Chapter 4
Orientation of axially ligated imidazoles in heme-proteins

4.1 Introduction

Heme proteins are of particular interest since they have a wide range of biological functions, including oxygen transport and storage (Hb, Mb), electron transport (cytochromes), and catalysis (catalases, peroxidases). The versatility in the function of the heme group arises in particular from the different possibilities of axial ligation and interactions with the protein surrounding. The heme-iron is typically hexa-coordinated but sometimes it can also be penta-coordinated. Four coordination places are occupied by the heme nitrogens. Among the two axial ligands can appear histidine, methionine, cysteine, and tyrosine or some small molecules as for instance water, O_2, CO, CO_2, NO, CN^- or other substrate molecules.

Since, heme-proteins belong to a widely spread group of proteins with different biological functions, it is interesting to understand how the apoproteins modulate and tune the properties of the hemes. For that purpose, synthetic polypeptides and proteins including heme as a cofactor were synthesized, recently (Mutter et al., 1988; Bryson et al., 1995; Choma et al., 1994; Rau & Haehnel, 1998; Gibney et al., 2000). Theoretical (Vangberg & Ghosh, 1999; Jewsbury et al., 1994; Harris et al., 1998; Rovira & Parinello, 1999), and experimental works (Walker, 1999; Safo et al., 1994; Nakamura et al., 1996; Walker et al., 1996; Shokhirev & Walker, 1998) on heme model systems demonstrate the influence of axial ligands on spectral properties and redox potentials of hemes (Wallace & Clark-Lewis, 1992; Lloyd et al., 1995; Pond et al., 1999). It was found that not only the type but also the conformation and orientation of the axial ligands can have an influence on the heme properties.

We were particularly interested to investigate heme-proteins that have axially ligated histidines, and to examine the factors that determine the conformation and orientation of the imidazole rings relative to each other and relative to the heme. Our interest for this group of proteins arises from the fact that different conformations can shift the heme redox potential (Walker et al., 1986), which we wanted to calculate in the frame of this doctoral work by evaluating the electrostatic interactions. In that context knowledge about the orientation of axially coordinated histidines could be useful, specially for the modeling of the artificial Cb. Furthermore, the orientation of the coordinated histidine is considered to have a strong influence on function and spectroscopic properties of hemes (Walker, 1999), and can control the coordination of substrates to heme-proteins (Menyhard & Keseru, 1998).

Experimental data on heme model systems and molecular mechanics studies suggests that the porphyrin ring conformation depends on the orientations of axial ligands with bulky substituents. In complexes with parallel orientation of two planar axial ligands the porphyrin ring remains planar. For porphyrin complexes, which have two planar axial ligands in perpendicular orientation the porphyrin ring is almost always distorted from planarity (Walker, 1999; Safo et al., 1994; Shelnutt et al., 1998; Shelnutt et al., 2000).

Experimental data for $[Fe(TMP)(5\text{-}MeHIm)_2]ClO_4$ (Munro, 1999) and theoretical quantum-chemical DFT calculations on $[Fe(por)(py)_2]$ and $[Fe(por)(py)_2]^+$ systems (Ghosh et al., 1999) have shown that there is no preference for parallel or perpendicular mutual orientation of axially coordinated planar ligands. The conclusion is that the two

conformational isomers are almost isoenergetic. The energy balance between the two forms is the result of crystal field stabilization effects favoring the parallel form and steric effects caused by substituents on axial ligands and on the porphyrin that favor the perpendicular form. The estimates of two opposite energetic effects are both less than 3 kcal/mol (Munro et al., 1997). Moreover, experimental and theoretical investigations on model systems (Momot & Walker, 1997; Shokhirev et al., 1997; Polam et al., 1997) demonstrate, that there is no rotation barrier for imidazole ligands axially coordinated to porphyrin without bulky substituents. It means that the mutual interactions between two axial histidines, or their interactions with the porphirin atom skeleton is negligible.

In heme model systems, the orientation of axial ligands can depend on crystal field stabilization effects or on steric effects caused by bulky substituents on axial ligands and on the porphyrin (Walker, 1999; Munro et al., 1999; Safo et al., 1997). In heme-proteins, there are a couple of different factors, which can influence the orientation of coordinated imidazoles. Among them are hydrogen bonds between the imidazole NδH group and H-bond acceptors of the protein, non-bonded interactions of the imidazole ring with the protein backbone and side chains, non-bonded interactions of the imidazole with the porphyrin atom skeleton and the side chains of cysteines covalently bound to heme (in cytochrome *c* heme-proteins). Also, electrostatic interactions and the influence of charged amino acid side chains should be considered.

