

## Structure of the Heme d of *Penicillium vitale* and *Escherichia coli* Catalases\*

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**A heme d prosthetic group with the configuration of a *cis*-hydroxychlorin  $\gamma$ -spirolactone has been found in the crystal structures of *Penicillium vitale* catalase and *Escherichia coli* catalase hydroperoxidase II (HPHII). The absolute stereochemistry of the two heme d chiral carbon atoms has been shown to be identical. For both catalases the heme d is rotated 180 degrees about the axis defined by the  $\alpha$ - $\gamma$ -meso carbon atoms, with respect to the orientation found for heme b in beef liver catalase. Only six residues in the heme pocket, preserved in *P. vitale* and HPHII, differ from those found in the bovine catalase. In the crystal structure of the inactive N201H variant of HPHII catalase the prosthetic group remains as heme b, although its orientation is the same as in the wild type enzyme. These structural results confirm the observation that heme d is formed from protoheme in the interior of the catalase molecule through a self-catalyzed reaction.**

The vast majority of heme proteins contain iron protoporphyrin IX (heme b) as the prosthetic group, but in recent years an increasing number of naturally occurring porphyrins with different covalent structures has been emerging (1). Among those, chlorins and isobacteriochlorins, which contain a partially saturated porphyrin macrocycle, have received special attention. In particular, two proteins isolated from *Escherichia coli*, the terminal oxidase that predominates at low levels of oxygen (1) and the catalase HPHII (2), have been shown to contain two structurally related chlorins (Fig. 1) that have been generically termed heme d. Spectroscopy data on the demetallized and esterified prosthetic groups of both enzymes were consistent (3) with the presence of a  $\gamma$ -spirolactone at the saturated pyrrole ring III of the macrocycle in the C-6 position and a vicinal C-5 hydroxy group, although the relative orientation of these two

groups was shown to be different in the two proteins (2). In the terminal oxidase, the two substituents have the more stable *trans* configuration (Fig. 1A, *ii*), while in the prosthetic group of HPHII they have the *cis* configuration (Fig. 1A, *i*). The absolute stereochemistry of the two chiral centers of the HPHII heme d has never been reported.

Additional data on the cytochrome *d* terminal oxidase (4) suggested that the actual form of the prosthetic group present in the interior of the enzyme was the corresponding *trans*-diol (Fig. 1A, *iv*), and that lactonization occurred spontaneously during isolation of the heme. By analogy with this system, and due to the facile formation of the  $\gamma$ -lactone ring (3, 5), it was also assumed that the corresponding *cis*-diol (Fig. 1A, *iii*) was the actual species in the active site of HPHII (2, 6). The enzymes responsible in *E. coli* for the conversion of protoheme into heme d have never been identified, and this led Timkovich and Bondoc (1) to propose that the formation of *cis*-heme d may be catalyzed by HPHII itself. According to this hypothesis, Loewen *et al.* (7) reported the conversion of the protoheme cofactor to heme d in the presence of a source of hydrogen peroxide, using heme b containing recombinant HPHII.

For a number of fungal catalases, the presence of heme prosthetic groups with altered covalent structure has been inferred from their electronic spectra. The optical spectra of catalases from *Penicillium vitale* (8) and *Neurospora crassa* (9) have been suggested to arise from a chlorin-like structure. In other organisms, *e.g.* *Penicillium chrysogenum* (10), the electronic spectrum of the ferric enzyme resembles that of a chlorin derivative ( $\lambda_{\max} = 405, 590, 720$  nm), although the authors do not mention this peculiarity in their report. To our knowledge, no relationship with the bacterial heme d structure had been proposed for the prosthetic group of these fungal catalases.

Catalase (hydrogen peroxide:hydrogen peroxide oxidoreductase, EC 1.11.1.6) is present in virtually all aerobic organisms where it dissociates hydrogen peroxide into molecular oxygen and water. The most common form of the enzyme is a homotetramer with one porphyrinic prosthetic group per subunit. The three-dimensional structures of five of these heme-containing catalases have been reported: *P. vitale* (PVC)<sup>1</sup> (Protein Data Bank code 4CAT; polyglycine coordinates from a structure determination at 2.0-Å resolution, see below) (11), beef liver (BLC) (Protein Data Bank codes 7CAT and 8CAT; both structures at 2.5-Å resolution) (12) *Micrococcus lysodeikticus* (MLC)

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<sup>1</sup> The abbreviations used are: PVC, *Penicillium vitale* catalase; BLC, beef liver catalase; MLC, *Micrococcus lysodeikticus* catalase; PMC, *Proteus mirabilis* catalase; HPHII, hydroperoxidase II.

