Factors Determining the Orientation of Axially Coordinated Imidazoles in Heme Proteins†

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Received March 1, 2001; Revised Manuscript Received April 30, 2001

ABSTRACT: Factors determining conformations of imidazole axially coordinated to heme in heme proteins were investigated by analyzing 693 hemes in 432 different crystal structures of heme proteins from the Protein Data Bank (PDB), where at least one histidine is ligated to heme. The results from a search of the PDB for protein structures were interpreted with molecular force field computations. Analysis of data from these crystal structures indicated that there are two main factors that determine the orientations of imidazole ligated to heme. These are the interactions of imidazole with the propionic acid side chains of heme and with the histidine backbone. From the analysis of the crystal structures of heme proteins, it turned out that the hydrogen bonding pattern is often not decisive, though it is probably used by nature to fine-tune the orientation of imidazole axially ligated to heme. We found that in many heme proteins the NδH group of imidazole ligated to heme can assume a number of different hydrogen bonds and that in mutant structures the orientation of the ligated imidazole often does not change significantly, although the mutant altered the hydrogen bonding scheme involving the imidazole. Data from crystal structures of heme proteins show that there are preferred orientations of imidazoles with respect to heme. Generally, the NδH group of imidazole is oriented toward the propionic acid groups of the heme. In some cases, the NδH group of imidazole is close to only one of the propionic acid groups, but it is practically never oriented in the opposite direction. The imidazole also adopts a preferred orientation with respect to its histidine backbone such that the plane of the imidazole ring is practically never parallel to the Cα−Cβ bond of its histidine backbone. For a given conformation of histidine backbone with respect to heme, as well as imidazole with respect to histidine backbone, the orientation of the imidazole with respect to heme is uniquely determined, since the three orientations depend on each other. Hence, the interaction of the imidazole with the backbone also influences the orientation of the imidazole with respect to the heme. Force field computations are in agreement with experimental data. With this method, we showed that there is an energy minimum when the NδH group of the imidazole is oriented toward the propionic acid groups and that there are minima of energy for orientations where the imidazole ring is orthogonal to the plane defined by the Cα−Cβ and Cβ−Cγ bonds of the histidine. The computations also demonstrated that these interactions are mainly of electrostatic origin. By taking into account these two major factors, we were able to understand the orientations of axially coordinated imidazoles for all groups of heme proteins, except for the group of cytochrome *c* peroxidase. In this group, the orientation of the imidazole is determined by a strong hydrogen bond of the NδH group with Asp235.

There are numerous proteins that contain heme as a cofactor. Many of them serve as redox-active groups to transfer electrons between different cofactors, which may be within the same protein or between different associated protein complexes. Heme proteins can serve also for transport and storage of small molecules such as oxygen in myoglobin (Mb)[1] and hemoglobin (Hb), respectively. The heme iron is typically hexacoordinated, but it sometimes can be pentacoordinated. Four coordination places are occupied by the pyrrole nitrogens of the porphyrin atom skeleton. Among the fifth and sixth ligands, the axial ligands, of heme iron in proteins are the amino acids histidine, methionine, cysteine, and tyrosine.

Because of the different biological functions of heme proteins, there are very important and interesting questions about the role of the protein in modulating the properties the iron−porphyrin cofactor. To understand the influence

† This work was supported by the Deutsche Forschungsgemeinschaft SFB 498, Project A5, the GRK 80/2, and the GRK 268, by the Fond der Chemischen Industrie with the BMFT, and by the Serbian Ministry of Science and Technology (Grant 02E09). S.D.Z. was supported by a DAAD fellowship.

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[1] Abbreviations: bis-Cc, cytochrome *c* with a bishistidine-ligated heme; Cb, cytochrome *b*; CcPo, cytochrome *c* peroxidase; Hb, hemoglobin; Mb, myoglobin; mono-Cc, cytochrome *c* with a monohistidine-ligated heme; PR, propionic acid group of heme; PRA, propionic acid at pyrrole ring A of heme; PRD, propionic acid at pyrrole ring D of heme.

10.1021/bi010428q CCC: $20.00
Published on Web 06/08/2001

of the protein environment on properties of the heme, recently synthetic peptides and proteins were synthesized (*1*−*5*). The axial ligands and their bonding geometry are very important in modulating the properties of the iron−porphyrin cofactor. By replacement of the axial ligands in heme proteins with different natural and non-natural residues, it was shown that axial ligands have a strong influence on spectral properties and redox potentials (*6*−*8*). Theoretical (*9*−*16*) and experimental (*17*−*30*) works on heme model systems demonstrate the influence of axial ligands on the properties of heme.

However, not only the type but also the conformation of the axial ligands can influence the properties of heme. In many heme proteins, one or even both of the axial ligands are histidines. The orientations of histidines, axially ligated to heme, are considered to have a strong influence on the function of heme cofactors in proteins. Different conformations can shift the redox potential (*31*), and they can control the coordination of substrates to heme proteins (*32*).

It was shown that the spectroscopic properties of ferriheme model complexes can depend on the mutual orientation of axially coordinated planar ligands. These complexes with parallel and perpendicular orientations of the axial ligands can have different spectroscopic properties, depending on the character of the electronic ground state (*17*). The same behavior with respect to the spectra is also characteristic for ferriheme proteins (*17*).

With experimental data on heme model systems and molecular mechanics studies, it was shown that the orientations of axial ligands have an influence on the porphyrin ring conformations. The planarity of a metalloporphyrin can be distorted by interaction with axial ligands. In complexes with a parallel orientation of two planar axial ligands, the porphyrin ring remains planar. For porphyrin complexes, which have two planar axial ligands in a perpendicular orientation, the porphyrin ring is almost invariably distorted from planarity (*17*, *18*, *33*, *34*).

It was shown that DFT calculations can successfully be used for transition metal complexes, including the first row transition metal complexes (*15*, *35*−*39*). DFT calculations on $[Fe(por)(py)_2]$ and $[Fe(por)(py)_2]^+$ showed that for Fe(II) there is no difference in the preference for parallel or orthogonal orientations of axially coordinated pyridines, but that for Fe(III) the orthogonal orientation is more stable (*40*).

Recently, $[Fe(TMP)(5\text{-}MeHIm)_2]ClO_4$ has been obtained in two distinct crystalline forms with different relative axial ligand orientations: one with an almost parallel and one with an almost perpendicular orientation (*41*). Hence, one can conclude that the two conformational isomers are energetically nearly equivalent. The energy balance between the two forms is the result of crystal field stabilization effects favoring the parallel form and steric effects that favor the perpendicular form. Estimates of the two opposing energetic effects are both less than 3 kcal/mol.

In heme model systems, the orientation of axial ligands can depend on crystal field stabilization effects or on steric effects caused by substituents on axial ligands and on the porphyrin (*17*, *41*, *42*). In heme proteins, the heme does not possess bulky substituents, but there is the protein environment, which can have an influence on the orientation of the axial ligands.

It can be supposed that a number of different factors can have an influence on the orientations of axially coordinated imidazoles in heme proteins. Among these are hydrogen bonds between the imidazole NδH groups and hydrogen bond acceptors of the protein, nonbonded interactions of the imidazole ring with the protein backbone and side chains, and nonbonded interactions of the imidazole with the porphyrin atom skeleton and the side chains of cysteines, which are bound covalently to the porphyrin ring in cytochrome *c* heme proteins.

To determine which of these factors can have a significant influence on the orientation of imidazoles ligated to heme in heme proteins, our approach in this work was first to analyze the influence of the hydrogen bonding pattern of imidazole axially coordinated to heme, and to investigate the heme−histidine conformations in the Protein Data Bank (PDB) (*43*) to see if specific orientations are preferred. It has been already shown that analysis of crystal structures of proteins can provide information about interactions in proteins (*44*, *45*). Searching in the PDB for crystal structures, we found in 432 heme proteins a total of 693 hemes to which at least one histidine is ligated. We discriminated among six groups of heme proteins, namely, myoglobin (Mb), hemoglobin (Hb), cytochrome *c* peroxidase (CcPo), two types of cytochrome *c* (mono-Cc and bis-Cc), and cytochrome *b* (Cb). In the mono-Cc (bis-Cc) group, the heme possesses one (two) histidine as an axial ligand. In crystal structures of heme proteins, the imidazole plane of axially coordinated histidines is to a good approximation orthogonal with respect to the heme plane. We monitored the orientation of imidazole from histidine ligated to heme relative to the heme, relative to the second imidazole in bishistidine-ligated hemes, and the orientation of these imidazoles with respect to their histidine backbone conformation and the mutual correlations between these orientations. This way, we found the conformational characteristics that are common for the different groups of heme proteins that we considered as well as the specific differences between them.

We have found that some orientations of axially coordinated imidazoles relative to the heme are preferred. The available experimental and theoretical data on heme model systems show that the interactions of imidazoles with the porphyrin atom skeleton or the mutual interactions of two axially coordinated imidazoles cannot be responsible for the preferred orientations observed from crystal structures of heme proteins. For model systems of imidazole heme complexes with nonhindered porphyrins, which do not possess bulky substituents, practically no barrier of rotation was found for axial imidazole ligands (*46*−*49*).

From these data, it was clear that there are other factors, which should be investigated in an effort to explain the orientations of imidazoles found in crystal structures. By searching the PDB, we analyzed the hydrogen bonding pattern of the axially ligated imidazoles. We used a molecular force field to evaluate interactions of the imidazole ring of histidine ligated to heme with the porphyrin atom skeleton, with the propionic acids, and, if available, with cysteines covalently bound to heme. We also investigated the influence of the histidine backbone on the orientation of the imidazole relative to the heme. For several groups of proteins, where these considerations could not provide an explanation, we checked the particular structures for specific hydrogen bonds

of the Nδ*H* group of the imidazole ring of histidine with components of the protein.