We were interested to find the general and the most significant factors determining the axial imidazole orientation. In our approach, we first analyzed the influence of the hydrogen bonding pattern of imidazoles axially coordinated to heme, since it is crystallographically known that ligated histidines are almost in all cases H-bonded. Then, we made data mining in the protein data base (PDB) (Berman et al., 2000), to see if specific orientations are preferred. It has been already shown that analyzing the crystal structures can provide information about interactions in proteins (Gallivani & Dougherty, 1999; Zarić et al., 2000). We used a molecular force field to evaluate interactions of the imidazole ring of histidine ligated to heme with the porphyrin atom skeleton, the propionic acids and if available with the cysteines covalently bound to heme. We also investigated the influence of the histidine backbone on the orientation of the imidazole relative to the heme. In few cases, we found that some other influences, as for instance a specific hydrogen bond is responsible for a particular orientation of axially ligated imidazole rings.

4.2 Methods

4.2.1 Data mining in the PDB

Searching in the PDB of crystal structures we found among 432 different heme-proteins, a total of 693 hemes to which at least one histidine is ligated. The selected heme-proteins were clustered in six groups. We found 138 mono-histidine ligated hemes in 133 proteins of the myoglobin (Mb) group, 323 mono-histidine ligated hemes in 121 proteins of the hemoglobin (Hb) group, 72 mono-histidine ligated hemes in 72 proteins of the cytochrome *c* peroxidase (CcPo) group, 99 mono-histidine ligated hemes in 68 proteins of cytochrome *c* (mono-Cc) type, as well as 39 bis-histidine ligated hemes in 17 proteins of cytochrome *c* (bis-Cc) and 26 bis-histidine ligated hemes in 21 proteins of the cytochrome *b* (Cb) type. The list of the PDB codes for different groups of heme-protein is given in Appendix F. In the cytochrome *c* groups, the two cysteines are covalently bound to the heme, making thioether bonds with substituents on the pyrrole rings B and C (see Fig. 4.1).

By searching in the PDB database, we monitored the orientation of ligated histidines relative to heme, relative to the second histidine (in bis-histidine ligated hemes). We also investigated the orientation of axially ligated imidazoles with respect to their histidine backbone conformation and the position and orientation of the histidine backbone relative to the heme plane. On this way, we found the conformational characteristics, which are common for the different groups of heme-proteins as well as the specific differences between them.

Characterizing hydrogen bonds involving imidazole axially ligated to heme. We assumed that the atom pair Nδ1–Hδ1 of a ligated histidine forms the hydrogen bond, if there is a suitable hydrogen bond acceptor atom at a distance closer than 3.5 Å from the Nδ1 atom of the imidazole ring. We were searching in the PDB database for such H-bond acceptors. The most probable H-bond partner of the NδH group of coordinated imidazole is a CO group of the protein backbone. However, other protein polar groups can also participate in these H-bonds. Since, a number of them can be found around the hemes in heme-proteins, we suspected that for the other imidazole orientations, different from the native one, there are also hydrogen bond partners, available. To find the other possibilities for the hydrogen bonding, we rotated the imidazole ring around its Nε2-Fe bond with the heme, without changing other parts of the crystal structure. We considered that another hydrogen bond can be formed, if the distance of Nδ1 to the corresponding hydrogen bond acceptor atom is smaller than 4.0 Å. A slightly larger 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. The obtained data were presented in graphical form where the inverse of the atom pair distances with the possible hydrogen bonding partners where given as a function of the pseudo-torsion angle α, characterizing the imidazole orientation. The inverse of this atom pair distance was chosen, since it provides a rough measure of the electrostatic interactions, which are relevant for the strength of the hydrogen bond.

Characterizing the orientation of ligated imidazole relative to heme. Analyzing the common parameters of the histidines ligated to heme, we found that, the N(His)-Fe bond length is close to 2.0 Å, the bond angle N(His)-Fe-N(heme) is roughly 90^0 and the coordinated imidazole rings are nearly orthogonal to the corresponding heme planes. We investigated the orientation of the histidine axially ligated to heme by monitoring the following torsion angles (Figure 4.1) in the PDB crystal structures of heme-proteins:

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 axis of the Nε2-FE 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 the torsion angle β (Cα-Cβ-Cγ-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-Cβ-FE-CHA).
4. The mutual orientation of the imidazole planes of two ligated histidines (1 and 2) is characterized by the difference of the corresponding torsion angles $\Delta\alpha = |\alpha_2 - \alpha_1|$.

