Crystal Structure of Cholesterol Oxidase Complexed with a Steroid Substrate: Implications for Flavin Adenine Dinucleotide Dependent Alcohol Oxidases†,‡

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ABSTRACT: Cholesterol oxidase from *Brevibacterium sterolicum* is a flavin-dependent enzyme that catalyzes the oxidation and isomerization of 3β-hydroxy steroids with a double bond at Δ^5–Δ^6 of the steroid ring backbone. The crystal structure of the free enzyme in the absence of a steroid substrate has previously been determined. In this paper we report the crystal structure of the complex of cholesterol oxidase with the steroid substrate dehydroisoandrosterone, refined at 1.8-Å resolution. The final crystallographic *R*-value is 15.7% for all reflections between 10.0- and 1.8-Å resolution. The steroid is buried within the protein in an internal cavity which, in the free enzyme crystal structure, was occupied by a lattice of water molecules. The conformations of a number of side chains lining the active-site cavity have changed in order to accommodate the steroid substrate. A loop region of the structure between residues 70 and 90 differs significantly between the substrate-free and substrate-bound forms of the enzyme, presumably to facilitate binding of the steroid. The hydroxyl group of the steroid substrate is hydrogen-bonded to both the flavin ring system of the FAD cofactor and a bound water molecule. FAD-dependent cholesterol oxidase shares significant structural homology with another flavoenzyme, glucose oxidase, suggesting that it might also be a member of the glucose–methanol–choline (GMC) oxidoreductase family. Although there is only limited sequence homology, a superposition of these two structures reveals a conserved histidine residue within hydrogen-bonding distance of the active-site water molecule. This histidine residue is completely conserved in the known sequences of GMC oxidoreductases and may act as the general base in the oxidation reaction. The structure of the substrate complex suggests possible mechanisms for the oxidation reaction, although the isomerization reaction cannot be explained by an analysis of the complex.

A wide range of soil bacteria share the ability to survive when grown on a medium of cholesterol, the degradation of which provides their sole source of carbon and energy (Stadtman *et al.*, 1954; Fukuda *et al.*, 1973). The first enzyme utilized in this degradation pathway is cholesterol oxidase (3β-hydroxysteroid oxidase, EC 1.1.3.6). This enzyme is bifunctional, catalyzing both the oxidation of Δ^5-ene-3β-hydroxysteroids with a *trans* A–B ring junction to the corresponding Δ^5-3-ketosteroid, with the reduction of oxygen to hydrogen peroxide, and also the isomerization to the Δ^4-3-ketosteroid (Figure 1). Although the enzyme exhibits a broad range of steroid specificities, the presence of a 3β-hydroxyl group is essential for activity (Smith & Brooks, 1975; Kamei *et al.*, 1978; Inouye *et al.*, 1982). The highest enzyme activity is observed using cholesterol as a substrate. The enzyme from the soil bacterium *Brevibacterium sterolicum* is used in the clinical determination of serum cholesterol. This secreted enzyme is a flavin-dependent monomeric oxidase containing 1 molecule of the cofactor flavin adenine dinucleotide (FAD) per molecule of protein. In contrast, cholesterol oxidases from *Nocardia rhodochrous*, *Streptomyces griseocarneus*, and *Streptomyces violascens* lack FAD or any other coafactor (Cheetham *et al.*, 1982; Kerenyi *et al.*, 1975; Arima *et al.*, 1969). Very limited kinetic data on steroid binding are available and most of the work has been carried out for the enzyme from *Nocardia* (Smith & Brooks, 1975, 1977; Hesslink *et al.*, 1989; Cheetham *et al.*, 1982).

A study of the specific binding interaction of a large and predominantly hydrophobic substrate has intrinsic interest. It is also a first step in surveying the features of steroid binding sites which mediate different substrate specificities. Only recently have structures of steroid binding enzymes been reported. Ghosh *et al.* (1991) have determined the structure of 3α,20β-hydroxysteroid dehydrogenase, an enzyme which oxidizes the hydroxyl group at both ends of androstane and pregnane steroid derivatives.

The crystal structure of cholesterol oxidase from *B. sterolicum* (ATCC21387) has previously been determined and refined at 1.8-Å resolution (Vrielink *et al.*, 1991). Since a full sequence of the enzyme was not available at the time this work was being carried out, the model was built using partial peptide sequence fragments and the sequence from the homologous FAD-dependent enzyme from *Streptomyces* sp. (Ishizaki *et al.*, 1989). The high quality of the diffraction data enabled the sequence of the enzyme to be determined directly from the electron density map. The refined model revealed a large active-site cavity which, in the absence of a bound steroid substrate, was filled with an ordered lattice of water molecules. The architecture of the active site and the location of various residues provided some indication as to the mechanism of steroid binding and catalysis. Subsequent to this work, a full gene sequence for the enzyme has become available (Ohta *et al.*, 1991) and has been incorporated into the model. To obtain further information about substrate recognition and catalysis it was necessary to determine the structure of the enzyme with a bound steroid substrate.

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‡ The coordinates of the rerefined cholesterol oxidase structure and the cholesterol oxidase–dehydroisoandrosterone complex have been deposited with the Brookhaven Protein Data Bank under Accession Numbers 3COX and 1COY, respectively.

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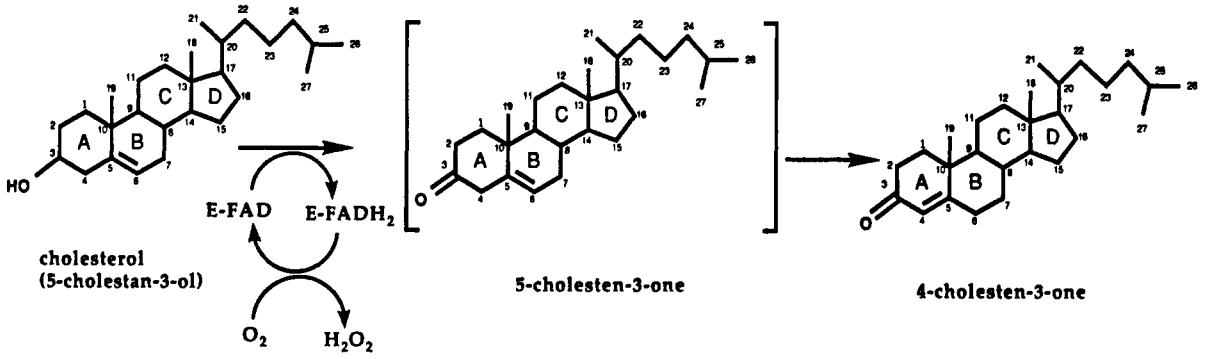

FIGURE 1: Enzymatic reaction catalyzed by cholesterol oxidase with cholesterol as the steroid substrate. The steroid numbering scheme referred to in the text is shown.