(13) (at 1.5 Å resolution), HPII catalase from *E. coli* (6) (Protein Data Bank code not yet available) and *Proteus mirabilis* (PMC) (Ref. 14; Protein Data Bank codes 1CAE and 1CAF, at 2.2- and 2.8-Å resolution, respectively). PVC and HPII catalases contain a C-terminal domain of about 150 residues, with a “flavodoxin-like” topology that is absent in BLC, MLC, and PMC. These smaller catalases, lacking a “nucleotide binding” domain, can bind a molecule of NADPH (14), while no nucleotides have been found to bind to PVC or HPII. The three-dimensional structure of native PVC was reported at 2.0-Å resolution with an agreement *R* factor of 31.4% for a partial, x-ray, sequence (11). The resulting electron density map was not sufficient, despite of the resolution, to precise the chemical modifications present in the heme prosthetic group and suggested by the spectroscopic data. For HPII the 2.8-Å resolution structure (*R* factor 20.1%) supported the presence of a modified heme in the enzyme, although its conformation could not be defined (6).

In this work we describe the configuration of the heme *d* group now determined in the crystal structures of PVC at 1.8-Å resolution for both the native enzyme and the complex with 3-amino-1,2,4-triazole (with *R* factors of 16.7 and 14.8%, respectively) and in the structure of the HPII catalase with azide, at 2.2-Å resolution (*R* factor 18.1%). Comparison of the heme environment in PVC and HPII catalase with the mammalian BLC reveals common peculiarities of the heme *d* pockets. The configuration of the heme *b* group found in the crystal structure, at 2.2-Å resolution (*R* factor 18.5%), of the essentially inactive N201H variant of HPII (7), in which the distal Asn<sup>201</sup> residue has been replaced by a histidine, is also considered.

#### MATERIALS AND METHODS

**PVC Data Collection and Refinement**—Crystals of both native and 3-amino-1,2,4-triazole (AT)-PVC complex were grown by ultracentrifugation, as previously reported for the native enzyme (11). Purified PVC (15) was reacted with the catalase inhibitor AT, following the Chang and Schroeder protocol (16) with some modifications (concentration of AT was 80 mM and protein concentration was 3 mg/ml). After 6 h of reaction the catalytic activity decreased to 2–3% of the initial value. X-ray data from AT-PVC crystals were collected using synchrotron radiation at the EMBL outstation in Hamburg with a MAR Research image plate as detector. Data from native PVC crystals were collected using an Raxis image plate detector and CuK<sub>α</sub> radiation from rotating anode x-ray sources. Both data sets were processed with the package DENZO (17). Refinement was started using the current model of native PVC (11) and carried out with the restrained least-square Hendrickson-Konnert minimization as implemented in the CCP4 suite of programs (18). Maximum likelihood refinement method with program REFMAC<sup>2</sup> was also used to improve the quality of the final models. The current *R* factor for AT-PVC is 14.8% (*R*<sub>free</sub> = 19.4%) for 139,766 reflections in the resolution shell 30–1.8 Å, and for native PVC it is 16.7% for 137,997 reflections in the resolution shell 20–1.8 Å. In both cases the two subunits in the asymmetric unit have been independently refined. For AT-PVC more than 1500 solvent molecules, and for native 1100 solvent molecules, have been explicitly included. The quality of the electron density maps allowed us to define with confidence most of the protein primary sequence, which has not yet been chemically determined. A detailed report of the structure determination will be presented elsewhere. The presence of the inhibitor 3-amino-1,2,4-triazole in the PVC structure does not seem to introduce any significant change in the heme *d* conformation. To avoid redundancies we have consistently used, in this work, the best refined PVC atomic coordinates obtained for the complex.