The cis-conformation defines the zero point value of the corresponding torsion angle. The positive rotation sense of the torsion angles α, β and γ is a opposite to clockwise. Geometrically, it can be shown that these three torsion angles are not independent and fulfill the relation:

$$\gamma = \alpha + \beta \, . \qquad (4.1)$$

The data obtained 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 of 10^0 width. To obtain a clearer representation of the data, the values of the angular distribution functions were connected by continuous lines.

4.2.2 Molecular force field computations

To interpret the most probable orientation of histidine ligated to heme, we calculated the energies of different imidazole–heme conformations using CHARMM22 (MacKerell et al., 1998), by setting the dielectric constant to $\varepsilon = 1$, as it is typically used for this force field. We varied the torsion angle α continuously and monitored the interaction of the imidazole ring with the heme. Different groups of atoms and interaction types were considered, in order to find the most relevant ones. In the case of cytochrome *c*, the interaction of the imidazole with the covalently bound cysteines were also considered. Instead of modeling the idealized histidine-heme systems, we rather used the atomic coordinates from appropriate crystal structures of heme-proteins, but we evaluated only the interaction energy between the imidazole ring and the heme and covalently bound cysteine atoms. In these computations, two propionic groups of heme were considered to be negatively charged if not otherwise stated.

The influence of the protein backbone conformation was evaluated calculating the interaction energy of the imidazole ring of histidine with its protein backbone. In this computation, to avoid artifacts from the bare charges, the C-terminus was amidated and N-terminus methylated. Among the three torsion angles: β_{-1} (N-Cα-Cβ-Cγ), β (Cα-Cβ-Cγ-Nδ1) and β_{+1} (Cβ-Cγ-Nδ1-Cε1) between the imidazole ring and the protein backbone of histidine, which can influence the histidine conformation relative to its backbone (see figure 4.1), the central angle β is the most relevant one. Therefore, we fixed the value of the torsion angle β and energy minimized all other degrees of freedom, including also the torsion angles β_{-1} and β_{+1}. We obtained an unconstrained histidine conformation, for each value of angle β. Varying the torsion angle β, we evaluated the energies of different conformations, where the torsion angle β was fixed and all other degrees of freedom were allowed to relax. Finally, the interaction energy of the imidazole ring of histidine with its protein backbone was graphically represented as a function of torsion angle β.

4.3 Results and Discussions

4.3.1 General overview of PDB data

Figure 4.2 exhibits the distribution of the pseudo-torsion angle α for all heme-proteins. The solid line shows data of all heme-proteins but excluding the data from the Hb and Mb families. Since, the Hb and Mb groups are very large, consisting of proteins with a high degree of sequence homology, we presented them separately as dashed dotted line, scaled down by the factor 4. To stress that the two groups of cytochrome *c* heme-proteins, mono-Cc (dashed line) and bis-Cc (dotted line), have the angle α around 0^0, they are shown individually. In many crystal structures of heme-proteins the value of the angle α is close to 0^0, where the projection of imidazole Nδ-H bond vector on the heme plane is placed between the two propionic groups PRA ($+45^0$) and PRD (-45^0). It is obvious that in most

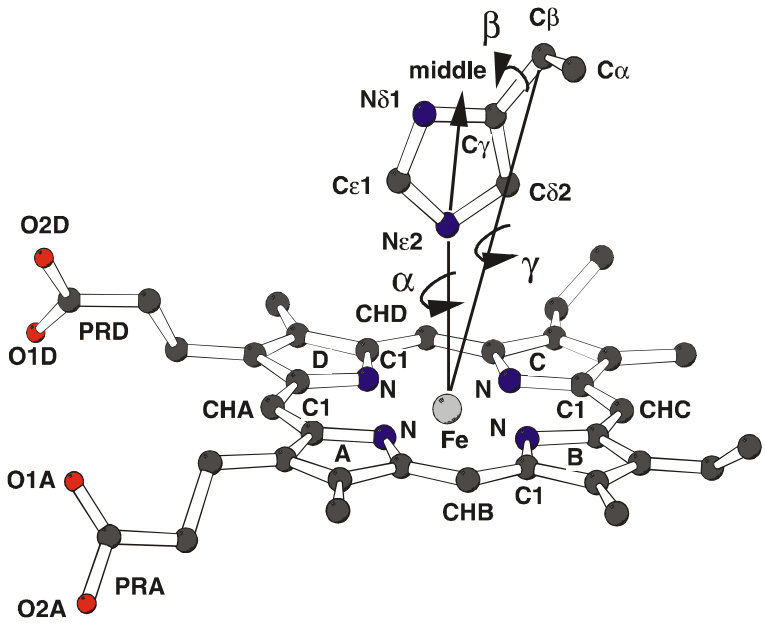

Figure 4.1. Definition of the three torsion angles α, β and γ used to characterize the conformation of imidazole ligated to heme. The pseudo-torsion angle α(Cε1-Nε2-Fe-CHA) with rotation axis Nε2-Fe denotes the orientation of the Nδ1H-group of the imidazole relative to the center of the two propionic acid groups PRA and PRD defined by the atom CHA. The torsion angle β(Cα-Cβ-Cγ-Nδ1) characterizes the orientation of the imidazole ring relative to the Cα-Cβ 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-Cβ-FE-CHA). For the structure displayed the values of the torsion angles are $\alpha \approx 0^0$, $\beta \approx 180^0$ and $\gamma \approx 180^0$.