METHODS

Data Mining in the PDB

We found in the PDB 697 hemes in 432 different crystal structures of proteins, where at least one histidine is ligated to heme. For the sake of simplicity, we did not sort out protein structures possessing the same or nearly the same sequence. Since we are interested in general rules governing the relative orientation of imidazole axially ligated to heme and not in relative weights of statistical distributions from different groups of heme proteins, this approach is acceptable. The selected heme proteins were clustered in six groups. We found 138 monohistidine-ligated hemes in 133 proteins of the myoglobin (Mb) group (Table S1 of the Supporting Information), 323 monohistidine-ligated hemes in 121 proteins of the hemoglobin (Hb) group (Table S2 of the Supporting Information), 72 monohistidine-ligated hemes in 72 proteins of the cytochrome *c* peroxidase (CcPo) group (Table S3 of the Supporting Information), 99 monohistidine-ligated hemes in 68 proteins of the cytochrome *c* (mono-Cc) type (Table S4 of the Supporting Information), and 39 bishistidine-ligated hemes in 17 proteins of the cytochrome *c* (bis-Cc) type (Table S5 of the Supporting Information) and 26 bishistidine-ligated hemes in 21 proteins of the cytochrome *b* (Cb) type (Table S6 of the Supporting Information). In heme proteins of the cytochrome *c* group, the pyrrole rings of heme, which are opposite the propionic acid groups, are bound to cysteines covalently.

Characterizing Hydrogen Bonds Involving Imidazole Axially Ligated to Heme. Hydrogen bonds of the Nδ1−Hδ1 group of histidines axially ligated to heme in different crystal structures were detected by searching for suitable hydrogen bond acceptor atoms, which were closer than a cutoff distance of 3.5 Å to the Nδ1 atom of the imidazole ring. We were also searching for possibilities that the Nδ*H* group of an imidazole ring axially ligated to heme in heme proteins may form hydrogen bonds with other partners occurring for imidazole orientations, which differ from the native protein structure. For that purpose, the imidazole ring was rotated around the Nε2−Fe bond that it forms with heme without changing other parts of the crystal structure. We considered that another hydrogen bond can be formed, if the cutoff distance to the corresponding hydrogen bond acceptor atom is shorter than 4.0 Å for at least one orientation of the imidazole ring. Here, a slightly larger cutoff distance was chosen to account for possible structural relaxation of the actual crystal structure, which is not considered here but may go along with the formation of a hydrogen bond that differs from the crystal structure. To demonstrate the possibility of forming such hydrogen bonds for different orientations of the imidazole ring relative to the heme, we plotted the inverse of the corresponding atom pair distances for the possible hydrogen bonding partners that we found as a function of the pseudo-torsion angle α (defined below) characterizing the imidazole orientation. We chose the inverse of this atom pair distance since it provides a rough measure of the electrostatic interactions, which are relevant for the strength of the hydrogen bond.

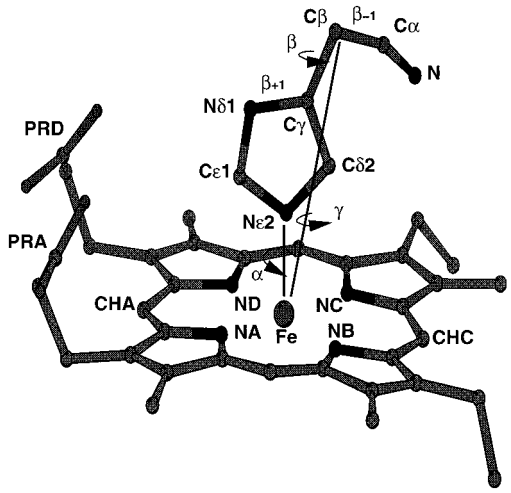

FIGURE 1: Definition of the three torsion angles (α, *β*, and *γ*) used to characterize the conformation of imidazole ligated to heme. The pseudo-torsion angle α (Cε1−Nε2−Fe−CHA) with the Nε2−Fe rotation axis denotes the orientation of the Nδ1H group of the imidazole relative to the center of the two propionic acid groups PRA and PRD defined by the CHA atom. The torsion angle *β* (Cα−C*β*−C*γ*−Nδ1) characterizes the orientation of the imidazole ring relative to the Cα−C*β* bond of the histidine backbone. The orientation of the histidine backbone with respect to the center of the propionic acids of the heme is measured by the pseudo-torsion angle *γ* (Cα−C*β*−FE−CHA). For the structure that is displayed, the values of the torsion angles are as follows: $\alpha \approx 0°$, $\beta \approx 180°$, and $\gamma \approx 180°$. The torsion angles obey the relation $\gamma = \alpha + \beta$.

Characterizing the Orientation of Ligated Imidazole Relative to Heme. The orientations of the histidines axially ligated to heme were derived from the PDB crystal structures of heme proteins. The N(His)−Fe bond length of histidines ligated to heme is close to 2.0 Å. With few exceptions, the N(His)−Fe−N(heme) bond angle is close to 90°. The imidazole rings of the histidine ligands are nearly orthogonal to the corresponding heme planes. In particular, we paid attention to the following torsion angles characterizing the orientation of the histidine relative to the heme (see Figure 1).

(1) The orientation of the imidazole ring with respect to the propionic acids of the heme is measured by the pseudo-torsion angle α (Cε1−Nε2−FE−CHA). This angle describes the imidazole ring rotation around the Nε2−FE axis forming the bond of the imidazole with the heme iron.

(2) The orientation of the imidazole ring of the histidine relative to the polypeptide backbone is characterized by torsion angle *β* (Cα−C*β*−C*γ*−Nδ1).

(3) The orientation of the histidine backbone with respect to the propionic acids of the heme is measured by the pseudo-torsion angle *γ* (Cα−C*β*−FE−CHA).

(4) The relative orientation of the imidazole planes of two histidines (1 and 2), which are axial ligands at the heme, is characterized by the difference of the corresponding torsion angles ($\Delta\alpha = |\alpha_2 - \alpha_1|$).

As usual, the zero-point value for the definition of the torsion angles corresponds to the cis conformation. The rotation sense of torsion angles α, *β*, and *γ* is considered positive; if applied to the heme, it moves the propionic acid group PRD toward PRA via the acute angle, as indicated by the arrows in Figure 1. Note that the two propionic acid groups of heme can be discriminated by the different bonding

patterns of heme substituents at pyrrole rings B and C opposite PRD and PRA, respectively.

Due to the sp^2 hybridization of the Cγ atom, the four atoms (Cβ, Nδ1, Cδ2, and Cγ) of histidine are in the same plane. Thus, torsion angle β also defines the relative orientation of the imidazole plane with the plane defined by atoms Cα, Cβ, and Cγ. Therefore, the three torsion angles (α, β, and γ) defined above (Figure 1) characterize the relative orientation of only three different planes, which are given by atoms Cα, Cβ, and Cγ (histidine backbone), atoms Nϵ2, FE, and CHA (plane orthogonal to heme), and the imidazole plane. Hence, these torsion angles are not independent and fulfill the relation

$$\gamma = \alpha + \beta \quad (1)$$

This relationship allows computation of the probability distribution of one of the three angles, if the distributions of the other two angles are given. Let us, for instance, assume that the probability distributions for angles α and β are known. Then we can express the distribution in γ as a convolution of distributions $a(\alpha)$ and $b(\beta)$ according to

$$g(\gamma) = \int_0^{2\pi} a(\gamma-\beta)b(\beta)\,d\beta \equiv \int_0^{2\pi} a(\alpha)b(\gamma-\alpha)\,d\alpha \quad (2)$$

Despite the simple relation among the three probability distributions, it will be necessary to visualize all three distributions to understand the relationships between the torsion angles and their consequences.

The data obtained in this way were grouped according to the different heme protein families, and the corresponding torsion angle distributions were represented graphically by generating histograms with 36 bins, each with a width of 10°. To obtain a clearer representation of the data, the values of the angular distribution functions were connected by continuous lines.

Molecular Force Field Computations

To understand the preference for the orientation of histidine ligated to heme, we calculated the energies of different imidazole heme conformations using CHARMM22 (*50*) with a dielectric constant ϵ of 1 as is typically used for this force field. We varied the above-defined torsion angle α continuously and monitored the interaction of the imidazole ring with the heme considering different groups of atoms and interaction types. In the case of cytochrome *c*, we also considered the interaction of the imidazole with the covalently bound cysteines.

To investigate the influence of the protein backbone conformation, we assessed the interaction of the imidazole ring of histidine with its protein backbone. To model the histidine backbone as part of a larger piece of protein backbone and to avoid artifacts from bare charges, the C-terminus was amidated and the N-terminus methylated. There are three torsion angles between the imidazole ring and the protein backbone of histidine, which can be varied to change the histidine conformation relative to its backbone. These are β_{-1} (N−Cα−Cβ−Cγ), β (Cα−Cβ−Cγ−Nδ1) and β_{+1} (Cβ−Cγ−Nδ1−Cϵ1). For a definition of the corresponding atoms and rotation axes, see Figure 1. The center torsion angle β is the most relevant for characterizing the different orientations of the imidazole ring. Therefore, the energy dependence of the histidine backbone conformation is monitored with respect to this torsion angle. To obtain an essentially unconstrained histidine conformation, we evaluated the energy dependence on torsion angle β for fixed values of β, while all other degrees of freedom, in particular, also torsion angles β_{-1} and β_{+1}, were allowed to relax by applying energy minimization with respect to all degrees of freedom except torsion angle β.

RESULTS AND DISCUSSION

In this study, we investigated the orientation of the imidazole ring plane of histidine axially ligated to heme in heme proteins by considering the crystal structures of the PDB, as well as by performing molecular force field computations in relation to characteristics of the heme conformation and hydrogen bonding pattern. We identified six groups of heme proteins, where the heme is ligated with at least one histidine. Four groups of heme proteins contain 632 monohistidine-ligated hemes, namely, myoglobin (Mb), hemoglobin (Hb) (except one mutant), cytochrome *c* peroxidase (CcPo), and a subset of cytochrome *c* (mono-Cc), whose PDB codes are listed in Tables S1−S4 of the Supporting Information. Two groups of heme proteins possess 65 bishistidine-ligated hemes. These are a subset of cytochrome *c* (bis-Cc) and all cytochrome *b* (Cb) heme proteins. Their PDB codes are listed in Tables S5 and S6 of the Supporting Information. We investigated the influence of hydrogen bonds formed by the NδH group of imidazole axially coordinated to heme. We generated distributions of torsion angles from the PDB of crystal structures of these heme proteins, which are appropriate for characterizing histidine heme conformations. To understand the results, we subsequently correlated them with molecular force field calculations.