**HPII and N201H Variant Data Collection and Refinement**—Crystals of HPII and of the N201H variant were obtained as described (6). These crystals diffracted at least to 2.0-Å resolution but showed substantial decay during x-ray exposure, and complete data for both the wild type enzyme and the variant at 2.2-Å resolution could only be collected using synchrotron radiation at the EMBL outstation in Hamburg. Data from the wild type enzyme were processed using the package MOSFLM (19), while the package XDS (20) was used for the variant. Automatic refine-

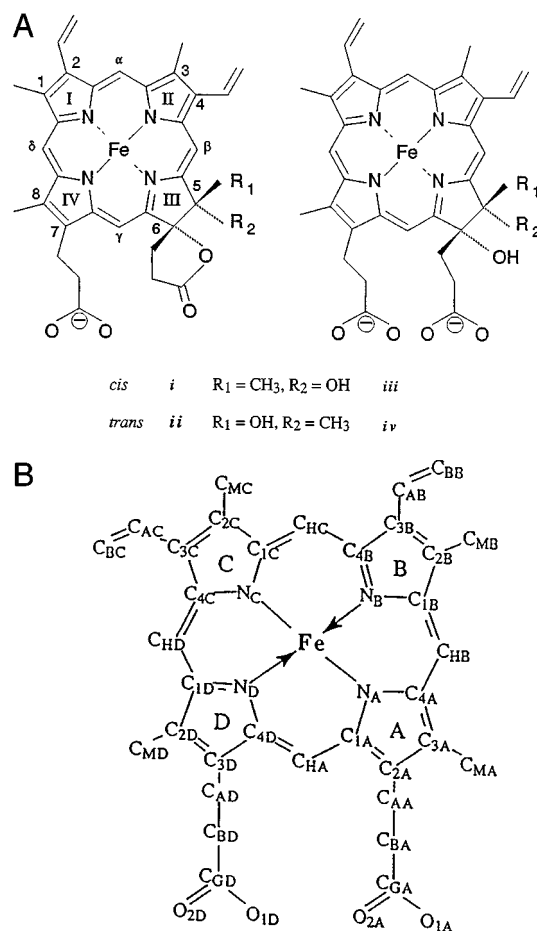


FIG. 1. A, structures of the *cis*- (*i*) and *trans*- (*ii*) heme *d* hydroxy- $\gamma$ -spirolactones and the related *cis*- (*iii*) and *trans*- (*iv*) diol compounds. The absolute configuration shown for the two chiral carbon atoms of *i* is the one obtained in this work from the crystal structure of PVC and HPII catalase. The absolute configuration of the two *trans*-heme *d* derivatives related to the cytochrome *d* terminal oxidase (*ii*, *iv*) is at present unknown, and the one shown in the figure is only for the purpose of comparison. B, heme *b* structure with the Protein Data Bank atomic nomenclature used in the Introduction. The orientation of the heme shown (defined by the relative positions of vinyl groups in pyrrolic rings I and II) corresponds to the one found in beef liver catalase when the heme is viewed from the distal side.

ment steps were done with the program XPLOR (21) and were alternated with inspection and manual rebuilding using the graphic program TURBO (22). The final crystallographic agreement *R* factors are, for the wild type and the N201H variant, respectively, 18.1% for 95,744 reflections in the resolution shell 8.0–2.2 Å and 18.5% for 120,748 reflections also in the resolution shell 8.0–2.2 Å. Strict noncrystallographic symmetry, relating the four subunits contained in the asymmetric unit of the crystal, has been maintained through the whole refinement. 322 solvent molecules/subunit have been added for the wild type, and 351 have been added for the variant.

#### RESULTS

The heme *d* group characterized in the active sites of the refined crystal structures of both PVC and HPII catalase possesses the structure of the *cis*-hydroxy  $\gamma$ -spirolactone (Fig. 1A, *i*), as can be clearly inferred from the electron density maps (Fig. 2). The absolute configuration of the two  $\beta$ -carbon atoms of the macrocycle bearing the hydroxy and the spirolactone substituents is S and R, respectively. In the two enzymes, the electron density of the methyl and vinyl side chains of the pyrrole rings I and II indicates that the orientation of the heme *d* is mostly inverted (see below) with respect to that found for the heme *b* group in BLC (12), while the electron density for the  $\gamma$ -spirolactone and the hydroxy heme substituents clearly ap-

<sup>2</sup> G. Murshudov, A. Vagin, and E. Dodson, manuscript in preparation.