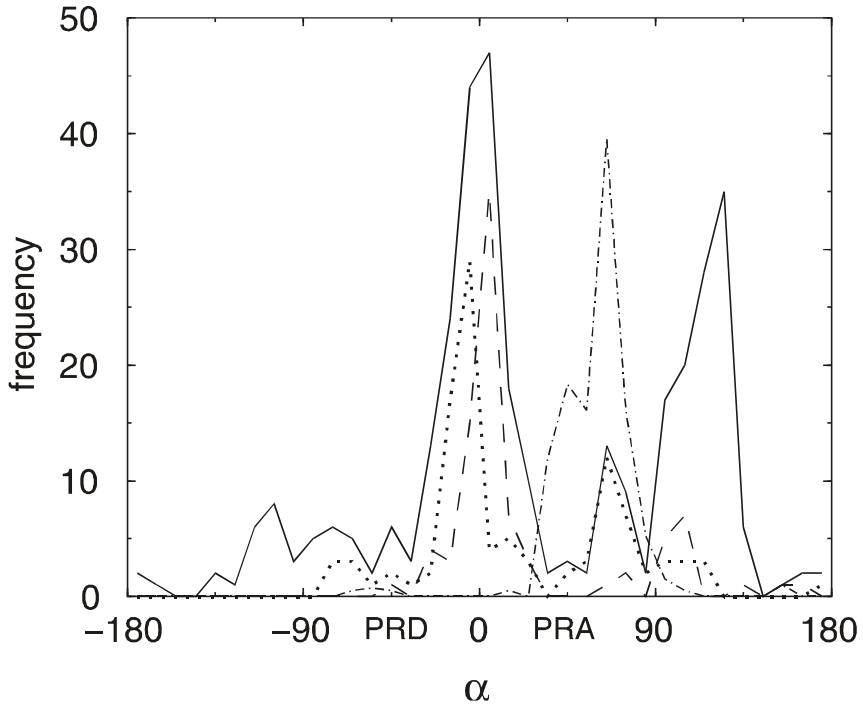

Figure 4.2. Distributions of the torsion angle α of the imidazole heme conformation for different groups of heme-proteins from the PDB. Solid line: imidazoles of all heme-proteins with exception of the Mb and Hb groups (Tables F3-F6 in Appendix F); dashed line: heme-proteins of the mono-Cc group; dotted line: heme-proteins of the bis-Cc group; dashed-dotted line: proteins of the Mb and Hb groups (Tables F1 and F2 in Appendix F) scaled down by the factor four. The approximate orientation of the propionic acid groups PRA and PRD is marked on the x-axis.

crystal structures the Nδ1H group of imidazole is oriented toward the center of both propionic acids ($\alpha = 0^0$) or at least toward one propionic acid group ($\alpha = 45^0$). For a large number of structures, which belong to the group of cytochrome *c* peroxidase (CcPo), the angle α is around 120^0. Further, in the Hb and Mb proteins the angle α adopts values between 30^0 and 70^0 (figure 4.2, dashed-dotted line). In a small number of crystal structures the angle α can also take other values, but there are very few structures with α value close to $\pm 180^0$. Therefore, we suspect that there is an attractive interaction between the imidazole ring and the propionic acid groups, which favors such orientations of imidazole relative to the heme. However, since the orientation of the imidazole was not in all cases toward the propionic groups, it was obvious that there are also other factors, which should be considered.

4.3.2 Hydrogen bonding scheme of imidazole ligated to heme

The Nδ1 nitrogen atom of histidine coordinated to heme is always protonated acting as donor group in a hydrogen bond. For more than 98% of all histidines coordinated to heme, we found suitable hydrogen bonding partners less than 3.5 Å away from the Nδ1 nitrogen. For the remaining 2% a suitable hydrogen bonding partner atom was more than 3.5 Å away from the Nδ1 nitrogen. In 7.5% of all cases, a water oxygen atom is the hydrogen bonding partner. The protein backbone CO group is the most probable hydrogen bonding partner of the imidazole Nδ1H group. Thereby, the residue type and number involved in this hydrogen bond varies, depending on the considered protein structure. In this section, the results obtained from the PDB data mining will be discussed.