Imidazole Heme Conformations: General Survey of PDB Data

The imidazole ring plane is practically orthogonal to the heme plane. Therefore, a single torsion angle is sufficient to characterize the imidazole heme conformation. For that purpose, we were choosing torsion angle α (Cϵ1−Nϵ2−FE−CHA) (see Figure 1). The zero value of this torsion angle corresponds to the imidazole ring conformation, where the Nδ−H bond vector of imidazole projected on the heme plane is placed between the two propionic acids groups PRA and PRD. The orientation of the acidic groups of the propionates relative to the heme may vary due to the flexibility of their chains. As a rough measure of their orientation projected on the heme plane, we consider the corresponding pyrrole nitrogens, which are located at angular values α of 45° and −45°, respectively.

Generally, the resolution of protein crystal structures is too low to allow direct discrimination between imidazole ring orientations of a histidine that differ by 180°, because the difference in the electron densities of nitrogen and carbon atoms is too small. Therefore, the orientation of an imidazole ring in a protein crystal structure is determined according to the possible hydrogen bonding schema involving the two nitrogen atoms (Nδ1 and Nϵ2). However, for an imidazole axially ligated to heme, this ambiguity in the orientation of the imidazole ring can be lifted for higher-resolution crystal

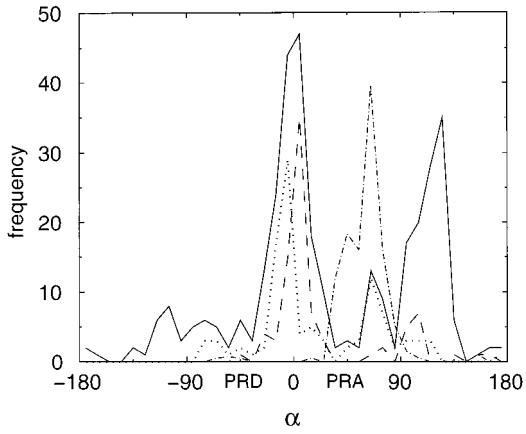

FIGURE 2: Distributions of the torsion angle α of the imidazole heme conformation for different groups of heme proteins from the PDB: (—) imidazoles of all heme proteins with the exception of the Mb and Hb groups (Tables S3−S6), (− − −) heme proteins of the mono-Cc group, (•••) heme proteins of the bis-Cc group, and (−•−) proteins of the Mb and Hb groups (Tables S1 and S2) scaled down by a factor of 4. The approximate orientation of the propionic acid groups PRA and PRD is marked on the *x*-axis.

structures, since from the two imidazole ring fractions between the Cγ atom connecting to the backbone and the Nϵ2 atom binding to the heme iron the ring fraction involving two atoms (Nδ1 and Cϵ1) is more bulky than the other involving only the Cδ2 atom (Figure 1).

Figure 2 shows the distribution of the pseudo-torsion angle α for all heme proteins with at least one histidine ligated to heme. The solid line represents data for all heme proteins but excluding data from the Hb and Mb groups. The two groups of cytochrome *c* heme proteins, namely, mono-Cc (dashed line) and bis-Cc (dotted line), are displayed by individual distributions. The distribution of the pseudo-torsion angle α was considered separately for the heme proteins of the Hb and Mb groups (dashed dotted line, scaled down by a factor of 4), since they constitute two large groups with a high degree of sequence homology and with a number of structures, which have nearly identical sequences.

In many of the crystal structures of heme proteins, the value of angle α is close to 0°, but there are also a number of structures where α is ∼120° and for the Hb and Mb groups α is between ∼30° and ∼70° (Figure 2, dashed−dotted line). In a small number of crystal structures, α can also adopt other values, but there are practically no structures of heme proteins with a value of α close to ±180° (Figure 2). Since the propionic acid groups are located at approximately 45° (PRA) and −45° (PRD), it is obvious that in most crystal structures the NδH group of imidazole is oriented toward both propionic acids (α = 0°) or at least toward one propionic acid group (α = 60°), but practically never in the opposite direction (α = ±180°). Therefore, we suspect that there is an attractive interaction between the imidazole ring and the propionic acid groups, which influences the orientation of imidazole relative to the heme.

However, since the orientation of the imidazole was not in all cases toward the propionic acid groups (with an angle α of ∼0°), it was obvious that there are other factors that are influencing the orientation of the imidazole. To understand these factors, we investigated the interactions of the imidazole with the propionic acid groups, with the backbone of the corresponding histidine, with covalently bound cysteines, and with possible hydrogen bonding partners.

Hydrogen Bonding Scheme for Imidazole Axially Coordinated to Heme

The Nδ1 atom of histidine axially coordinated to heme is protonated and can therefore act as donor group in a hydrogen bond. For more than 98% of all imidazoles axially coordinated to heme in the considered crystal structures of heme proteins, we found suitable hydrogen bonding partners less than 3.5 Å away from the Nδ1 atom. For the remaining 2%, the hydrogen bonding partner atom was more than 3.5 Å away from the Nδ1 atom. In 7.5% of the considered ligated histidines, a water oxygen atom is the hydrogen bonding partner. The most probable hydrogen bonding partner of the NδH group of the imidazole was found to be a CO group from the protein backbone. The residue number and type involved in this hydrogen bond vary, depending on the variation in sequence and structure of the group of heme proteins being considered. In the following, we will discuss the results that we obtained from data mining in the PDB.

Hydrogen Bonds of Axially Coordinated Imidazoles in Myoglobin and Hemoglobin. Myoglobin and hemoglobin are heme protein families whose members possess a very high degree of sequence and structure identity. Consequently, one can expect that hydrogen bonds involving axially coordinated imidazoles will generally show the same pattern. For practically all members of the Mb and Hb heme protein families, the backbone CO group that is four residues below the coordinated histidine forms a hydrogen bond with the NδH group of the imidazole ring. Generally, in both heme protein families, the propionic acid group PRA points toward the imidazole ring of the axially ligating histidine, whereas the propionic acid group PRD points away from the imidazole ring.

(1) Mb. For most proteins of the Mb group, the Hδ atom of His93, which is axially ligated to heme, forms simultaneously hydrogen bonds with Leu89 and Ser92. The part of the Leu89-Ala90-X91-Ser92-His93 (where X is Gln, Glu, or Asn) sequence involved in hydrogen bonding is largely conserved. The bifurcated hydrogen bond of the imidazole can be asymmetric and in this way allows a large variation in the orientation of the imidazole plane relative to the heme, giving rise to α values ranging from 20° to 78°. Hence, the bifurcated hydrogen bond can be considered to be a means of fine-tuning the orientation of the imidazole ring relative to the heme over a larger angular regime. But, for most Mb crystal structures, the value of the pseudo-torsion angle α is close to 45°. This is just the orientation where the NδH group of the imidazole ring points toward the propionic acid group PRA, whose oxygen atoms are oriented toward the imidazole ring.

Effects of mutation of conserved residue Ser92 to Ala, Val, and Leu were studied in pig Mb by Smerdon et al. (*51*). They found that the binding affinity for O_2, CO, and CN ligands increased with the mutations. But, although the hydrogen bonding pattern near the heme changes and the NδH group of His93 loses one hydrogen bond, the imidazole ring of His93 does not change its orientation remarkably. Also, in the Mb structure classified as PDB entry 1rse, Ser92

was mutated to Asp. Again, the orientation of the imidazole ring did not change and the pseudo-torsion angle $\alpha = 50°$, remaining close to the most probable value for the native proteins. In contrast to Ser92, Asp92 does not make a hydrogen bond with the imidazole ring of His93. That is presumably due to repulsive electrostatic interactions of the acidic group of Asp92 with the propionic acid group PRA, which is pointing toward the imidazole ring. In a few crystal structures of Mb from sea hare, residue 91 is a phenylalanine. Its carbonyl backbone oxygen atom makes the only hydrogen bond with His95, which is axially ligated to heme. The pseudo-torsion angle α characterizing the orientation the imidazole ring adopts in this case is between 64° and 78°, slightly larger than the most probable value of 45°. This change in orientation is probably due to a repulsive interaction of the negatively charged acidic groups between Glu94 and the propionic acid PRA. As a consequence, the propionic acid group moves away from the imidazole ring, which diminishes its influence on the orientation of the imidazole.

(2) Hb. Also, in the Hb family of heme proteins, the NδH group of His87 (or His92) ligated to heme forms a hydrogen bond with the backbone CO group at sequence position 83 (or 88) four residues away from the ligating histidine. The residue type involved in hydrogen bonding varies between Leu, Phe, Val, and Lys, although it is predominantly Leu. In contrast to proteins of the Mb family, there is no bifurcating hydrogen bond, since in most Hb structures the corresponding serine is missing. The pseudo-torsion angle α is $\sim$65°.

In some Hb structures (PDB entries 1eca, 1ecd, 1ecn, and 1eco), the heme is placed upside down in the binding pocket, which means that it is rotated around the CHA−CHC axis (see Figure 1) by 180°. Consequently, the pseudo-torsion angle α adopts a value of −50° and the propionic acid group PRD is oriented toward the imidazole ring of the ligating histidine. In these Hb structures that contain serine at position 86 and phenylalanine at position 83, the hydrogen bond with the Oγ atom of Ser86 is stronger than with the backbone oxygen of Phe83. Also in the Hb structure classified as PDB entry 1ith, residue Ser93 is a neighbor of the ligating histidine His94. Nevertheless, also in this structure, only Lys90 forms a hydrogen bond with His94.

In a few Hb structures, other residues are involved in hydrogen bonding with the NδH group of the ligated histidine. In the Hb structures classified as PDB entries 1vhb and 2vhb, His85 forms a strong hydrogen bond with the acidic oxygen of Glu137 instead with the backbone CO group of Ile81. The value of the pseudo-torsion angle α (150°) is here unusually large. In a mutant Hb structure from sea cucumber classified as PDB entry 1hlb, where two histidines (His104 and His73) are axially coordinated to heme, the Oη atom of Tyr114 forms a hydrogen bond with His104 ($\alpha = 125°$) and a water oxygen forms a hydrogen bond with His73 ($\alpha = -163°$). In the wild-type structure (PDB entry 1hlm), His104 forms the corresponding hydrogen bond with Leu100 and the value of the pseudo-torsion angle $\alpha = 40°$.