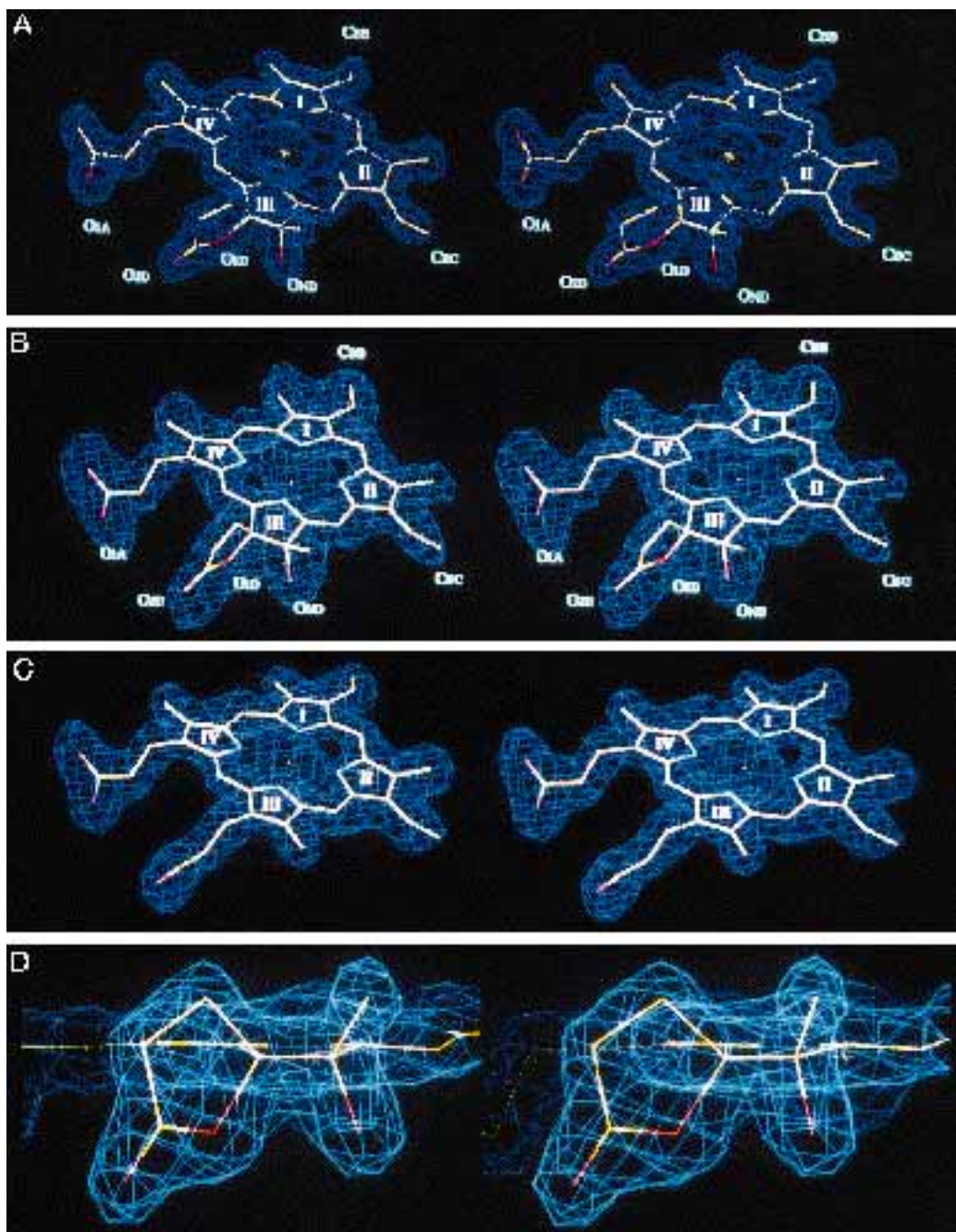


FIG. 2. Stereo views of ( $F_o - F_c$ ) omit maps of the heme groups of PVC (A), HPII (B), and the N201H variant (C). The structures of the prosthetic groups modeled inside the density are also shown. The proximal tyrosine ligand (not shown) resides in the lower part of the drawing (see Fig. 3), and the pyrrolic rings closer to the viewer are rings II and III. Ring III is modified in both PVC and native HPII but not in the N201H variant (see "Results"). D, detail of the heme *d* group of PVC. The quality of the electron density allows us to distinguish the relative positions of the methyl carbon and the hydroxylic oxygen atoms.

pears in the pyrrole ring opposite to the essential distal histidine, Fig. 3. Therefore, the heme *d* prosthetic group of PVC and HPII catalase is mainly the result of modification of the pyrrole ring III, in agreement with the biochemical data available for HPII (2).