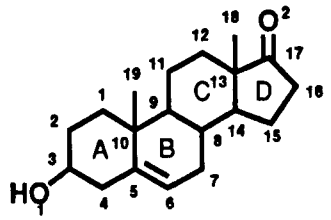

FIGURE 2: Chemical structure of the steroid dehydroisoandrosterone.

Here we report the structure of a complex of cholesterol oxidase with the steroid substrate dehydroisoandrosterone, refined to 1.8-Å resolution. This structure enables us to observe the specific interactions made between a steroid enzyme and a substrate. Cholesterol oxidase is a member of a family of flavoproteins which carry out oxidation reactions on alcohol substrates. The structure of this steroid protein complex provides an insight into the structural details of flavin–protein interactions and flavin-assisted oxidation.

EXPERIMENTAL PROCEDURES

Crystal Soak. Crystals of cholesterol oxidase from *B. sterolicum* were grown as previously described (Vrielink *et al.*, 1991) and stored in a buffer solution containing 1.3 M sodium/potassium phosphate at pH 7.0. Crystals were soaked in a solution prepared as follows: a solution containing 1.3 M sodium/potassium phosphate at pH 7.0 was diluted to 0.1 M, the pH was adjusted to pH 4.0 with orthophosphoric acid, and Triton X-100 was added to give a final concentration of 0.25 mg/mL. Dissolved oxygen was removed by nitrogen bubbling. To 1 mL of this solution was added 5 μL of 80 mM dehydroisoandrosterone (see Figure 2), dissolved in ethanol, to give a saturated substrate solution in a final ethanol concentration of 0.5% (v/v). Working in a glove box under a nitrogen atmosphere, crystals of cholesterol oxidase were soaked in this steroid solution for 7 h, with the crystals being transferred to a fresh solution every hour. The initial yellow color of the crystals, due to the oxidized FAD, disappeared over a period of several hours. These colorless crystals were used for X-ray diffraction studies.

Because the conditions used for the substrate soak were very different from those used originally for the crystals of the free enzyme, "control" data were collected in which crystals were treated with the identical conditions used for the substrate soak but the substrate itself was omitted. These data were analyzed to test the effect of transferring the crystals to a different buffer solution. The crystals soaked in this "control" solution remained yellow in color.

Data Collection and Processing. Selected soaked and colorless crystals were sealed into glass capillaries under a nitrogen atmosphere for X-ray diffraction measurements. In order to protect the reduced enzyme complex in the crystal against oxygen after mounting, 100 mM sodium dithionite was added to the solution present in one end of the capillary (Schreuder *et al.*, 1988). Data for the substrate complex were collected at the synchrotron radiation source, Daresbury, U.K., with an Arndt Wonacott oscillation camera (Enraf-Nonius, Delft). The wavelength of radiation used was 0.89 Å, and the crystals were cooled to 4 °C using a stream of dry air. The crystal morphology was such that data could be collected at a number of different positions along the crystal length. A complete data set to 1.8-Å resolution was collected using two crystals. The crystal remained in a colorless state throughout the data collection. The film data were processed using the program MOSFLM (A. G. W. Leslie, personal communication) with the initial crystal orientation determined using the program REFIX (Kabsch, 1988).

Table I: Summary of Data Collection and Processing for the "Control" and Dehydroisoandrosterone Soaks

	"control data"	substrate data
wavelength (Å)	1.54	0.89
resolution (Å)	1.8	1.8
temperature (°C)	18	4
number of crystals	1	2
number of reflections	175309	192439
independent reflections	46364	46169
% complete data	97.6	96.3
merging *R*-factor[a] (%)*	6.6	6.6
R-factor at 1.8 Å (%)	30.6	24.6

[a] $R_{merge} = 100 \times \Sigma_h\Sigma_i|I(h)_i - \langle I(h)\rangle|/\Sigma_h\Sigma_i\langle I(h)_i\rangle$ (summed over all observations *i*).

The "control" data were collected on an Enraf-Nonius FAST television area detector using graphite monochromatized CuKα radiation with no crystal cooling. Frames of 0.1° were collected with a crystal to detector distance of 80 mm. Images from the area detector were evaluated on-line using the program MADNES (Messerschmidt & Pflugrath, 1987). One crystal was used to collect a complete data set to 1.8-Å resolution. Details of the data collection and processing are presented in Table I. The unit cell dimensions for the crystals used in collecting both sets of data were indistinguishable from those reported by Vrielink *et al.* (1991).