Hydrogen bonds of axially coordinated imidazoles in myoglobin and hemoglobin. Since, Mb and Hb proteins show a very high degree of sequence and structure identity, the hydrogen bond pattern involving coordinated histidines is very similar throughout the whole group. It is always the backbone CO group, four residues in sequence below the coordinated histidine, that forms a hydrogen bond with the Nδ1H group of the imidazole ring. Also, in both heme-protein families the propionic group PRA points toward the axially ligated imidazole ring, whereas the propionic group PRD points away from it.

Mb: For most proteins of the Mb group the Hδ atom of coordinated His93, forms simultaneously hydrogen bonds with Leu89 and Ser92. The part of the sequence Leu89-Ala90-X91-Ser92-His93, with X=Gln, Glu, or Asn involved in hydrogen bonding is largely conserved. The bifurcated hydrogen bond of the imidazole is often asymmetric, and can be considered to be a mean for fine tuning the orientation of the imidazole ring over a larger angular regime, from $\alpha = +20^0$ to $+78^0$. But, for most Mb crystal structures the value of the pseudo-torsion angle α is close to 45^0. This is exactly the orientation where the imidazole Nδ1H group and the propionic acid group PRA point toward each other.

Effects on mutation of the conserved residue Ser92 to Ala, Val, and Leu were studied in pig Mb by Smerdon et al. (1993). They found that the binding affinity for O_2, CO, and CN ligands increased with the mutations. But, although the H-bond network in heme binding pocket including the proximal His97 changes and the Nδ1-H group of the ligated His93 looses one H-bonding partner, the imidazole ring of His93 does not change its orientation remarkably. Also in the Mb structure with PDB code 1rse, Ser92 was mutated to Asp. Again the orientation of the imidazole ring did not change and the torsion angle is $\alpha = 50^0$, remaining close to the most probable value for the native Mb. Replacing Ser92 by Asp92, the negatively charged side chain of Asp92 moves away from the imidazole ring of His93, due to

the electrostatic repulsive interactions with the propionic group PRA, which is pointing toward the imidazole ring. Thus, Asp92 does not form an H-bond with His93 in contrast to Ser92. In a few crystal structures of Mb from sea hare, residue 91 is a phenylalanine. Here only the carbonyl backbone oxygen makes the only hydrogen bond with His95 axially ligated to heme. The torsion angle α characterizing the orientation of the imidazole ring adopts in this case values between 64^0 and 78^0, slightly larger than the most probable value of 45^0 for Mb. This change in the orientation is probably due to a repulsive interaction of the negatively charged acidic group of the glutamate Glu94 and the propionic acid group PRA. As a consequence, the propionic acid group moves away from the imidazole ring, what diminishes its influence on the orientation of the imidazole.

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 the most Hb structures the corresponding serine is missing. The pseudo-torsion angle α is around 65^0.

In the binding pocket of some Hb structures (PDB code 1eca, 1ecd, 1ecn, 1eco), the heme is turned around the CHA-CHC axis (see figure 4.1) by 180^0. Accordingly, the pseudo-torsion angle α adopts the value of -50^0 and now the propionic group PRD is oriented toward the coordinated imidazole ring. In these Hb structures containing serine at position 86 and phenylalanine at position 83, we found that the ligated His87 makes a stronger hydrogen bond with the Oγ oxygen of Ser86 than with the backbone oxygen of Phe83. These few structures are the only exceptions among the Hb structures with bifurcated H-bonds. Also in the Hb structure with PDB code 1ith, Ser93 residue is neighbor of the ligated His94. However, in this structure only the backbone CO group of Lys90 forms a hydrogen bond with His94. In some structures, instead of the Ser93, threonine is found at the same position. Nevertheless, we did not find that it forms an H-bond with His.

In a few Hb structures other residues are involved in hydrogen bonding with the NδH group of the ligated histidine. In the Hb structures with PDB codes 1vhb and 2vhb, instead of the backbone CO group of Ile81, the acidic oxygen of Glu137 forms a strong hydrogen bond with His85. Consequently, the torsion angle $\alpha = 150^0$ is here unusually large. In a mutant Hb structure from sea cucumber with PDB code 1hlb, where two histidines (His104 and His73) are axially coordinated to heme, the Oη oxygen of Tyr114 forms a hydrogen bond with His104 ($\alpha = 125^0$) and a water oxygen forms a hydrogen bond with His73 ($\alpha = -163^0$). In the correspond wild type structure (PDB code 1hlm), His104 forms the H-bond with Leu100 and the value of the pseudo-torsion angle is $\alpha = 40^0$.