In all heme-containing catalases whose structures have been reported, the heme prosthetic groups are well buried inside the tetramer, about 20 Å from the nearest molecular surface (6, 11–14). Therefore, the presence of heme *d* and the 180° rotation, with respect to BLC, of the prosthetic group in PVC and HPII can be assumed to be accommodated by peculiarities that are probably common to the heme pockets of both proteins. While the pyrrolic rings III and IV of heme *b* are symmetric with respect to a 180° rotation about the axis defined by the

$\alpha$ - $\gamma$ -meso carbon atoms, the ring I and ring II methyl and vinyl substituents exchange positions. Thus the observed preference in the orientation in which heme *b* binds to a particular heme protein is determined by the different contacts that these substituents make with the polypeptide chain. For PVC and HPII, the presence of a  $\gamma$ -spirolactone ring and an additional hydroxy group makes the heme *d* more asymmetric. Nevertheless, the N201H variant of HPII, which possesses an unmodified heme *b* group, exhibits the same heme orientational preference as the wild type enzyme and opposite to the one found in BLC. It is therefore logical to assume that the different contacts of the methyl and vinyl groups in one heme orientation or in the reverse are the main determinant of the orientation of the heme inside the pocket. Heme-contacting residues that appar-

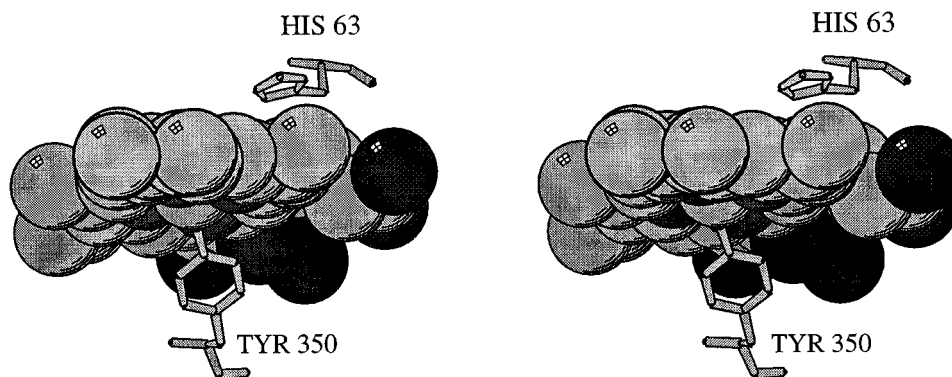


FIG. 3. **Van der Waals representation of the heme *d*.** The proximal tyrosine ligand (Tyr<sup>350</sup> in PVC) is shown to emphasize the orientation toward the proximal side of the ring III hydroxylic and lactonic oxygens, which can interact with the aromatic ring of the tyrosine. The modified ring is far from the essential histidine (His<sup>63</sup> in PVC).

ently govern the heme orientation in PVC (Ile<sup>41</sup>, Val<sup>209</sup>, Pro<sup>291</sup>, and Leu<sup>342</sup>) are preserved in HPII (Ile<sup>114</sup>, Ile<sup>279</sup>, Pro<sup>356</sup>, and Leu<sup>407</sup>) and show marked differences from the structurally equivalent residues in BLC (Met<sup>60</sup>, Ser<sup>216</sup>, Leu<sup>298</sup>, and Met<sup>349</sup>, respectively). However, none of the substitutions appears to introduce by itself a strong steric hindrance, and therefore, individually, they would not provide a definitive explanation for the observed heme inversion. It is also possible that other differences could be at play during the initial binding of the heme groups to catalase.