Model Building and Refinement of the Structures. Before the substrate-soaked data were analyzed, the structure for the free enzyme was rebuilt incorporating the correct gene sequence throughout (Ohta *et al.*, 1991). This revised model was refined to give a final *R*-factor of 15.6% on all reflections between 10- and 1.8-Å resolution. A total of 49 residues were changed in the final model as compared to the model reported by Vrielink *et al.* (1991). Eighteen residues located either on the surface of the molecule or at loop regions have poor electron

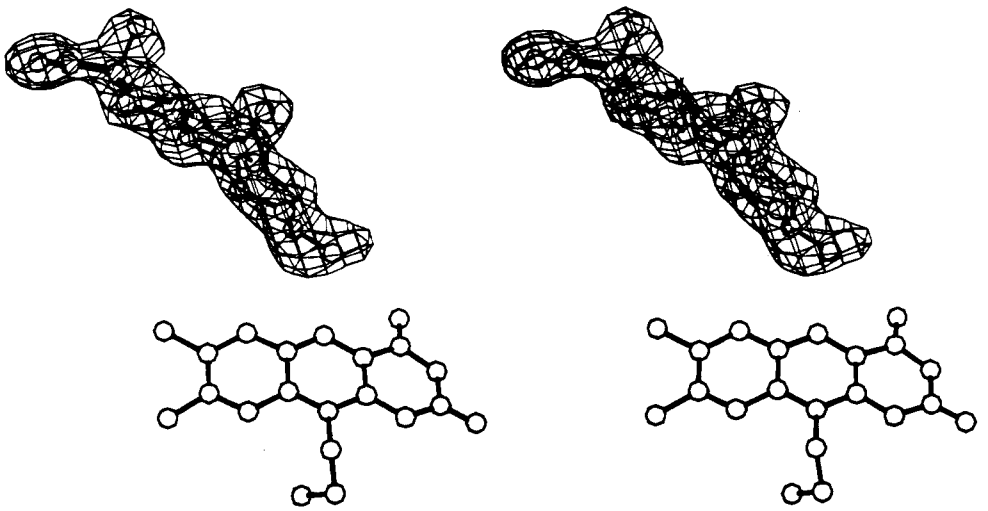

FIGURE 3: Stereo diagram showing the difference electron density, $(F_o - F_c)\alpha_c$, contoured at 2σ, for the substrate-bound structure in the region of the active site. The model structure factors and phases have been calculated after removal of the substrate. The flavin ring system has been included and the refined model for the steroid substrate, dehydroisoandrosterone, has been placed into the difference density.

density and either have been omitted from the model or have been modeled with incomplete side chains.

This refined model was used as the starting model for the initial difference electron density maps obtained for the "control" and substrate-soaked crystals. The difference electron density maps were calculated using coefficients $(3F_{obs} - 2F_{calc}) \exp(i\alpha_{calc})$ and $(F_{obs} - F_{calc}) \exp(i\alpha_{calc})$, respectively, where α_{calc} and F_{calc} values were obtained from the starting model. The structures for the "control" and substrate-soaked data were rebuilt from the starting model using an Evans & Sutherland computer graphics system, running FRODO software (Jones, 1978). Crystallographic refinements of the models were carried out using the program XPLOR (Brünger *et al.*, 1987). The bond length and angle parameters of the flavin ring system and steroid substrate were obtained from the Cambridge Structural Database (Allen *et al.*, 1983). The results of the refinement are summarized in Table II. The coordinates of the rerefined free enzyme structure and the substrate complex have been deposited with the Brookhaven Protein Data Bank under Accession Numbers 3COX and 1COY, respectively.

Table II: Crystallographic Refinement Statistics

	"control data"	substrate data
resolution (Å)	10–1.8	10–1.8
number of reflections	45729	44943
R-factor[a] (%)	17.3	15.9
average *B*-factor for the model (Å^2)	19.4	16.6
rms deviation in bond lengths (Å)	0.011	0.010

[a] $R\text{-factor} = 100 \times \Sigma|F_o - F_c|/\Sigma F_o$.

RESULTS

The free enzyme structure reported by Vrielink *et al.* (1991) was compared to the revised model into which the correct sequence had been incorporated. No significant structural changes were observed between these two structures, and the rms deviation between all main-chain atoms was 0.09 Å. The revised structure will be referred to as the free enzyme structure.

A careful comparison of electron density maps for the "control" and the free enzyme structures showed no convincing evidence of any structural changes. It could therefore be concluded that the soak conditions in the absence of steroid did not significantly affect the structure of the enzyme in the crystals.

The initial electron density difference map did not allow one to discriminate between the substrate (dehydroisoandrosterone) and the product (androstenedione) of the oxidation and isomerization reaction. Two sets of crystallographic refinement were carried out on the steroid-bound complexes: the first using a dehydroisoandrosterone substrate and the second using the androstenedione product. The differences between the two refined complexes were found to be too small to allow an unequivocal determination from the electron density map as to which form of the steroid had bound to the crystal. However, as the crystals were subjected to saturating concentrations of the substrate and the anaerobic conditions prevented the reduced enzyme from reoxidizing, it is assumed the substrate, and not the product, is present in the crystals.

In crystallographic refinement there is always a strong interaction between the occupancy and the atomic temperature factors. We were not able to separate these well enough to give an estimate of the occupancy. When refinement of the complex was carried out at full substrate occupancy, the temperature factors of the substrate are 37.6 Å^2, substantially higher than that of the surrounding protein atoms (14.1 Å^2). These observations suggest that the substrate is bound to the crystal at less than full occupancy.

Cholesterol oxidase presents an extreme for a substrate binding site. The substrate does not bind in a depression, cleft, or gorge but in an internal cavity which is totally sealed from the solvent, both when a steroid substrate is absent and when it is bound. In the free enzyme structure the internal cavity is occupied by a lattice of 13 water molecules. In the structure of the enzyme–substrate complex the steroid is buried within the cavity, displacing 12 of these bound water molecules. The difference electron density map of the dehydroisoandrosterone-bound crystals is shown in Figure 3. A four-ring steroid backbone can be modeled into the difference density with the hydroxyl group at C3 of the substrate positioned near to the flavin ring system. The clear density for the two axial methyl groups at C10 and C13 of the substrate allowed an unambiguous interpretation of the steroid orientation to be made. The cavity is not large enough to accommodate the eight-carbon "tail" of cholesterol, which is replaced by an oxygen atom in dehydroisoandrosterone. One may assume that when cholesterol is bound, the enzyme has the end of this tail hanging out. An overall view of the molecule, showing

FIGURE 4: Stereoview of the structure of the complex of cholesterol oxidase with bound steroid, dehydroisoandrosterone, drawn using the program MOLSCRIPT (Kraulis, 1991). The secondary structure elements of the protein are shown. The atoms for the FAD cofactor are shown with open circles and those for the steroid substrate are shown with filled circles.