Hydrogen bonds of axially ligated imidazoles in cytochrome c peroxidase. This is also very compact heme-protein-family, with a high degree of structure and sequence identity. In the native CcPo 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 subset, where Asp235 forms a hydrogen bond with the imidazole ring of the coordinated His175 and one subset, where Asp246 forms a hydrogen bond with 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 interval from 115^0 to 125^0 and from 100^0 to 125^0, respectively. In the CcPo structure with PDB code 1ccc Asp235 is mutated to alanine. The corresponding hydrogen bond disappears, since there is the negatively charged Asp235 is lacking. Instead of that, His175 makes the H-bond with a water molecule. As a consequence, the angle α becomes smaller ($\alpha = 100^0$). The change of the imidazole orientation is toward the

propionate PRA, but the effect is rather small, since the propionate charges are screened by the formation of salt bridge with Arg48. Also in the CcPo structures with the mutants Asp235Asn and Asp235Glu ($\alpha = 122^0$ and $\alpha = 97^0$, respectively) there are only minor changes in the orientation of the imidazole ring relative to heme.

Hydrogen bonds of axially ligated imidazoles in mono-histidine ligated cytochrome c. The c-type hemes are characterized by two covalently bound cysteines. The part of the sequence Cys-X-X-Cys-His, including the ligating histidine and two cysteines attached to the heme is highly conserved. Regarding the structure and sequence homology, 68 mono-histidine ligated cytochrome *c* (mono-Cc) heme-proteins can be divided in a few subgroups. The largest subgroup (mono-Cc124) contains cytochrome *c* isozyme 1 and 2, cytochrome c2 (formerly c550), cytochrome c4, cytochrome c551I, and cytochrome c1 from the cytochrome bc1 complex. The second subgroup (mono-Cc6) consists of cytochrome c6 (also called c553) 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. Their structure is a four-helix bundle with one mono-histidine coordinated heme.

Mono-Cc124: All members of this subgroup have a very similar tertiary structure. 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 9 residues (cytochrome c551I) 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 -25^0 and $+20^0$.

Mono-Cc6: These heme-proteins have a globular α-helical structure similar to the previous subgroup, but the 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 -25^0 and -10^0. Interestingly, in all of these structures there is also a proline, close in amino acid sequence, but too far away to form a hydrogen bond with the ligated histidine.

Mono-Cc': The coordinated histidine of these proteins is solvent accessible and the NδH group of the imidazole ring forms a H-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. The generally more favorable orientation of the imidazole ring toward the propionates is also sterically hindered by two other residues. Correspondingly, the torsion angle α is between 80^0 and 120^0 but in most cases it is close to 100^0.

Also cytochrome *f* belongs to the group of mono-histidine ligated cytochromes. This globular protein possesses 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 ($\alpha = 10^0$) that are water exposed.

Hydrogen bonds of axially ligated imidazoles in bis-histidine ligated cytochromes. They involve two groups of heme-proteins: cytochrome *b* (Cb) and bis-histidine ligated cytochrome *c* (bis-Cc). Each of these groups is very heterogeneous, containing heme-proteins with completely different three-dimensional structures. Therefore, their hydrogen bond patterns can be a quite different. Nevertheless, in the most cases the protein backbone oxygens take part in the H-bonds with coordinated His. For some of the coordinated histidines no hydrogen bonding partner was found. Since mutants are not available, it was not possible to conclude whether the hydrogen bonding scheme has an influence on the orientation of the imidazole

ring coordinated to heme. However, for the bis-Cc group, the coordinated histidines are mostly oriented toward the propionic acid groups.