The interactions of the modified pyrrolic ring in the heme *d* group are represented for both PVC and HPII in Fig. 4. A hydrogen bond is formed between the hydroxy group (OND) of heme *d*, acting as the hydrogen donor, and the O- $\gamma$  oxygen of a serine residue (sequence number 349 in PVC and 414 in HPII). This serine is in turn hydrogen-bonded to the carboxylate oxygen of an aspartic residue (sequence number 53 in PVC and 118 in HPII) from another subunit related by a molecular dyad axis (6, 11). These interactions must contribute to the stabilization of the heme *d* with the hydroxyl oxygen pointing toward the proximal side. In BLC (12) and MLC (13) the equivalent residue is an alanine (residue 356 in BLC). However, in PMC (14) a serine residue is maintained in this position, although heme *d* has not been reported. O2D is hydrogen-bonded to a water molecule (Fig. 4), which in turn is bound to the unmodified propionic group. O2D also interacts with the NE2 atom of a glutamine residue (residue 354 in PVC) that is present in most catalase sequences but is replaced by histidine in BLC (residue 361). In PVC, but not in the HPII model, O2D can also form a hydrogen bond with the guanidinium group of Arg<sup>357</sup>. On the distal side, the modified pyrrolic ring forms almost exclusively hydrophobic interactions with the side chains of Ala<sup>61</sup> and Val<sup>62</sup> in PVC (Ile<sup>126</sup> and Val<sup>127</sup>, respectively, in HPII). The existence of these interactions further confirms the orientation of the heme *d* ring III oxygens toward the proximal side (Fig. 4). In both enzymes, the heme *d* oxygens O1D and OND are close to the aromatic ring of the tyrosine axial ligand, with several interatomic distances shorter than 3.5 Å (Fig. 3). These polar interactions (23) could alter the electronic distribution in the aromatic ring, influencing the catalytic properties of heme *d* in catalases.

The structure of the heme *b*-containing N201H variant of HPII allows us to analyze the changes introduced in the protein when heme *d* is present in the interior of the catalase molecule. As indicated above, the orientation of the heme is also reversed with respect to BLC in the structure of the N201H variant. Heme inversion is therefore previous and perhaps unrelated to heme *d* formation. The main structural changes between the wild type enzyme, containing heme *d*, and the N201H variant

are in the side chains of residues Gln<sup>419</sup> and Ile<sup>126</sup> (Fig. 4). In the N201H structure, the negative charge of the ring III propionic group is neutralized by two salt bridges with the guanidinium group of Arg<sup>422</sup> (Fig. 4C). In wild type HPII, this guanidinium group interacts with the OE1 oxygen of Gln<sup>419</sup>, whose side chain experiences an important rearrangement (Fig. 4B). The torsion angle  $\chi_1$  of residue Ile<sup>126</sup>, situated in the distal side above the modified pyrrolic ring, changes from  $-56^\circ$  in N201H to  $168^\circ$  in the native structure, probably to avoid contacts with the heme *d* carbon atoms CMD and CAD that project outside the heme plane. In PVC the equivalent residue is an alanine, and no reorientation is required.

Neither extra solvent molecules nor cavities have been detected in the two heme *d*-containing catalases in the vicinity of the modified pyrrolic ring. In fact, no important volume changes between the heme *b* and heme *d* structures are expected, since the increment in volume due to the presence of an extra hydroxy group in the modified heme *d* is, at least in part, balanced by the formation of the covalent bond between O1D and C3D to give the cyclic  $\gamma$ -lactone.

#### DISCUSSION

The present crystallographic study of PVC and HPII catalase active sites has shown that the major orientation of the prosthetic group in these two enzymes differs by a  $180^\circ$  rotation about the  $\alpha$ - $\gamma$ -meso carbon axis with respect to that found for the heme *b* group in BLC. However, there is also evidence of heme rotational disorder in the pocket of these two catalases, a phenomenon that has been reported for other heme proteins like myoglobin (24) and cytochrome *b*<sub>5</sub> (25), from NMR studies. The protein residues responsible for the observed preferences in the orientation in which the heme is bound and the possible functional role for the existence of heme rotational disorder in those proteins are subjects of continuing debate.

The chemical nature of the heme *d* in PVC and HPII catalase has been determined, and the presence of a hydroxy group and a  $\gamma$ -spirolactone ring with a relative *cis* configuration in the active form of the prosthetic group has been confirmed. The absolute configuration of the two carbon atoms of the modified pyrrolic ring III of the macrocycle has also been determined. The heme *d* prosthetic group, which is formally the result of the cyclization of the *cis*-diol (Fig. 1A), has both hydroxy groups directed toward the proximal side of the enzyme, while it is generally accepted that the oxygen chemistry occurs on the distal side of the prosthetic group (7). This apparently contradictory observation raises an interesting question regarding the biosynthesis of the *cis*-heme *d*, which will have to be taken into consideration by any mechanism that tries to explain the formation of this derivative.