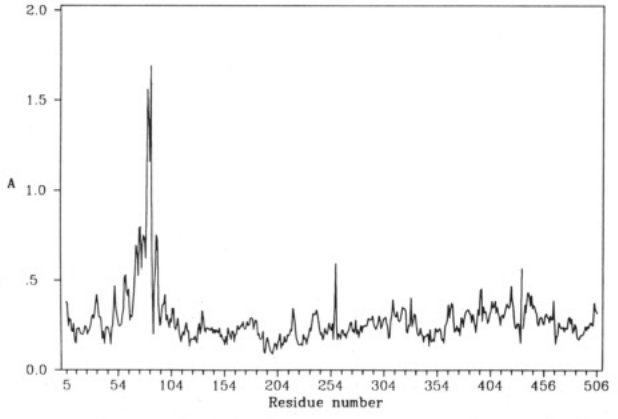

FIGURE 5: Plot showing the differences in the positions of the main-chain atoms between the native structure and the steroid-bound structure. Large differences are observed between residues 70 and 90.

Table III: Torsional Angle Changes of Active-Site Residues upon Steroid Binding

	native			substrate bound		
residue	χ_1 (deg)	χ_2 (deg)	χ_3 (deg)	χ_1 (deg)	χ_2 (deg)	χ_3 (deg)
Phe 359	−163	−54		−175	−79	
Tyr 446	−74	−12		−68	−65	
Met 325	−166	176	−92	−73	−157	82
Ile 218	−154	177		67	−155	
Phe 444	73	−83		57	−89	

the juxtaposition of both the cofactor and substrate, is shown in Figure 4.

The only significant changes in the position of the main chain upon steroid bindings are located between residues 70 and 90 (Figure 5). In the free enzyme structure, this region corresponds to a loop with relatively high atomic temperature factors between strands A2 and D1. The loop blocks the entrance to the active site cavity and presumably forms a lid which opens the surface of the molecule to allow the substrate to enter the internal cavity. In the steroid-bound structure, parts of this loop have poor electron density, particularly between residues 78 and 87 along the solvent-exposed side of the molecule, whereas side chains which point toward the interior of the protein have well-defined electron density. The side chain of Phe83 on this loop projects into the active-site cavity, blocking the entrance. On substrate binding this residue retains a similar side-chain conformation but has moved with the loop, resulting in an enlargement of the steroid binding cavity, while the side chain makes van der Waals contact with the steroid (Figure 6). The average temperature factors for this loop region are 37.4 Å^2 for main-chain atoms and 36.1 Å^2 for side-chain atoms, suggesting the high flexibility of this region of the structure.

The conformations of some of the side chains lining the active-site cavity have changed to accommodate the steroid (Table III). The conformation of the FAD cofactor is identical in the two structures, with the flavin ring system distorted from planarity. The FAD molecule makes similar hydrogen-bonding contacts to the protein and water molecules in both structures.

A single water molecule (H_2O541) remains in the active-site cavity upon binding of the steroid. In the free enzyme structure this water molecule is within hydrogen-bonding distance of the side chains of Asn485 and His447 and the nitrogen at position 5 of the flavin moiety. On substrate binding these interactions are retained, and in addition there is a hydrogen bond between the water molecule and O1 of the substrate (Figure 7 and Tables IV and V).

There are only two potential hydrogen-bonding atoms in the dehydroisoandrosterone substrate: O1 on the A ring and O2 on the D ring (see Figure 2). The oxygen, O2, on the D ring makes no polar interactions with the protein or buried water molecules. In contrast, the O1 hydroxyl group is within

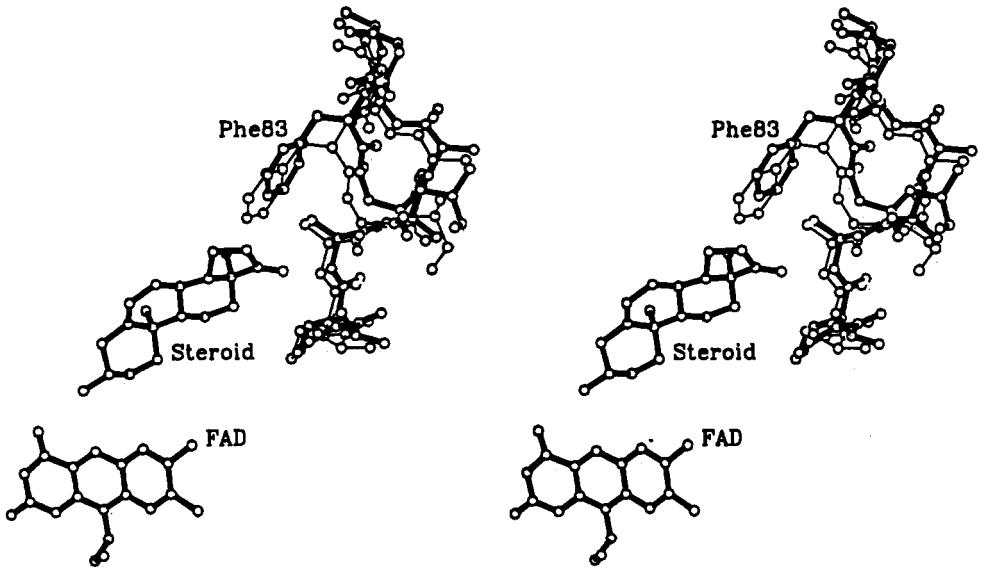

FIGURE 6: Stereo diagram showing the movement of the loop between residues 75 and 85. The substrate-bound complex is shown with thick lines and the free enzyme is shown with thin lines. The steroid and the FAD cofactor for the substrate-bound complex have been included. The side chain atoms past Cβ have been omitted for all residues except Phe83.