Hydrogen Bonds of Axially Ligated Imidazoles in Cytochrome c Peroxidase. The members of the family of CcPo heme proteins have also a high degree of structure and sequence identity. In the native CcPo heme proteins, the NδH group of the ligating imidazole forms a strong hydrogen bond with the acidic oxygen of an aspartate. There are two different subsets of CcPo heme proteins: one where Asp235 forms a hydrogen bond with the imidazole ring of the coordinated His175 and one where Asp246 forms a hydrogen bond with the imidazole ring of the coordinated His184. The corresponding values of the pseudo-torsion angle α characterizing the orientation of the imidazole ring relative to the heme are in the ranges of 115−125° and 100−125°, respectively. In the CcPo structure classified as PDB entry 1ccc, Asp235 is mutated to alanine. As a consequence, His175 ligating to heme has to find a new hydrogen bonding partner. Interestingly, the NδH group of the imidazole ring of His175 does not reorient to form a hydrogen bond with another residue of the protein but essentially maintains its orientation that it assumes in the nonmutated structure ($\alpha = 100°$) and forms a hydrogen bond with a water molecule. Also in the CcPo structures with mutants Asp235Asn and Asp235Glu ($\alpha = 122°$ and 97°, respectively), there are only minor changes in the orientation of the imidazole ring relative to heme.

Hydrogen Bonds of Axially Ligated Imidazoles in Monohistidine-Ligated Cytochrome c. We investigated the crystal structures of 68 monohistidine-ligated cytochrome *c* (mono-Cc) heme proteins from the PDB. In *c*-type cytochromes, the heme is covalently bound to the polypeptide chain by two cysteines, which bind at pyrrole rings B and C opposite the propionic acid groups. With regard to structure and sequence homology, we can discriminate three subgroups and some individual cytochrome *c* proteins that do not fit in one of these subgroups. The largest subgroup (mono-Cc124) contains cytochrome *c* isozyme 1 and 2, cytochrome c_2 (formerly c_{550}), cytochrome c_4, cytochrome c_{551l}, and cytochrome c_1 from the cytochrome bc_1 complex. The second subgroup (mono-Cc6) consists of cytochrome c_6 (also called c_{553}) heme proteins. The members in the first two subgroups are globular proteins with a large α-helical content. The cytochrome *c*′ (mono-Cc′) heme proteins constitute a third subgroup. The structure of these heme proteins is a four-helix bundle with one monohistidine-coordinated heme.

(1) Mono-Cc124. For all members of this subgroup, the tertiary structure is very similar. The carbonyl backbone oxygen of a proline, which is typically 12 or 18 residues away from the coordinated histidine, forms a hydrogen bond with the histidine. But in some cases, the proline can also be 70 (Cyt c_1 from the Cyt bc_1 complex) or only 10 residues away from the coordinating histidine. The residues of the polypeptide segment between the histidine and proline adopt a loop structure. The pseudo-torsion angle α characterizing the imidazole heme orientation adopts values between −25° and 20°.

(2) Mono-Cc6. These heme proteins have a globular α-helical structure similar to that of the previous group, but the level of sequence identity with the mono-Cc124 cytochromes is low. Here, the hydrogen bond is formed between the coordinated histidine and the backbone CO group of a glycine, arginine, or asparagine. The corresponding pseudo-torsion angle α adopts values between −25° and −10°. Interestingly, in all of these structures, there is also a proline too far away to form a hydrogen bond with the coordinated histidine.

(3) Mono-Cc′. In the crystal structures of these heme proteins, the coordinated histidine is solvent accessible and

the NδH group of the imidazole ring forms a hydrogen bond with a water molecule. The charges of the propionic acids are screened by three arginines, which prevent an orientation of the imidazole ring with the NδH group pointing toward the propionic acid groups. Correspondingly, the pseudo-torsion angle α is between 80° and 120°, but in most cases, it is close to 100°.

Also, cytochrome *f* belongs to the group of monohistidine-ligated cytochromes. The globular protein has predominantly β-strand structure. In this heme protein, the NδH group of the coordinated histidine forms a hydrogen bond with a water molecule and is oriented toward the propionic acid groups ($\alpha = 10°$).

Hydrogen Bonds of Axially Ligated Imidazoles in Bis-histidine-Ligated Cytochromes. They involve two groups of heme proteins, namely, cytochrome *b* (Cb) and bishistidine-ligated cytochrome *c* (bis-Cc). Each of these groups is very heterogeneous, since they consist of different subgroups of heme proteins with different three-dimensional structures. Since in the different subgroups no mutants are available, the hydrogen bonding scheme of the coordinated histidines does not vary in each of the subgroups. For some of the coordinated histidines, no hydrogen bonding partner was found. Hence, for this group of heme proteins, it was not possible to conclude whether the hydrogen bonding scheme has an influence on the orientation of the imidazole ring of histidines coordinated to heme. Nevertheless, the coordinated histidines exhibit a uniform orientation of their imidazole rings, whose NδH groups are generally oriented toward the propionic acid groups.

Influence of Hydrogen Bonds on the Imidazole Orientation in Heme Proteins. In myoglobin, the orientation of imidazoles axially ligated to heme varies to some extent through the bifurcated hydrogen bond involving the NδH group of imidazole. However, mutants where the hydrogen bonding partner of the axially ligated imidazole was exchanged did not show a significant effect on the orientation of the imidazole ring. In hemoglobin, only in one case did we find a significant change in the imidazole orientation, where the axially ligated imidazole forms a strong hydrogen bond with the side chain of a negatively charged glutamate. In CcPo heme proteins, the axially ligated imidazole generally forms a strong hydrogen bond with a negatively charged aspartate. After mutation of that residue to alanine, the NδH group of imidazole forms a hydrogen bond with a water, but the imidazole ring does not change its orientation significantly. In all CcPo heme proteins, the value of the pseudo-torsion angle α differs considerably from a value close to zero, corresponding to an orientation where the NδH group of imidazole points toward the propionic acid groups. With the exception of cytochrome *c′*, where the negative charges of the propionic acids are screened by positive charges of three arginines, the value of the pseudo-torsion angle α is close to zero in all mono-Cc structures. In summary, with very few exceptions, imidazole axially coordinated to heme exhibits only minor changes in the orientation relative to the heme while the hydrogen bonding scheme is varied.

Possible Hydrogen Bonds of Imidazoles Axially Ligated to Heme in Heme Proteins. To obtain a better understanding of the role of hydrogen bonds formed by axially ligated imidazoles in heme proteins, we investigated the possibilities that the NδH group may form a hydrogen bond that differs from the one assumed in the native structure. For that purpose, we rotated the imidazole ring of axially ligated histidines around its Nδ−Fe bond and monitored the distance of the Nδ atom to possible hydrogen bond acceptor atoms as a function of pseudo-torsion angle α. We studied the possible existence of these hydrogen bonds for a large number of different heme proteins. Typical results from these studies were collected in Table 1 and are displayed in Figure 3. Table 1 provides an overview of possible hydrogen bonds involving the axially ligated imidazole by displaying two typical examples for each of the six groups of heme proteins that we consider. For the 12 heme proteins that are displayed, we found four possible hydrogen bonds in four cases, three possible hydrogen bonds in another four cases, two possible hydrogen bonds in two cases, and just the hydrogen bond assumed in the crystal structure in two of the twelve heme proteins that were considered. We used a rather conservative distance criterion for the detection of hydrogen bonds. If it is assumed that the protein structure would readjust to make a different hydrogen bonding pattern possible, the number of possible hydrogen bonds might even be larger.

Part A of Figure 3 demonstrates the typical hydrogen bonding pattern of myoglobin. One can clearly see the existence of the bifurcated hydrogen bond with Leu89 and Ser92. In addition, there is a hydrogen bond with the propionic acid group PRA, which is more distant than the other two hydrogen bonding partners but can be relatively strong, since the PRA group is negatively charged giving rise to more long-range electrostatic interactions. Interestingly, the orientation in the native structure points directly toward this propionic acid group. In part B of Figure 3, there are four possible hydrogen bonding partners where the axially ligated histidine of the mono-Cc heme protein points in very different directions. The distance to the possible bonding partner Cys17 is even smaller than the one assumed in the crystal structure. However, as we will learn later, this hydrogen bond cannot be formed due to unfavorable interactions with the backbone of the ligated histidine. Hence, a hydrogen bond is formed with Pro30 such that the NδH group of the imidazole points toward the propionic acid groups. For the bis-Cc heme protein, the axially ligated His109 has two more hydrogen bonding partners (part C of Figure 3). Here, a hydrogen bond with Cys105 would not be prevented by unfavorable interactions with the histidine backbone. But, in the crystal structure, the NδH group of the axially ligated imidazole forms a hydrogen bond with Gly24. That again corresponds to an orientation where the NδH group of the imidazole points toward the propionic acid groups of the heme.