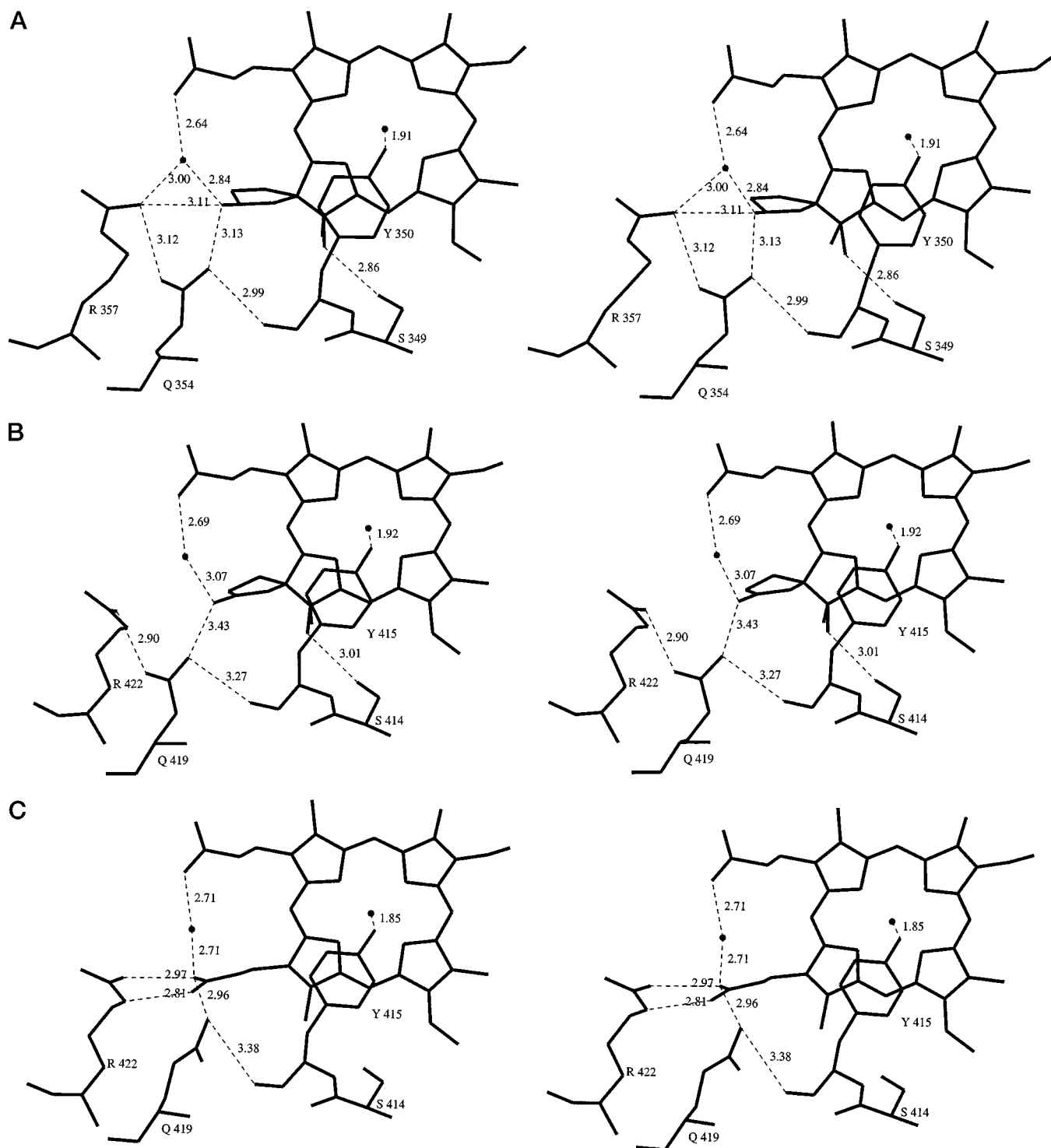


FIG. 4. Stereo views of the environment of the modified pyrrolic ring III of heme d in PVC (A) and HP11 (b) and of heme b in the N201H variant (C). Hydrogen bonds are represented by dotted lines, and the lengths are indicated (see "Results").