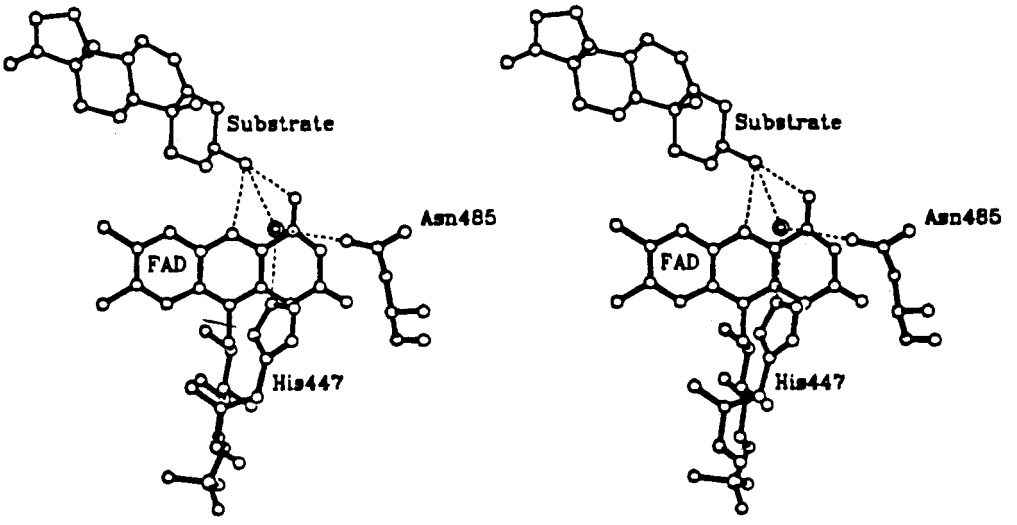

FIGURE 7: Stereo diagram showing the active site near the hydroxyl group of dehydroisoandrosterone. The side-chain atoms for His447 and Asn485 are included as well as H_2O541 (shown as a double circle). The hydrogen bonds between the side chains, water molecule, and steroid and are shown. The hydrogen-bond distances are given in Tables IV and V.

hydrogen-bonding distance of both the water molecule H_2O541 mentioned above and also atoms N5 and O4 of the flavin. There is a close contact between O1 and the main-chain nitrogen of residue 120 which would appear to be at an unfavorable angle for a hydrogen-bond interaction.

There are numerous van der Waals interactions between nonpolar steroid atoms and hydrophobic side chains including Tyr446, Pro344, Phe83, Val77, Leu375, and Pro76. As discussed above, three of these residues occur in the loop region where significant changes are observed upon steroid binding.

The active site of the enzyme contains one charged residue, Glu361, the side chain of which, in the free enzyme structure, has poorly defined electron density, suggesting multiple conformations. A comparison of the observed density for Glu361 in the free enzyme structure and the steroid-bound structure reveals that, upon steroid binding, the side-chain density for the glutamic acid becomes more clearly defined, although there is still clear evidence for more than one position for this side chain. The temperature factors for the side chain are relatively high (27 Å^2), suggesting there is still a large degree of mobility.

DISCUSSION

Cholesterol oxidase from *B. sterolicum*, like many other flavoenzymes, uses FAD as a cofactor and contains a topologically equivalent FAD binding domain that interacts with the AMP moiety of the cofactor. This domain includes a GXGXXG sequence motif near the amino terminus that is involved in binding to the phosphate group. In contrast, the substrate-binding domains show a variety of different topologies. The structure of the FAD-containing enzyme glucose oxidase (Hecht *et al.*, 1993) is similar to cholesterol oxidase not only in containing an FAD-binding domain but also in having a topologically similar substrate-binding domain. This similarity is sufficiently close to allow the structures to be superposed. A total of 162 α-carbon atoms in the FAD-binding domain can be superposed with a rms separation of 1.79 Å, while when both domains are considered, 313 α-carbon atoms can be superposed with a rms separation of 2.4 Å. Glucose oxidase contains an insertion of three α-helices between strands B3 and B4 of the substrate-binding domain of cholesterol oxidase. Furthermore, the loops which in cholesterol oxidase

Table IV: Contacts between Dehydroisoandrosterone and the Protein within a 4-Å Distance

substrate atom	target atom	distance (Å)	substrate atom	target atom	distance (Å)
O1	C4 FAD	3.60	C7	CG1 Val217	3.95
	C4a FAD	3.66			
	C5a FAD	3.88	C11	CZ Tyr446	3.94
	N5 FAD	2.91		OH Tyr446	3.19
	O4 FAD	2.89			
	CA Gly120	3.13	C12	CD Pro76	3.90
	N Gly120	3.17		OH Tyr446	3.22
	OE1 Glu361	3.81			
	H_2O541	3.20	C15	CD Pro366	3.97
C1	CE2 Tyr446	3.75	C16	CE2 Phe83	3.37
	CZ Tyr446	3.91		CZ Phe83	3.26
C2	C6 FAD	3.92	C18	CG2 Val77	3.37
	OE1 Glu361	3.78		CD2 Leu375	3.38
	H_2O541	3.57			
			C19	CB Ala363	3.57
C3	N5 FAD	3.70			
	C6 FAD	3.86	O2	CB Gln75	3.61
	N Gly120	3.69		CG Gln75	3.96
	CA Gly120	3.66		CDPro76	3.76
	H_2O541	3.99		N Pro76	3.92
				N Val77	3.28
C4	CA Gly120	3.82		CA Val77	3.83
	CD Pro344	3.81		CB Val77	3.42
	CG Pro344	3.59		CG2 Val77	3.61
				O Val77	3.91
C6	O Thr342	3.53			

Table V: Hydrogen-Bonding Network Centered at H_2O541 for the Native and Substrate-Bound Structures

		distance (Å)	
source atom	target atom	native	substrate bound
H_2O541	O1 substrate	–	3.20
	OE1 Glu361	4.12	3.73
	OE2 Glu361	2.98	3.66
	NE2 His447	2.93	2.85
	ND2 Asn485	2.86	2.76
	O4FAD	4.12	3.89
	N5FAD	3.21	3.10
	H_2O670	2.64	–

enclose the active-site cavity, in particular that between residues 46 and 94, are very much shortened in glucose oxidase. As a consequence the active site in glucose oxidase is completely exposed to solvent. Sequence comparisons of glucose dehydrogenase, choline dehydrogenase, glucose oxidase, and methanol oxidase have shown that homology exists between these enzymes, enabling a family of proteins, the glucose–methanol–choline (GMC) oxidoreductases, to be defined (Cavener, 1992). Of the members of this family of enzymes, only the structure of glucose oxidase is known. Sequence comparison based on the structural alignment of glucose oxidase and cholesterol oxidase shows a number of identical residues which are also conserved in the other members of the GMC family. These occur mainly in the FAD-binding domain, but a number of residues in the substrate binding domain that interact with the ribityl chain are also conserved. The structural homology observed between glucose oxidase and cholesterol oxidase indicates that cholesterol oxidase can also be included in the GMC oxidoreductase family.