Role of Propionic Acids in Imidazole Heme Conformation

Analyzing Data from the PDB. Since we suspected that the propionic acid groups may play an important role for the conformations of histidine ligated to heme, we investigated how the conformations of the two propionic acid groups attached to the heme correlate with the distribution of the torsion angle α. We discriminated five distinct conformations of the propionic acids: (i) both propionic acid groups pointing toward the ligated histidine (that conformation is depicted in Figure 1), (ii) both propionic acid groups in the heme plane, (iii) both turned away from the ligated histidine, (iv) only PRA pointing toward the ligated histidine,

Table 1: Possible Hydrogen Bonds of Imidazole Axially Ligated to Heme

	heme protein/resolution/ PDB entry/chain[a]	residue (atom type)/ ligated histidine[b]	distance (Å)[c]	angle α[d] (deg)	angle γ[e] (deg)
1	Mb/1.75 Å/1myg/B	Leu89 (O)/His93	2.72 (2.89)	97 (37)	
		Ser92 (Oγ)/His93	2.91 (2.93)	27	−30
		heme PRA (O2A)	3.64	37	
2	Mb/1.90 Å/5mba	Phe91 (O)/His95	3.07 (3.08)	79 (69)	−10
3	Hb/2.00 Å/1a0x	Leu83 (O)/His87	2.71 (2.71)	61 (61)	−25
4	Hb/1.40 Å/1ecn	Ser86 (Oγ)/His87	2.80 (2.84)	−30 (−50)	24
		Phe83 (O)/His87	3.03 (3.19)	−100 (−50)	
		Ser86 (O)	3.41	40	
		H_2O63	3.79	−40	
5	CcPo/2.20 Å/1ccp	Asp235 (Oδ1)/His175	2.90 (2.90)	124 (124)	
		Asp235 (Oδ2)	3.58	84	
		Met172 (O)	3.20	−156	−72
		Ala174 (O)	3.90	−86	
6	CcPo/1.60 Å/1arv	Asp246 (Oδ2)/His184	2.87 (2.89)	85 (95)	
		Asp246 (Oδ1)/His184	3.20 (3.25)	120 (95)	−72
7	mono-Cc/1.97 Å/1chh	Pro30 (O)/His18	2.73 (2.75)	−3 (7)	
		Cys17 (O)	2.55	−113	
		Gly29 (O)	3.49	−63	−93
		Cys14 (O)	3.59	167	
8	mono-Cc/1.80 Å/1cgo	H_2O 226/His120	2.91 (2.92)	112 (102)	
		Cys116 (O)	2.80	−158	−92
		H_2O270	3.63	62	
9	bis-Cc/2.16 Å/1czj	Gly24 (O)/His109	2.66 (2.66)	−3 (−3)	
		Cys105 (O)	3.21	−173	−96
		Cys108 (O)	3.91	−113	
10	bis-Cc/2.16 Å/1czj	Tyr73 (O)/His77	2.66 (2.68)	−1 (15)	
		Tyr73 (Oη)	3.77	105	−86
		Phe76 (O)	2.99	−115	
11	Cb/1.50 Å/1cyo	Gly42 (O)/His39	2.75 (2.75)	−96 (−96)	
		Gly41 (O)	3.78	−46	40
12	Cb/1.50 Å/1cyo	Phe58 (O)/His63	2.71 (2.71)	−114 (−114)	
		Val61 (O)	3.73	−34	11
		heme PRA (O2A)	3.93	36	
		H_2O509	3.31	−4	

[a] Group of heme proteins to which the considered protein belongs (Mb, Hb, CcPo, mono-Cc, bis-Cc, or Cb), resolution of crystal structure, PDB entry, and chain considered. [b] Residues (type and number) and corresponding atom types in parentheses involved in a hydrogen bond with the axially coordinated histidine. The residue listed first is the actual hydrogen bonding partner in the crystal structure. [c] Distance of closest approach of non-hydrogen atom pairs, which could possibly be involved in a hydrogen bond with the Nδ*H* group of histidine axially ligated to heme. The distances were varied by rotating the imidazole ring with respect to the pseudo-torsion angle α. The distance of the actual hydrogen bond is given in parentheses. [d] Pseudo-torsion angle α of closest approach as defined in footnote c. For a definition of the angle α, see the legend of Figure 1. The angle α in the actual crystal structure is given in parentheses. [e] For a definition of the pseudo-torsion angle γ, see the legend of Figure 1.

and (v) only PRD pointing toward the ligated histidine. The corresponding distributions of the torsion angle α for all histidine-ligated heme proteins of the bis-Cc and Cb groups are depicted in Figure S1A of the Supporting Information for propionic acid conformations i−iii and in Figure S1B for propionic acid conformations iv and v (see the Supporting Information). From Figure S1A, it becomes obvious that the Nδ*H* group of imidazole ligated to heme is with high probability located between the propionic acid groups of the heme, if both propionic acid groups point toward the imidazole. Also in the case where the propionic acid groups are located in the heme plane or below (types ii and iii), there is still a preference for this orientation. But no clear difference in the angular dependence could be observed between conformations, where both propionic acid groups are in the heme plane (ii) or turned away (iii) from the ligated imidazole. If the propionic acid groups are on different sides of the heme plane, the Nδ*H* group of the imidazole ligated to heme preferentially points toward the propionic acid group, which is on the same side of the heme plane (see Figure S1B of the Supporting Information). There are a number of exceptions where the pseudo-torsion angle α adopts a value larger than 90° or smaller than −90°, if only one propionic acid group points toward the imidazole ring, which is axially coordinated to heme. These exceptions appear mainly for heme conformations, where the propionic acid group PRA points toward the imidazole. The majority of the corresponding heme proteins belong to the cytochrome b_5 subgroup.

Force Field Computations on Interactions of Imidazole with Propionic Acid of Heme. To understand the influence of the propionic acid groups on the imidazole orientations relative to the heme in more detail, we performed specific force field computations using CHARMM22 (*50*). Although the aim of these force field computations is to elucidate the general principles of imidazole−heme interaction, we considered atomic coordinates from appropriate crystal structures of heme proteins instead of using coordinates from idealized model structures. However, we evaluated only the energy of the interaction between the imidazole ring and the heme atoms. For these computations, the two propionic acid groups of heme were considered to be negatively charged unless denoted otherwise.

We considered the crystal structures of two cytochrome c_3, PDB entries 1aqe (*52*) and 2cth (*53*). In both proteins, the hemes are axially coordinated by two histidines. In 1aqe, the axially ligated histidines are His109 and His77. In this structure, the propionic acid groups of the heme are both located on the same side of the heme plane as His109. Hence,

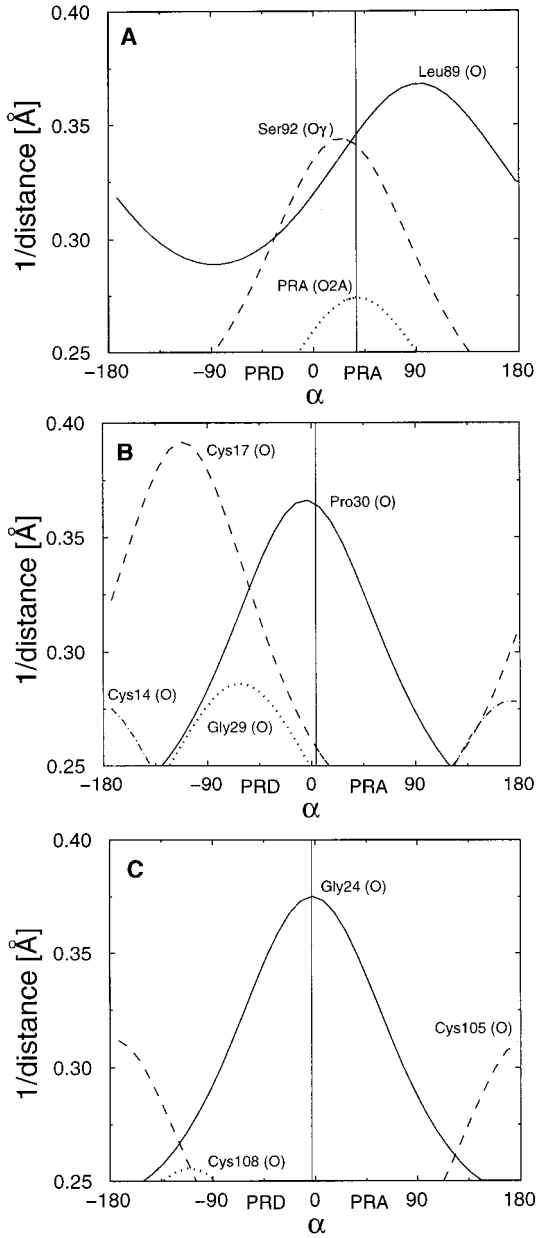

FIGURE 3: Possible hydrogen bonds of imidazole axially coordinated to heme in heme proteins. The hydrogen bonds of axially coordinated imidazole are studied by rotation of the imidazole ring around its Nδ−Fe bond and monitoring the distance between the Nδ1 atom and possible hydrogen bond acceptor atoms. In the figure, the inverse of this distance, which is a rough measure of the hydrogen bonding strength, is displayed as a function of the pseudo-torsion angle α as defined in Figure 1. Only hydrogen bonding partners whose minimum distance to the Nδ atom is smaller than 4.0 Å are considered. The vertical solid line marks the value of the pseudo-torsion angle α of the crystal structure. (A) Heme protein Mb (PDB entry 1myg, chain B). Possible hydrogen bonds are formed with the backbone oxygen of Leu89, with the side chain oxygen of Ser92, and with the acidic group of propionic acid PRA. For the optimal orientations, the corresponding values of α are 97°, 27°, and 37°, respectively. The orientation assumed in the crystal structure is at α = 37°. (B) Heme protein mono-Cc (PDB entry 1chh). Possible hydrogen bonds are formed with the backbone oxygens of Pro30, Cys17, Gly29, and Cys24. For the optimal orientations, the corresponding values of α are −3°, −113°, −63°, and 167°, respectively. The orientation assumed in the crystal structure is at α = 7°. (C) Heme protein bis-Cc (PDB entry 1czj). Possible hydrogen bonds are formed between the axially ligated His109 and the backbone oxygens of Gly24, Cys105, and Cys108. For the optimal orientations, the corresponding values of α are −3°, −173°, and −113°, respectively. The orientation assumed in the crystal structure is at α = −3°.