The electron density indicates that in both PVC and HP11 catalase, the heme modification occurs only in the pyrrolic ring opposite to the essential distal histidine. Thus the presence of a small percentage of rotationally inverted heme in the active sites of both PVC and HP11 catalase implies the existence of some ring IV modified heme in the interior of both. Experiments performed with the extracted prosthetic group of HP11 (7) showed the presence of a small fraction of an unidentified product, chromatographically distinct from the ring III modified heme d, which could correspond to this second heme d-type derivative.

The identification of a heme b group in the crystal structure of the inactive N201H variant of HP11 agrees with the hypothesis (1, 7) that heme b is not converted *in vivo* into heme d by a specialized hydroxylating system and subsequently incorporated into PVC or HP11 catalase. Rather, the heme b chromophore binds to the active site of the enzyme, where it is converted into the heme d derivative during the initial cycles of the catalytic turnover.

The high structural similarity of the heme binding pocket among catalases from different prokaryotic and eukaryotic sources does not imply the presence of the same prosthetic

group in all of them. The changes necessary to accommodate the modified heme d chromophore in the interior of the pocket are relatively small. Through the analysis of several catalase sequences we have observed that most of the residues that participate in the stabilization of the heme d are also present in other catalases not containing this prosthetic group. It is therefore unclear whether the presence of heme d is related only to those residues in the pocket or if some more distant effects also have a significant contribution.

What is the functional role of heme d in catalases? At present this question does not have a definite answer, and one can only speculate. PVC and HPII catalase exhibit, apart from the different heme d prosthetic group, other structural and functional peculiarities that are not shared by other well characterized catalases, which may or may not be related to the presence of heme d in the active site of these two enzymes. In particular, the presence of the extra C-terminal domain and heme d may be correlated, as they are both attributes of PVC and HPII.

BLC isolated from natural sources always contains a relatively large amount of bile pigments from the oxidative degradation of the heme prosthetic group in the interior of the enzyme (26). In contrast, PVC and HPII catalase contain almost exclusively heme d or, in certain cases, a small fraction of unmodified heme b. Heme d may be more resistant to the oxidative damage that heme b experiences during the catalytic turnover in BLC.

The catalytic cycle of a normally operating catalase involves two states of the enzyme, the resting Fe(III) state and the so-called compound I, which is the result of the reaction with one molecule of peroxide and is oxidized 2 equivalents above the resting enzyme. Compound I reacts with another molecule of hydrogen peroxide to give molecular oxygen and water, returning to the resting enzyme and so completing the catalytic cycle. However, under certain conditions, compound I of some catalases like BLC can be reduced by one electron to give compound II, which possesses an intermediate oxidation state between that of compound I and the resting enzyme. In turn, compound II can react with another molecule of peroxide to give compound III, a catalytically inactive intermediate. It is believed that the function of the bound nucleotide in BLC is to protect the enzyme from the formation of compound III, thus avoiding the inactivation of the enzyme (27). Interestingly, PVC and HPII catalase that possess the extra C-terminal domain with a "flavodoxin-like" topology lack the ability to bind NADPH. Nevertheless, HPII catalase compound I shows an extraordinary resistance to reaction with one electron donors to give compound II (28). According to the currently accepted electronic structure of the porphyrin macrocycle (29), the partial saturation of one of the pyrrole rings does not destroy the aromatic character of a chlorin-like derivative. One consequence of this is that the Fe(III)/Fe(II) reduction potentials of d-type hemes fall within the normal range observed for heme b derivatives (1). However, one cannot exclude the possibility that in the interior of the protein a substantial difference exists in the reduction potentials of the higher oxidation states involved in the catalytic turnover of catalase, which could explain the observed resistance of HPII catalase to form compound II. Other authors have noted the propensity of hydroxychlorin

derivatives to form hydrogen bonds and have suggested that this property may contribute to their biological function by helping to anchor them within the interior of the heme protein (30). Finally, if none of these correlations can be proved to be true, one can still consider the presence of heme d in PVC, HPII catalase, and very likely catalases from other sources as the equivalent to a neutral mutation in the primary sequence of an enzyme. In this hypothesis, heme d would be a "mutated" prosthetic group, whose modification would not affect the function of catalase. However, the fact that the structure of the heme d chromophore is well preserved in evolutionary distant organisms, like in the prokaryotic HPII catalase and the eukaryotic PVC, seems to argue against this possibility.

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