Flavoenzymes are involved in a wide range of catalytic reactions. The flavin moiety is able to carry out diverse chemistry and can thus be involved in a number of different catalytic mechanisms, the common feature being a transfer of electrons between the substrate and the flavin ring system (Ghisla & Massey, 1989). This diverse chemistry is dictated by the substrates involved and also by the protein environment around the flavin ring system.

Flavoenzymes are involved in two different types of alcohol oxidation reactions, one involving α-hydroxy acid substrates where the C–H bond to be oxidized is activated due to the adjacent functional group, and the second involving unactivated alcohols. The class of enzymes using α-hydroxy acid substrates has been extensively studied, and the reaction is believed to proceed by abstraction of the α-hydrogen as a proton from the C–H bond to form a carbanion (Walsh *et al.*, 1971; Porter *et al.*, 1973). Enzymes using unactivated alcohol substrates have been thought to catalyze a radical mechanism involving the transfer of a proton and two single electron entities from the substrate to the oxidized form of the flavin (Silverman *et al.*, 1980; Sherry & Abeles, 1985), although a hydride transfer mechanism may also occur (Ghisla & Massey, 1989). Members of the GMC oxidoreductase family all oxidize unactivated alcohols. The similarity in both structure and catalytic activity suggests that the members of this group of flavoproteins share a common enzymatic mechanism.

In cholesterol oxidase the steroid substrate is held in position by van der Waals contacts along the length of the steroid ring system and, at the A ring, by the water molecule H_2O541, which is also hydrogen-bonded to the side chains of His447 and Asn485 (Figure 7). The flavin ring also appears to play a role in positioning the substrate in the cavity through hydrogen bonds between O1 of the substrate and O4 and N5 of the cofactor. The side chain of His447 is further anchored in place by hydrogen-bond interactions involving the δ-nitrogen atoms of both Asn321 and Asn323.

The interactions seen between cholesterol oxidase and dehydroisoandrosterone are consistent with a radical mechanism similar to that proposed to occur for methanol oxidase (Sherry & Abeles, 1985). Figure 8 shows how such a mechanism may occur for cholesterol oxidase. A single electron is transferred to the flavin, followed by the transfer of the proton from C3 to N5. The distance between C3 of the steroid and N5 is 3.7 Å, but C3 is axially directed at N5 of the flavin and slight movements within the structure could facilitate this transfer. A transfer of a second electron to the flavin would result in a net negative charge on the flavin which, through resonance, could be positioned on O2. The hydrogen bond between O2 and N-Phe487, located at the N terminus of the C-terminal helix, suggests a stabilization of this charge by the helix dipole (Hol *et al.*, 1978). The transfer of two electrons and one proton to the flavin results in a net positive charge on O1 of the steroid. The O1 proton is, however, within hydrogen-bonding distance from H_2O541, which is, in turn, within hydrogen-bonding distance of NE2-His447. This water molecule could therefore act as a bridge to shunt the proton between the steroid and His447. The distances observed between H_2O541 and O1 of the steroid and NE2-His447 (see Table V) support the possibility of such a proton shunt.

In this mechanism the positive charge is accommodated by the side chain of His447 and the negative charge by the flavin ring, stabilized by the helix dipole. Interestingly, the superposition of the structures of cholesterol oxidase and glucose oxidase reveals a histidine (His516) in a position similar to that occupied by His447 in cholesterol oxidase. The sequence alignment described by Cavener (1992) shows that this residue is conserved in all four GMC oxidoreductases, suggesting that the histidine residue might play an essential role in the catalytic mechanism of the enzyme. Glucose oxidase has another active-site histidine (559) which has been proposed by Hecht *et al.* (1993) to act as the general base accepting the proton from