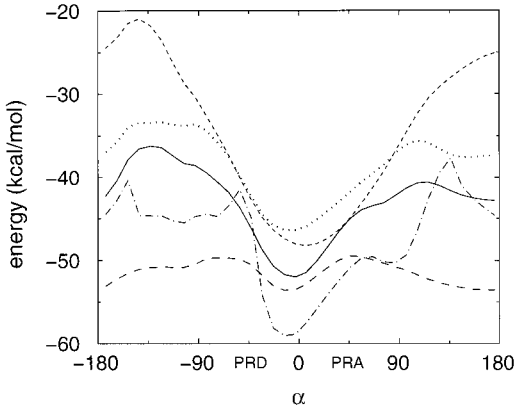

FIGURE 4: Calculated energies of the interaction of imidazole with heme for the torsion angle α. Both propionic acid groups point toward the imidazole. The interactions were calculated with the CHARMM22 (*50*) force field. The coordinates of heme were taken from the crystal structure of cytochrome *c* (PDB entry 1aqe) of the bis-Cc group. The imidazole of His109 was considered. The propionic acids were considered to be bare and unprotonated, if not otherwise stated: (—) total energy of the interaction between imidazole and heme, (···) electrostatic interaction only, (- - -) interaction of the Nδ1−Hδ1 and Cε1−Hε1 polar groups of imidazole with the carboxyl groups of the two propionic acid groups of heme, (−·−) interaction between imidazole and heme with hydrogen bonded-propionic acid groups (for more details, see the text), and (− − −) interaction between imidazole and heme with both propionic acid groups protonated.

this structure is a good example for the two cases, where both propionic acid groups are pointing toward or away from an axially ligated imidazole. In Figure 4, we show the calculated energies of the interaction of the imidazole ring of His109 with heme as a function of the pseudo-torsion angle α (Cε1−Nε2−FE−CHA) characterizing the orientation of the imidazole relative to the heme. Note that both propionic acid groups point toward the imidazole of His109. In agreement with most cytochrome *c* crystal structures, the total interaction energy (solid line) clearly favors conformations with the angle α close to zero, where the NδH group of the imidazole points toward the center of the two propionic acid groups. In the crystal structure, the corresponding value of the angle α is 7°. The main part of that interaction is of electrostatic origin (dotted line in Figure 4). This interaction involves predominantly the polar groups of the imidazole (Nδ1−Hδ1 and Cε1−Hε1) and the atoms of the carboxyl groups (COO^-) of the two propionic acid groups (short dashed line in Figure 4). The influence of the propionic acid groups decreases only slightly, if they are hydrogen bonded (dashed−dotted line in Figure 4). The hydrogen bonding pattern is modeled by considering the cytochrome *c* structure (PDB entry 1aqe), where one crystal water (H_2O209) bridges two oxygen atoms from different propionic acids and the other water molecule is placed to model the hydrogen bond, which the propionic acid PRA forms with Tyr19. The conformation of the two water molecules was subsequently energy minimized. The influence of the propionic acid groups on the orientation of a ligated imidazole becomes small, if the propionic acid groups are neutralized by protonation, which is, however, unlikely for these acidic groups (long dashed line in Figure 4). The electrostatic potential of the propionic acid groups of heme may also be shielded by salt

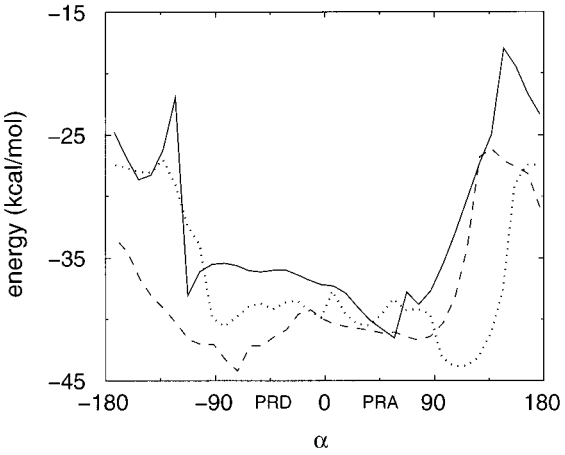

FIGURE 5: Calculated energies of the interaction of imidazole with heme as a function of torsion angle α. Both propionic acid groups are bare and unprotonated and point away from the imidazole. The solid line shows results from His77 ligated to heme of cytochrome c_3 structure 1aqe, where the imidazole of His77 is on the opposite side of the heme plane from the two propionic acid groups. The dashed line shows results from His35 ligated to heme of cytochrome c_3 structure 2cth, where PRD points toward the imidazole of His35 and PRA points away. The dotted line also exhibits results from cytochrome c_3 structure 2cth, but here the imidazole of His52 on the opposite side is considered, where PRA points toward the imidazole ligated to heme and PRD points away.

bridges, which can be formed with arginine for instance. In that case, the electrostatic interactions with the imidazole ring should be reduced less, albeit still significantly, as compared to the case of protonated propionic acid groups.

For His77 in cytochrome c_3 (PDB entry 1aqe) (*52*), on the other side of the heme plane both propionic acid groups are turned away. Here, the electrostatic interactions with the imidazole ring are much weaker such that the angular interval available for the imidazole heme conformations may extend from approximately −90° to 90° (total interaction, solid line in Figure 5). Nevertheless, in the crystal structure, the corresponding value of the angle α is 13°.

The case where one propionic acid group is located above and one below the heme plane can be studied by considering the cytochrome c_3 (PDB entry 2cth) (*53*), where PRD points toward His35 (dashed line in Figure 5) and PRA points toward His52 (dotted line in Figure 5). Due to the attractive interactions of the imidazole with the propionic acid groups, the energy minimum at negative (positive) values of angle α is deeper for His35 (His52). This is partially corroborated by the corresponding values of the angle α, which the two histidines adopt in the crystal structure. The value of the angle α is −63° for His35, but it is −14° for His52; i.e., the ligated imidazole remains in an orientation where the Nδ1H group approximately points toward the center of the two propionic acid groups.

Influence of the Histidine Backbone on the Orientation of Imidazole Relative to Heme

Analyzing Data from the PDB. We suspected that the histidine backbone may impose steric constraints on the orientation of the imidazole ring. This can also influence the orientation of the imidazole ligated to heme, since the histidine backbone has a specific orientation with respect to the heme. In Figure 6, the distribution of the histidine backbone angle β (Cα−Cβ−Cγ−Nδ1) is displayed for all noncoordinated histidines (dashed line, scaled down by a factor 6) and for all histidines coordinated to heme with the exception of the Mb and Hb groups of heme proteins (solid line). The distribution of the histidine backbone angle β of all noncoordinated histidines exhibits two maxima, which are situated at approximately 90° and −90°. The distribution of the backbone angle of coordinated histidines assumes also a maximum at approximately ±180°. A closer inspection of the structures of heme proteins contributing to this additional maximum shows that most of these heme proteins belong to the CcPo group. Hence, there is a specific reason that for this group of heme proteins the angle β assumes such an unusual value. Investigation of structures from the CcPo group showed that it is due to a strong hydrogen bond, which determines the orientation of the imidazole ring relative to the heme. See below about a more detailed discussion of the CcPo group of heme proteins. The heme proteins of the Mb (Hb) group, dotted (dash-dotted) line in Figure 6, contribute only to β angles close to 60° (+90°) and not to angles close to −90°.

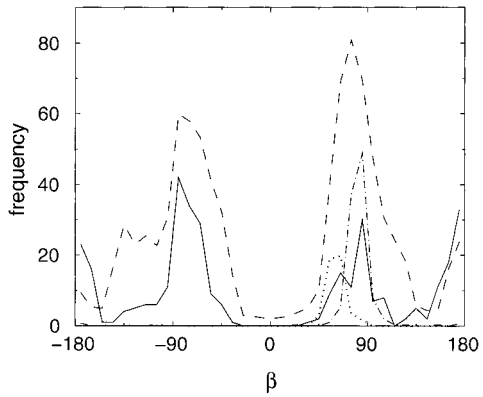

FIGURE 6: Overview of imidazole histidine backbone angles β derived from the PDB: (− − −) data from all noncoordinated histidines of the PDB scaled down by a factor of 6, (−) data from all heme proteins possessing hemes with axially coordinated histidines with the exception of the Mb and Hb groups, and [··· (−·−)] data from the Mb (Hb) group scaled down by a factor of 4.

Force Field Computations on Interactions of Imidazole with the Backbone. The influence of the histidine backbone on the orientation of its imidazole ring can easily be calculated with a molecular force field. In Figure 7, we show the energy profile of the backbone angle β generated with the CHARMM22 (*50*) force field, as described in the Methods. The total energy (solid line) exhibits two minima at β values of approximately −90° and 90°, which have well depths of 5.5 and 2.5 kcal/mol, respectively, and two maxima at approximately 0° and ±180°. The location of these minima and maxima is in agreement with the distribution of the backbone imidazole angle β, derived from the noncoordinated histidines in the PDB (Figure 6). In contrast to our initial supposition, we found no significant steric effects. The dominant part of the interaction of the imidazole ring with its histidine backbone is of electrostatic nature (Figure 7, dashed line), but van der Waals interactions are needed to make the minimum at −90° shallower and simultaneously the minimum at 90° deeper (dotted line). The torsion angle energy is slightly enhancing the difference between the minima and maxima (dashed−dotted line).

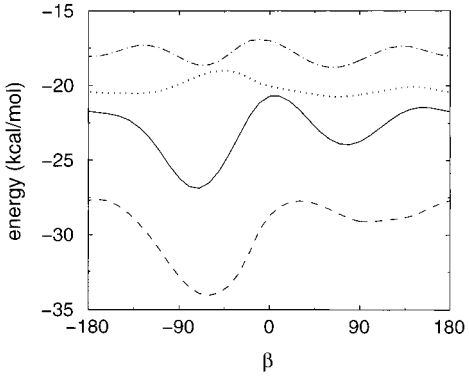

FIGURE 7: Calculated energies of the interaction between imidazole and its histidine backbone. The interaction energy is calculated with the CHARMM22 (*50*) force field and displayed as a function of the torsion angle β: (—) total energy, (− − −) electrostatic energy, (···) van der Waals (Lenard-Jones) energy, and (−·−) torsion energy term.