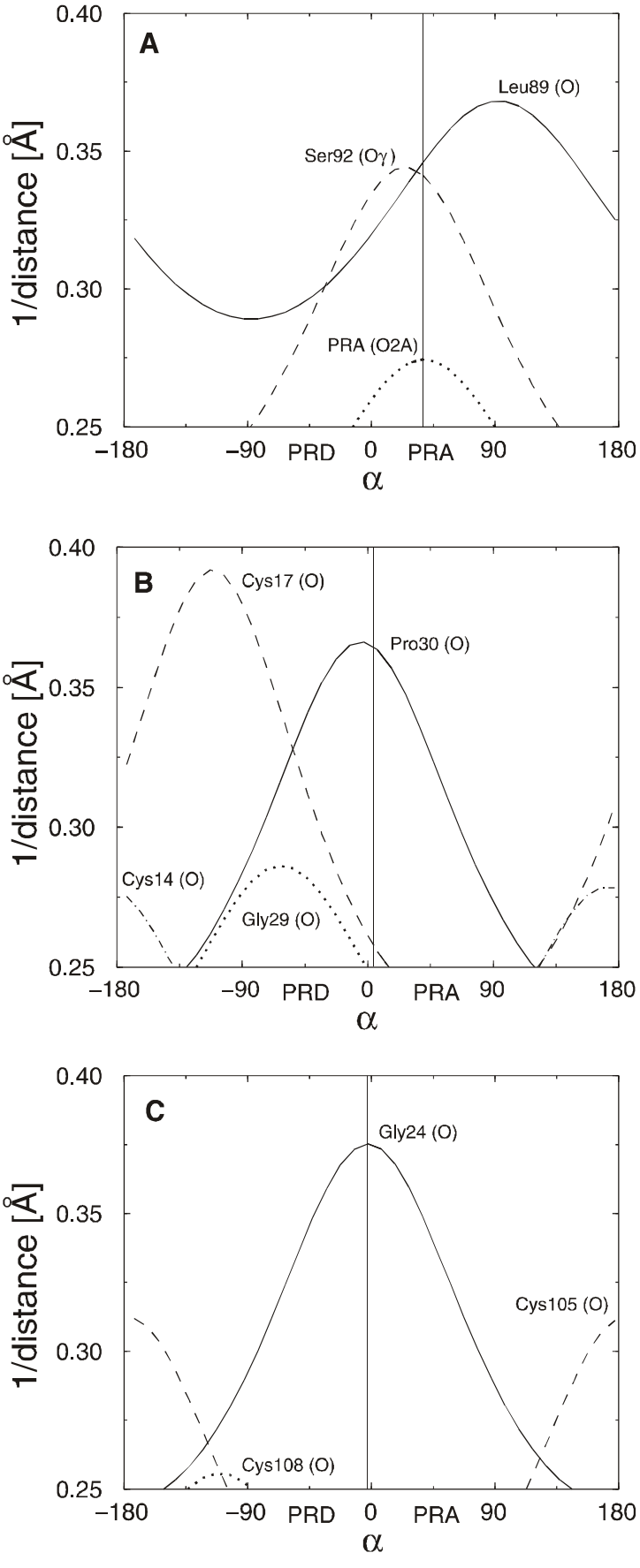

Figure 4.3. Possible hydrogen bonds of imidazole axially coordinated to heme in heme-proteins. The hydrogen bonds of axially coordinated imidazole are studied by rotating the imidazole ring around its Nδ-Fe bond and monitoring the distance between the Nδ1 atom and possible hydrogen bond acceptor atoms. 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 4.1. 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.

Part A: heme-protein Mb (PDB code 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 97^0, 27^0, and 37^0, respectively. The orientation assumed in the crystal structure is at $\alpha = 37^0$.

Part B: heme-protein mono-Cc (PDB code 1chh). Possible hydrogen bonds are formed with the backbone oxygens of Pro30, Cys17, Gly29, and Cys24. For optimal orientations the corresponding values of α are -3^0, -113^0, -63^0, and 167^0, respectively. The orientation assumed in the crystal structure is at $\alpha = 7^0$.

Part C: heme-protein bis-Cc (PDB code 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 -3^0, -173^0, and -113^0, respectively. The orientation assumed in the crystal structure is at $\alpha = -3^0$.

Table 4.1: Possible H-bonds of imidazole axially ligated to heme.

#	heme-protein / resolution/ PDB code / chain [a]	residue(atom type) / ligated histidine [b]	distance [Å] [c]	angle α [d] [degree]	angle γ [e] [degree]
1	Mb / 1.75 Å / 1myg / B	Leu89(O) / His93 Ser92(O γ) / His93 heme-PRA(O2A)	2.72 (2.89) 2.91 (2.93) 3.64	97 (37) 27 37	-30
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 Phe83(O) / His87 Ser86(O) H_2O63	2.80 (2.84) 3.03 (3.19) 3.41 3.79	-30 (-50) -100 (-50) 40 -40	24
5	CcPo / 2.20 Å / 1ccp	Asp235(Oδ1) / His175 Asp235(Oδ2) Met172(O) Ala174(O)	2.90 (2.90) 3.58 3.20 3.90	124 (124) 84 -156 -86	-72
6	CcPo / 1.60 Å / 1arv	Asp246(Oδ2) / His184 Asp246(Oδ1) / His184	2.87 (2.89) 3.20 (3.25)	85 (95) 120 (95)	-72
7	mono-Cc / 1.97 Å / 1chh	Pro30(O) / His18 Cys17(O) Gly29(O) Cys14(O)	2.73 (2.75) 2.55 3.49 3.59	-3 (7) -113 -63 167	-93
8	mono-Cc / 1.80 Å / 1cgo	H_2O 226 / His120 Cys116(O) H_2O270	2.91 (2.92) 2.80 3.63	112 (102) -158 62	-92
9	bis-Cc / 2.16 Å / 1czj	Gly24(O) / His109 Cys105(O) Cys108(O)	2.66 (2.66) 3.21 3.91	-3 (-3) -173 -113	-96
10	bis-Cc / 2.16 Å / 1czj	Tyr73(O) / His77 Tyr73(Oη) Phe76(O)	2.66 (2.68) 3.77 2.99	-1 (15) 105 -115	-86
11	Cb / 1.50 Å / 1cyo	Gly42(O) / His39 Gly41(O)	2.75 (2.75) 3.78	-96 (-96) -46	40
12	Cb / 1.50 Å / 1cyo	Phe58(O) / His63 Val61(O) heme-PRA(O2A) H2O509	2.71 (2.71) 3.73 3.93 3.31	-114 (-114) -34 36 -4	11

