors of the atoms and the occupancies of the aurovertins were refined in a separate run, in which temperature factors were restricted to less than 100 Å² were removed. The positions of individual atoms were refined with TNT, restraining the coordinates of the model to those of F_{1}-ATPase (for details, see ref. 28). 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Nonetheless, aurovertin B is bound to $\beta_E$ and its binding site is not compromised by this structural difference.

The three $\alpha$-$\beta$ pairs differ in their catalytic interfaces (17). In the region of the aurovertin binding site, the interface between $\alpha_{TP}$ and $\beta_{TP}$ is tightly packed, the one between $\alpha_E$ and $\beta_E$ is wide open, and the interface between $\alpha_{TP}$ and $\beta_{TP}$ is partially open (Fig. 4). In both $\beta_E$ and $\beta_{TP}$, aurovertin binds with the rigid spacer of conjugated double bonds lying almost parallel to the interface with the adjacent $\alpha$-subunits, but at a slight inclination so that the pyrone ring projects into the interface region. The pyrone ring is close to $\alpha_{TP}$ at the $\alpha_{TP}$-$\beta_{TP}$ interface, but in the $\alpha_E$-$\beta_E$ interface the subunits are too far apart to allow an equivalent interaction between $\alpha_E$ and the pyrone ring projecting from $\beta_E$.

**Amino Acids that Interact with Aurovertin B.** The interactions between aurovertin B and amino acids in the nucleotide binding domain of $\beta_E$ and $\beta_{TP}$ are mainly hydrophobic (Fig. 2 C–F). In both subunits, atom C8 of the aurovertin makes a van der Waals interaction with $\beta$-Leu342, atoms C11 and C12 interact with the side-chain of $\beta$-Ile344, atom C21 forms a hydrophobic contact with C$_g$ and C$_d$ of $\beta$-Pro350, and atom C24 is close to the side-chain of $\beta$-Leu351. There are also two potential hydrogen bonds to the C-terminal domain of the $\beta$-subunit between N$_e$ of $\beta$-Gln411 and O25 of aurovertin B, and between N$_e$ of $\beta$-Arg412 and O19. In addition, the pyrone ring makes a staggered stacking interaction with aromatic ring of $\beta$-Tyr458. The O17 of the aurovertin bound to $\beta_{TP}$ is in van der Waals contact with
This interaction is not present in the aurovertin binding site of \( \beta_{TP} \).

The involvement of \( \beta\text{-Arg412} \) in binding aurovertin B was anticipated from analysis of aurovertin resistant mutants of the \( E.\ coli \) \( F_1\text{-ATPase} \) where the equivalent arginine had been replaced by histidine, cysteine, or tryptophan (12, 13). These substitutions probably result in local conformational changes that reduce the affinity for the antibiotic. The Arg to Cys substitution would prevent possible hydrogen bond formation with O19 of aurovertin. Similar explanations can be invoked in the naturally aurovertin insensitive \( B.\ firmus \) OF4 (14) and \( B.\ PS3 \) (3), where \( \beta\text{-Arg412} \) is replaced by phenylalanine, and in \( M.\ gallisepticum \) (15), where it is substituted by asparagine. In \( B.\ PS3 \) and \( M.\ gallisepticum \), \( \beta\text{-Tyr458} \) is also changed to arginine and phenylalanine, respectively. Therefore, at least in \( B.\ PS3 \), the stacking interaction with the pyrone cannot form. In \( M.\ gallisepticum \), \( \beta\text{-Gln411} \) is replaced by arginine (15), which would be expected to modify the interaction between the protein and O25 of aurovertin. Whether this would weaken or strengthen aurovertin binding is unclear.

Although aurovertin B is not bound to \( \beta_{DP} \), the binding sites for the aglycone ring and the attached spacer appear to be conserved (Fig. 2G). However, the interface with the adjacent \( \alpha_{DP}\text{-Gln396} \) subunit is much tighter, and in consequence \( \alpha_{DP}\text{-Gln396} \) is in van der Waals contact with \( \beta_{DP}\text{-Tyr458} \), effectively blocking the binding site for the pyrone ring (Fig. 2H).

**Mechanism of Inhibition.** It is clear from the \( F_1\text{-aurovertin} \) structure that aurovertin cannot bind at the catalytic \( \alpha_{DP}\text{-}\beta_{DP} \) interface. If, as is thought, the crystal structure of bovine \( F_1\text{-ATPase} \) represents the ADP-inhibited state of the enzyme (17), the catalytic site in \( \beta_{DP} \) occupied by ADP, can be considered to be equivalent to a “tight” site in Boyer’s binding change mechanism (18). Similarly, the catalytic site in \( \beta_{TP} \)
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FIG. 4. Locations of the aurovertin B binding sites in bovine F_{1}-ATPase. The assembly is viewed from above (toward the membrane domain of intact F_{1}F_{0}-ATPase) showing the central α-helical coiled-coil structure in the γ-subunit and the C-terminal domains of α-subunits (residues 380–510) and β-subunits (residues 364–474) arranged in alternation around the coiled-coil. Aurovertin B molecules are bound to subunits β_{DP} and β_{E}. In β_{DP}, the pyrone ring is shown to interact with α_{E}, whereas in β_{E} it is unable to make an equivalent interaction with α_{E}, which is too far away. In β_{DP}, the aglycone pocket is present, but aurovertin B cannot bind to this subunit because of the interaction with α_{E}.

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