The constraints imposed on the histidine backbone angle β have a consequence for the distribution of the orientation of the imidazole ring plane with respect to the heme described by the angle α, since the pseudo-torsion angle γ, which characterizes the orientation of the histidine backbone toward the heme, assumes specific values. This is indeed the case, since γ fulfills the exact relationship $\gamma = \alpha + \beta$ as explained in the Methods. At the two energy maxima ($\beta = 0°$ and $\pm 180°$) of the interaction of imidazole ring with its histidine backbone (Figure 7), the plane defined by histidine atoms Cα, Cβ, and Cγ, connecting the backbone with the imidazole ring, is nearly parallel to the imidazole ring plane (see, for instance, Figure 1, where $\beta = \pm 180°$). Hence, the projections of the Cα−Cβ bond vector of the histidine and the Nδ1−H bond vector of the imidazole on the heme plane are parallel when $\beta = \pm 180°$ and antiparallel when $\beta = 0°$. As a consequence, the imidazole orientations, where $\gamma \approx \alpha$ or $\gamma \approx \alpha \pm 180°$, are prohibited.

Imidazole Heme Conformations for the Different Groups of Heme Proteins

Analyzing the influence of the propionic acid groups and the histidine backbone on the orientation of the imidazole ring, we showed that these two factors are very important for the orientation of the imidazole, and we assumed that most of the observed orientations can be explained by considering these two factors. First, by the interaction of the imidazole with its histidine backbone, the angle β can be in two disconnected angle intervals, which are located at approximately 90° and −90° (Figure 6). It is clear that the orientation of the imidazole with respect to the heme (defined by the angle α) depends on the orientation of the corresponding histidine backbone relative to the heme as described by the angle γ (see Figure 1). Second, since there is an exact relation between the angles α, β, and γ (eq 1), the interaction of the imidazole with the propionic acid groups determines to which of the two allowed regimes, at 90° or −90°, the angle β belongs. Hence, the angle β adopts the value which allows the imidazole ring to orient such that its Nδ1H group points toward the propionic acid groups of the heme. By considering these two factors, we managed to explain the orientations of the imidazoles in all groups of heme proteins, except CcPo. In the CcPo group, we found a strong hydrogen bond, which determines the orientation of the imidazole (see below for more details).

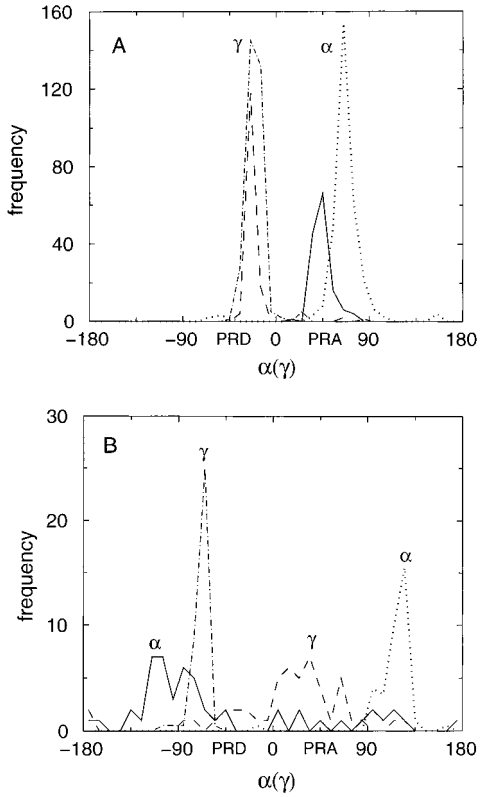

FIGURE 8: Distribution of the torsion angles α and γ for different heme proteins. The torsion angle α characterizes the orientation of the imidazole relative to the heme represented by solid and dotted lines. The torsion angle γ characterizes the orientation of the histidine backbone relative to the heme depicted by dashed and dashed−dotted lines. (A) Solid and dashed lines depict the angle distributions of heme proteins from the Mb group. Dotted and dashed−dotted lines depict data for the Hb group of heme-proteins. (B) Solid and dashed lines depict data for the Cb group of heme proteins. Dotted and dashed−dotted lines depict data for the CcPo group of heme proteins.

Since specific orientations of the imidazole rings of histidines ligated to heme are characteristic for different types of heme proteins, the distributions of the torsion angle α describing this orientation are shown for the six groups of heme proteins that are considered, in Figure 2 for mono-Cc (dashed line) and bis-Cc (dotted line), in Figure 8A for the Mb group (solid line) and Hb group (dotted line), and in Figure 8B for the Cb group (solid line) and for the CcPo group (dotted line).

Cytochrome c Groups. Evidently, in most of the cytochrome *c* structures, the Nδ1H group of the imidazole ring of histidine is situated directly between the two propionic acid groups PRA and PRD (Figure 2, dashed and dotted lines). These groups of heme proteins represent a large portion of all structures where the angle α is close to 0°. In these structures, the value of the angle β is approximately 90° or −90° (see Figure 9). Due to eq 1, the value of the angle γ characterizing the orientation of the histidine backbone relative to the heme adopts a value close to 90° or −90°. For proteins of the two cytochrome *c* groups that are considered, the orientation of the histidine backbone with respect to the heme and the interaction of the imidazole with its histidine backbone does not prevent the imidazole from adopting an orientation toward the heme, where the angle α

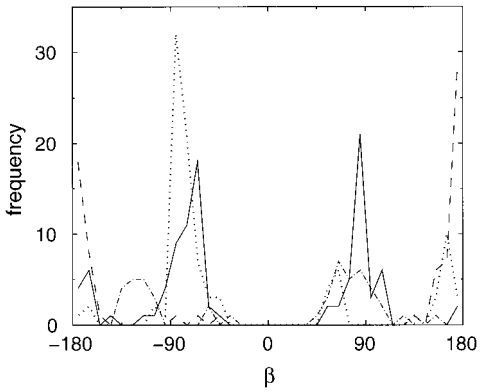

FIGURE 9: Distribution of the torsion angle β characterizing the orientation of the imidazole ring relative to its histidine backbone for different groups of heme proteins derived from the PDB: (—) bis-Cc, (− − −) CcPo, (•••) mono-Cc, and (−•−) Cb.

is approximately 0° or ±180°. It is clear that orientations of imidazoles with the angle α close to 0° are determined by the interactions with the propionic acid groups.

In all cytochrome *c* heme proteins, two cysteines are covalently bound to heme at the pyrrole rings opposite the propionic acid groups. However, we found neither by analysis of structures of heme proteins in the PDB nor by force field computations a significant influence from these cysteines on the orientation of the imidazoles axially coordinated to heme.

Myoglobin and Hemoglobin Groups. The distribution of imidazole heme pseudo-torsion angle α characterizing the orientation of the ligated imidazole relative to the heme is shown in Figure 8A for the Mb and Hb groups of heme proteins. The imidazole heme torsion angle α adopts almost exclusively positive values (solid line for Mb and dotted line for Hb). The Mb and Hb groups are very homogeneous with respect to sequence and structure. With the exception of a few mutants, from Mb and Hb, the Nδ*H* group of the imidazole ring points toward the propionic acid group PRA, which is on the same side of the heme plane as the imidazole ring, whereas the other propionic acid group PRD is located on the opposite side of the heme plane. Hence, the expected interaction of the imidazole ring with PRD should be weaker than with PRA. At the same time, the orientation of the imidazole ring is stabilized by a hydrogen bond of the imidazole Nδ*H* group with a backbone CO group in an orientation of $\alpha \approx 45°$ for Mb and $\alpha \approx 65°$ for Hb. For more details, see the preceding discussion about the hydrogen bonding scheme. In the top part of Figure 10, a typical crystal structure of Hb [PDB entry 1aox (*54*)] with an α value of 67° is displayed in the neighborhood of the heme.

The histidine backbone heme orientations for the Mb and Hb groups are also shown in Figure 8A (dashed line). Since for the Mb and Hb heme proteins the backbone Cα−Cβ bond vector is directed toward the center of the propionic acid groups of the heme ($\gamma \sim 0°$), the preferred orientation of the imidazole ring, where the Nδ*H* group is pointing toward the center of the propionic acid groups ($\alpha = 0°$), is severely hampered by the interaction of the imidazole ring with its histidine backbone. The distribution of the backbone angle γ has its maximum slightly shifted toward negative values (Figure 8A). Hence, it is not surprising to observe that for the Mb and Hb heme proteins the preferred orientation of

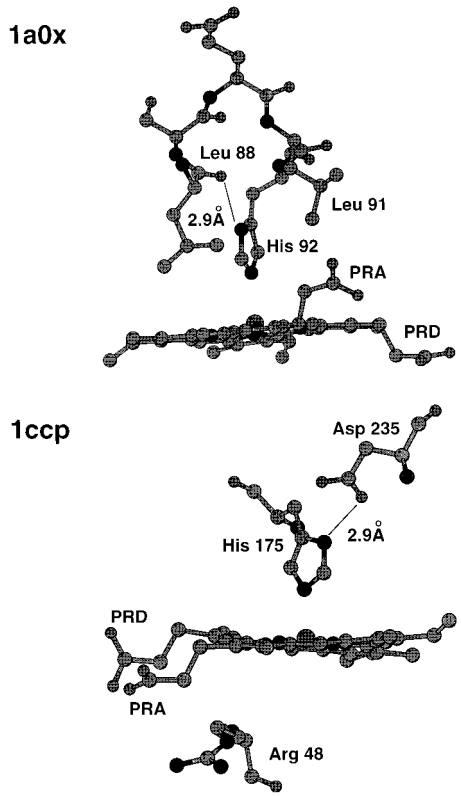

FIGURE 10: Examples of heme proteins, where the Nδ*H* group of an imidazole ligated to heme is not oriented toward the center of the propionic acid groups. The top part displays the heme neighborhood in the crystal structure of hemoglobin 1a0x, where the orientation of the His92 backbone prevents the generally preferred orientation of the imidazole of His92 toward the center of the propionic acid groups. The hydrogen bond of the imidazole Nδ*H* group with the backbone carbonyl of Leu88 is only of secondary importance. The bottom part displays the heme neighborhood in the crystal structure of cytochrome *c* peroxidase 1cpp, where the imidazole of His175 is oriented differently, because the Nδ*H* group is forming a strong hydrogen bond with the negatively charged Asp235.

the imidazole heme angle α is in the angle interval closer to 90° than to −90°.