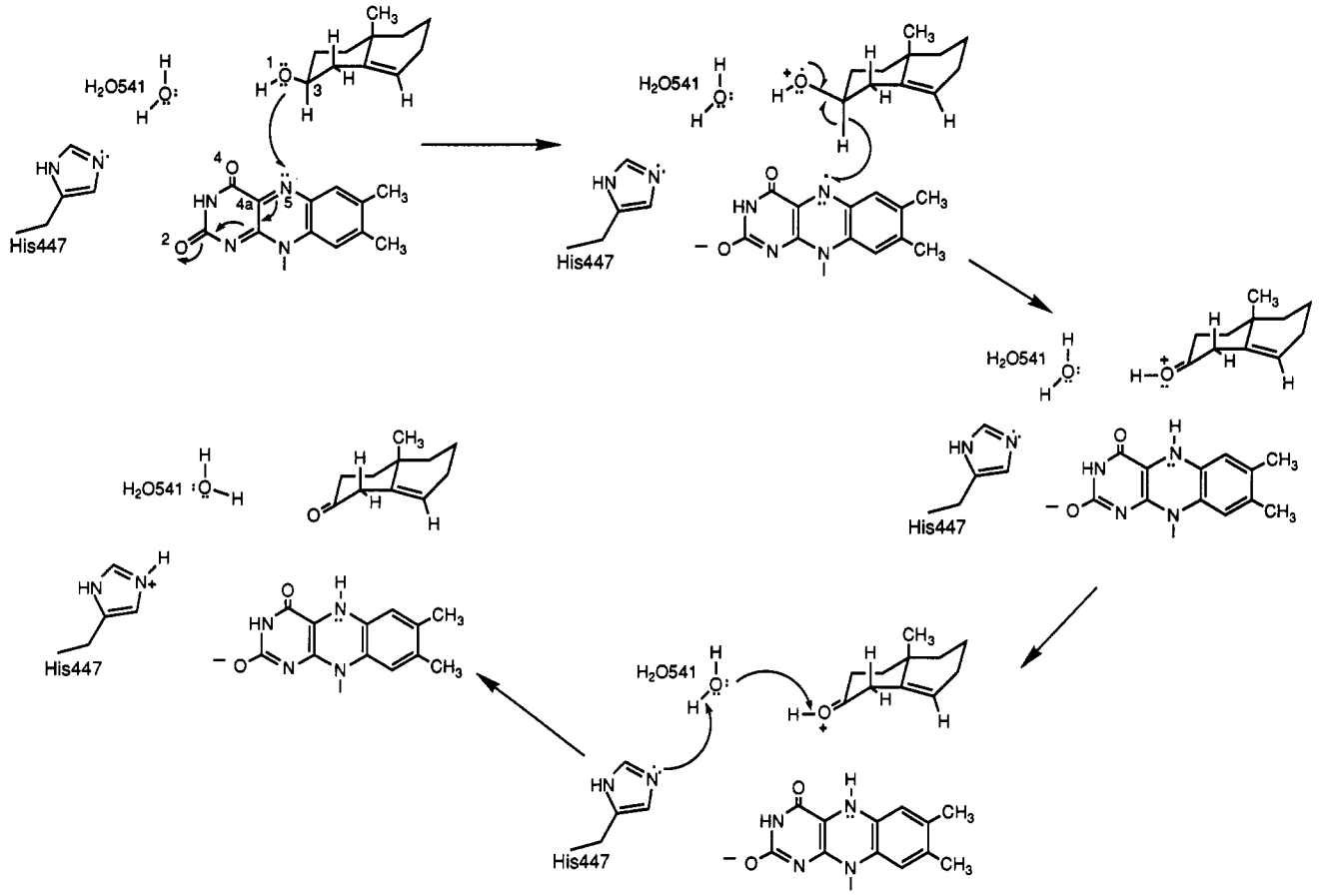

FIGURE 8: Possible radical mechanism for the oxidation of the steroid substrate. The A and B rings of the steroid and the flavin moiety of FAD are shown. The side chain of His447 and the bound water molecule, H_2O541, are included. The numbering of some of the atoms for the flavin ring and the substrate is included.

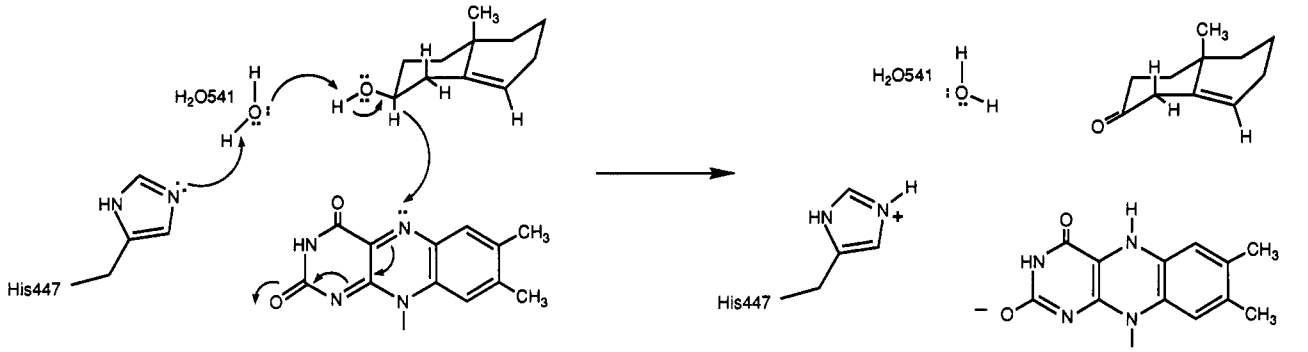

FIGURE 9: Hydride transfer mechanism for the oxidation of cholesterol oxidase. In this mechanism the hydrogen is transferred to the flavin ring as a hydride.

the 1-hydroxyl group of glucose. The structural superposition reveals an asparagine (485) which is hydrogen-bonded to the important water molecule, H_2O541 in cholesterol oxidase, at the analogous position to His559. Furthermore, in the other sequences of the GMC oxidoreductases this histidine is not conserved (Cavener, 1992), although a small adjustment to the published sequence alignment would place an asparagine at this position in the three other members of the GMC oxidoreductase family. The lack of conservation of His559 in the GMC oxidoreductases suggests that this residue does not act as the general base.

Biochemical data on glucose oxidase have suggested the involvement of a carboxylate group in substrate binding (Bright & Appleby, 1969). The structure has shown a glutamate residue (412) hydrogen-bonded to His559. Protonation of this glutamate residue would break the hydrogen bond to His559, causing a rearrangement of the active-site geometry and in this way inhibiting an enzyme–substrate complex from forming. Interestingly, the analogous asparagine (485) in cholesterol oxidase is also hydrogen-bonded to a glutamic acid side chain (361). However, this glutamic acid residue does not superpose with that of Glu412 in glucose oxidase; it is positioned two residues away on β-strand B4.

An alternative to the radical mechanism is the hydride transfer mechanism shown in Figure 9. The resulting end product is identical to that shown in Figure 8, with the flavin accommodating a negative charge and one proton and His447 accommodating the second proton. Here the abstraction of a proton from O1 that is transferred to His447 via H_2O541 facilitates the transfer of a hydride to the flavin moiety. This oxidation could be considered a concerted mechanism, whereas a radical mechanism might progress by a stepwise procedure. From the structure of the complex we are unable to distinguish between these two possibilities.

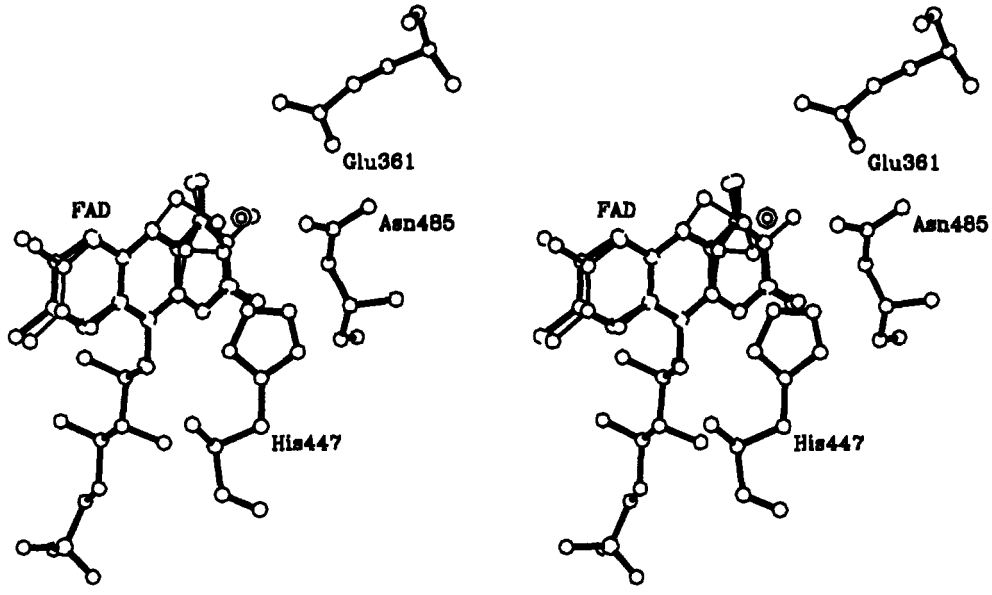

FIGURE 10: Stereo diagram showing the superposition of the structure of the flavin 4a-hydroperoxide model compound (Bolognesi *et al.*, 1978) with the flavin ring system of the substrate-bound structure. Some of the residues which line the active site of the enzyme are also shown. The water molecule, H_2O541, is shown as a double circle. The protein and flavin are shown with thick lines and the flavin 4a-hydroperoxide model is shown with thin lines.

The presence of Glu361 in the active site suggests an alternative general base to His447 in the oxidation reaction. However, the distance between the carboxyl oxygen atoms of Glu361 and H_2O541, and the fact that an analogous residue is not found in the same location in glucose oxidase, suggests that this residue is unlikely to be involved in the oxidation reaction.

Although the structure of the complex contains all the elements needed to account for the proposed oxidation mechanisms, this is not true for the isomerization step. The enzyme Δ^5-3-ketosteroid isomerase from the soil bacterium *Pseudomonas testosteroni* carries out the identical isomerization step to that of cholesterol oxidase (Talalay & Wang, 1955). The crystal structure of Δ^5-3-ketosteroid isomerase has been reported at 6-Å resolution (Westbrook *et al.*, 1984) and a higher resolution structure is in progress (Westbrook, personal communication). The mechanism of isomerization adopted by this enzyme has been shown to involve an intramolecular, cis diaxial transfer of the 4β proton to the 6β position by way of an enolic intermediate (Batzold *et al.*, 1976). The presence of Glu361 in the active site of the enzyme supports this suggestion, as this residue might act as the base which abstracts the axial hydrogen at C4 of the steroid.

In the uncomplexed structure, the side-chain electron density for Glu361 indicated that it may be mobile. The electron density for the complex, although showing slightly better defined density for the side chain of Glu361, places the carboxylate oxygen atoms over 3.5 Å from C4 of the steroid substrate. Modeling studies of the steroid and the glutamic acid side chain within the observed density indicate, however, that a structural change of the steroid during isomerization might bring them closer together, allowing for attack on the C4 axial hydrogen.

After the oxidation step the conformation of the A ring will change due to the formation of an sp^2 from an sp^3 hybridized center at C3. Thus the distance between C4 and the side-chain oxygens of Glu361 will differ from that seen in the present model. Inspection of the steroid-bound structure fails to reveal any residue which could act as a proton donor near C6. The present complex shows a distance of 5.8 Å between C6 and the side chain of Glu361. The glutamic acid could act as both the proton donor and the proton acceptor, but for this to occur the steroid would have to move by approximately 3.0 Å in order to place the glutamic acid side chain close enough for attack at C6.

Oxidases share a common feature in that they are regenerated by molecular oxygen, yielding H_2O_2. For the case of flavoenzymes the mechanism of reoxidation is believed to go through a flavin–peroxide adduct at C4a of the flavin ring system (Ghisla & Massey, 1989). A small molecule crystal structure of a flavin 4a-hydroperoxide model compound has been reported by Bolognesi *et al.* (1978). This structure shows deviations from planarity for the flavin ring system in the same way as has been observed in the structures of a number of flavoproteins (Karplus & Schulz, 1987; Schreuder *et al.*, 1989; Vrielink *et al.*, 1991; Hecht *et al.*, 1993). The structure of this 4a-hydroperoxide model was superposed onto the flavin ring system of cholesterol oxidase and is shown in Figure 10. This superposition shows that only C4a of the flavin changes significantly between the two structures, changing from an sp^2 hybridized center in the enzyme to an sp^3 center in the hydroperoxide model. This change in hybridization moves the oxygen atom attached to C4a to within hydrogen-bonding distance of NE2 of His447 and the side chain of Asn485. In order to accommodate the flavin–peroxide adduct, the active-site water molecule (541) must be displaced. The histidine (447), protonated from the oxidation reaction, will be in position to release its proton to the oxygen of the adduct, with the second proton coming from N5 of the flavin.

We have studied the structure of the FAD-containing enzyme cholesterol oxidase complexed with the steroid dehydroisoandrosterone to further understand the mechanism of both steroid oxidation and isomerization. The structural homology with glucose oxidase suggests that cholesterol oxidase will share a similar mechanism of oxidation with other enzymes in the GMC oxidoreductase family.

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