Cytochrome b Group. In heme proteins of the cytochrome *b* (Cb) group, both axial ligands of the heme are histidines. In these heme proteins, the imidazole rings of the ligated histidines are preferentially at an angle α of approximately −90°; i.e., the Nδ*H* group points away from both propionic acid groups, even though it is not too far from the propionic acid group PRD (solid line in Figure 8B). In this group of heme proteins, the backbone angle γ of the histidine relative to the heme is not far from $\gamma = 0°$ (dashed line in Figure 8B). Hence, the Nδ*H* group of the imidazole ligated to heme cannot be oriented toward the center of the propionic acid groups. Since the distribution of the backbone angle γ has its maximum slightly shifted toward positive values, is not surprising to observe that the preferred orientation of the imidazole with respect to the heme is preferentially at negative α values, close to −90°.

Cytochrome c Peroxidase Group. The heme proteins of the CcPo group are very homogeneous with respect to the sequences. The backbone Cα−Cβ bond vector of the histidine is oriented in the angle interval at $\gamma = -90°$ relative to the heme (Figure 8B, dashed−dotted line), which would not prevent an orientation of the Nδ*H* group of the imidazole

toward the center of the propionic acid groups of the heme. However, the distribution of the torsion angle α assumes its maximum at 120° (Figure 8B, dotted line). In this orientation, the imidazole ring can no longer interact strongly with the propionic acid groups. But at the same time, the interaction with the histidine backbone is unfavorable. This becomes obvious by inspecting Figure 9, which displays the distribution of the backbone angle β for different groups of histidine-coordinated heme proteins. For the heme proteins of the CcPo group, the backbone angle β adopts almost exclusively values close to ±180° (Figure 9, dashed line), which corresponds to a very unfavorable interaction of the imidazole ring with its histidine backbone. Since for the CcPo group of heme proteins the orientation of the imidazole ring ligated to heme cannot be explained by the interactions with the propionic acid groups and the histidine backbone, we suspect that other factors are determining the orientation of the imidazole in that case. We looked at the surrounding of the heme to find such factors.

In heme proteins of this group, an aspartate (Asp235), which is located at the heme edge opposite the two propionic acid groups, forms a hydrogen bond with the Nδ*H* group of the imidazole ligated to heme (see the bottom part of Figure 10). This hydrogen bond is particularly strong, since aspartate is normally charged negative and thus enforces this orientation of the imidazole. It is interesting to note that for CcPo heme proteins both propionic acid groups are located on the same side of the heme plane, but on the opposite side of the imidazole ring. That is obvious when inspecting the bottom part of Figure 10, which exhibits a typical crystal structure of CcPo [PDB entry 1ccp (*55*)] where α = 124°. This conformation of the propionic acid groups weakens the possibility of them having an influence on the orientation of the ligated imidazole. The acidic group of PRA forms a salt bridge with a positively charged arginine (Arg48), which partially neutralizes the negative charge of that propionic acid group and weakens its influence on the orientation of the ligated imidazole ring further. Hence, it is understandable that in CcPo heme proteins the imidazole ring adopts an orientation that does not depend on the propionic acid groups.

Mutual Orientation of Axially Coordinated Imidazoles

For the heme proteins, where both axial ligands at heme are histidines, it is of interest to monitor the mutual orientation of the participating imidazole ring planes measured by the difference of torsion angles $\Delta\alpha = |\alpha_2 - \alpha_1|$ as depicted in Figure 11. There are two different groups of heme proteins that possess two histidines as ligands, the Cb group and the bis-Cc group. For the bis-Cc group (solid line in Figure 11), the majority of imidazole planes ligated to heme are oriented parallel to each other. That is not astonishing, if we recall that for the whole group of cytochromes *c* the preferred orientation of the imidazoles is such that the Nδ*H* group points toward the center of the two propionic acid groups. For the group of cytochrome *b* heme proteins, the mutual orientation of the imidazole planes of the axially coordinated histidines does not show such a clear trend, although smaller values of the angle Δα are preferred (dashed line in Figure 11).

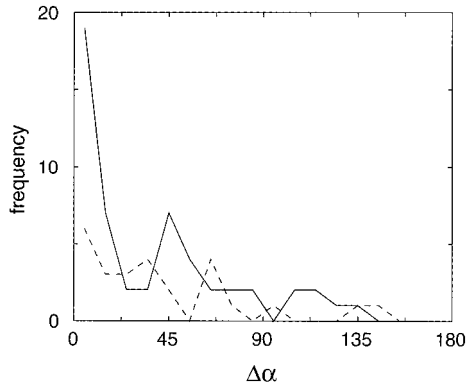

FIGURE 11: Relative orientation of the imidazole planes of histidines axially coordinated to heme, as measured by the torsion angle difference $\Delta\alpha = |\alpha_2 - \alpha_1|$. The distributions were derived from heme proteins of the PDB, where the heme is axially coordinated to histidines: (—) from the cytochrome *c* group (bis-Cc) and (– – –) from the cytochrome *b* group (Cb).

CONCLUSIONS

Factors determining conformations of imidazole axially coordinated to heme in heme proteins were investigated by analyzing the crystal structures from the PDB. We distinguished six groups of heme proteins, namely, myoglobin (Mb) (133 structures), hemoglobin (Hb) (121 structures), cytochrome *c* with one (mono-Cc) (68 structures) or two (bis-Cc) (17 structures) axially coordinated histidines, cytochrome *c* peroxidase (CcPo) (72 structures), and cytochrome *b* (Cb) (21 structures). In the Cb group of heme proteins, all hemes are coordinated by two axially ligated histidines.

By searching the PDB, we found that in most of the crystal structures the Nδ*H* group of the imidazole ligated to heme is oriented toward the propionic acid groups of the heme, and practically never in the opposite direction. These data indicate that there is an interaction of imidazole with the propionic acid groups. By molecular force field computations, the interaction of imidazoles with the propionic acid groups was evaluated. For the conformation where the propionic acid groups are both on the same side of the heme plane as the imidazole ring, the molecular force field computations exhibit a deep and broad energy minimum, which is mainly due to electrostatic interactions of the imidazole ring with the acidic groups of the propionic acids of the heme. However, this is also the preferred orientation for other conformations of the propionic acid groups, albeit less pronounced.

A second important influence that we found is due to the interaction of the imidazole ring with its histidine backbone, which is also mainly electrostatic in nature. This interaction prohibits conformations where the imidazole plane is oriented parallel to the Cα−Cβ bond of its histidine backbone. As a consequence, the orientation of the histidine backbone relative to the heme also determines the orientation of the imidazole with respect to the heme.

Considering these two factors, i.e., the interactions of imidazole with propionic acid groups and with histidine backbone, we were able to understand the orientation of axially coordinated imidazoles for the Mb, Hb, mono-Cc, bis-Cc, and Cb groups of heme proteins.

Since the propionic acids often prefer to remain charged, the acidic groups of the propionates may often be at the

protein surface. Hence, the Fe−CHA vector points toward the protein surface. The protein backbone carrying a histidine axially ligated to such a heme is also close to the protein surface and may therefore have a tendency to be oriented parallel with respect to the protein surface. Consequently, the pseudo-torsion angle γ should preferentially be close to $+90°$ ($-90°$). Due to interactions of the imidazole with the backbone, the torsion angle β should be in one of the two allowed regimes around $\beta = +90°$ or $-90°$. Thus, according to eq 1, the pseudo-torsion angle α can adopt a value around zero. This is in agreement with the fact that for the majority of heme proteins the Nδ-H bond vector points toward the center of the propionic acids.

Generally, we did not find that hydrogen bonds of the imidazole NδH group with the protein are very important for the imidazole heme conformation. In practically all cases, the NδH group is involved in a hydrogen bond. Often the acceptor group is the carbonyl group (CO) of the protein backbone, which is abundant everywhere in a protein such that it does not impose a serious constraint on possible orientations of the imidazole plane. In fact, we found that in many heme proteins the NδH group of an axially coordinated imidazole has different possibilities of forming hydrogen bonds, which differ from the hydrogen bond assumed in the actual crystal structure. Nevertheless, in mutation studies, where the native hydrogen bonding partner was exchanged, the orientation of the imidazole often did not change significantly. From that, we conclude that the hydrogen bonding pattern of axially ligated imidazole does not determine the overall orientation of imidazole. However, these small variations of the imidazole orientation may be used by nature to fine-tune the value of the heme redox potential.

We found one exception to the role of hydrogen bonds for the heme proteins of the CcPo group. In these proteins, the NδH group of the imidazole, which is axially coordinated to heme, is hydrogen bonded with an aspartate (Asp235). This hydrogen bond is very strong, because of the negative charge of the aspartate. It enforces an orientation of the imidazole ring, which deviates by ∼120° from the orientation generally preferred due to the interactions with the propionic acid groups.

In summary, the major factors influencing the conformation of imidazole axially coordinated to heme in heme proteins are the electrostatic interactions of the imidazole with the propionic acid groups of heme and the interactions of the imidazole ring with the histidine backbone atoms. In some cases, strong hydrogen bonds of the NδH group of the imidazole with negatively charged acidic residues can also be important. No other significant influence could be found. One may wonder why there is so little direct influence coming from the amino acids of the protein environment. Nevertheless, the protein determines the conformations of the propionic acids and of the histidine backbone, and thus, it indirectly has a significant influence on the conformations of imidazoles axially coordinated to heme.

The understanding of the mechanisms that determine the heme imidazole conformations by interactions with specific amino acids would open new avenues for the design of mutants of heme proteins and artificial heme proteins with specific ligand heme conformations. We demonstrated that porphyrin side groups have a strong influence on the orientation of imidazoles ligated to heme such that by synthesis of porphyrins with suitable substituents the orientation of a ligated imidazole can be controlled at will. Hence, via construction of mutated and artificial heme proteins and use of substituted porphyrins as cofactors in native or mutated proteins, it will be possible to obtain heme proteins with specifically designed ligand heme conformations and redox potentials of the heme whose values can be tuned at will.

ACKNOWLEDGMENT

We thank Dr. Martin Karplus for providing the program CHARMM.

SUPPORTING INFORMATION AVAILABLE

Tables of Protein Data Bank entries for heme proteins and a figure showing the distributions of torsion angle α for all bis-Cc and Cb heme proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI010428Q