[a] Group of heme-proteins to which the considered protein belongs to (Mb, Hb, CcPo, mono-Cc, bis-Cc, Cb), resolution of crystal structure, PDB code, and chain considered.

[b] Lists residues (type and number) and corresponding atom types in brackets involved in an H-bond with the axially coordinated histidine. The residue listed first is the actual hydrogen bonding partner in the crystal structure.

[c] Provides distance of closest approach of non-hydrogen atom pairs, which could possibly be involved in an H-bond with the NδH 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 brackets.

[d] Provides pseudo torsion angle α of closest approach as defined in footnote c. For a definition of the angle α see figure 4.1. The angle α in the actual crystal structure is given in brackets.

[e] For a definition of the pseudo torsion angle γ see figure 4.1.

Influence of hydrogen bonds on imidazole orientation in heme-proteins. The orientation of imidazoles axially ligated to heme can vary to some extend through the bifurcated hydrogen bond, as in Mb family. However, here the ligated imidazole is more or less oriented toward the propionic group PRA. We found a significant deviation from the imidazole ring orientation toward the propionates, only in the cases where the ligated imidazole forms a strong hydrogen bond with a negatively charged acidic amino acid. That could be the explanation for unusually large values of the torsion angle α in CcPo family and in a few other exceptions. 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 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 pattern is varied.

Possible hydrogen bonds of imidazoles axially ligated to heme in heme-proteins. As, it is explained in the method section, we rotated the imidazole ring of axially ligated histidines around the Nδ-Fe bond and monitored the distance of the Nδ atom to possible hydrogen bond acceptors as a function of the pseudo-torsion angle α. In that way, we were searching for the possibility that the NδH group may form a hydrogen bond different from one of the native structure. We studied that for a large number of different proteins, and two typical examples for each of the six groups of heme-proteins are displayed in Table 4.1. We found that in the most of the considered cases, there are several possibilities for making the H-bonds. Our distance criterion for the detection of hydrogen bonds was rather conservative. Considering the structural relaxation the number of possible H-bonds might become considerably larger.

Part A of Figure 4.3 demonstrates the typical hydrogen bonding pattern of myoglobin. The bifurcated hydrogen bond with Leu89 and Ser92 is easily recognizable. In addition there is a hydrogen bond with the propionic group PRA, which is more distant than the two other hydrogen bonds but can be relatively strong, since the PRA group is negatively charged. Interestingly the orientation of the axially ligated imidazole in the native structure points directly toward this propionic acid. In part B, four possible hydrogen bonding partners of the axially ligated histidine in the mono-Cc heme-protein (PDB code 1chh) are shown. The minimal distance to the possible bonding partner Cys17 is even smaller than the one assumed in the crystal structure. However, this hydrogen bond can not be formed due to unfavorable interactions of the imidazole ring with its backbone (see later). Instead, a hydrogen bond is formed with proline Pro30 such that the NδH group of the imidazole points toward the propionic acid groups. There are three possible hydrogen bond partners of the coordinated His109 in bis-Cc heme-protein (part C). Here, a hydrogen bond with Cys105 is not prevented by unfavorable interactions with the histidine backbone. Nevertheless, in the crystal structure the coordinated histidine forms a hydrogen bond with Gly24. Again that corresponds to an orientation, where the NδH group of the imidazole points toward the propionic acid groups of the heme.

4.3.3 Role of propionic acids

Analyzing data from the PDB. Since we suspected that the propionic groups may play an important role for the orientation of histidine ligated to heme, we analyzed how the distribution of the torsion angle α depends on the conformations of two heme propionates. We discriminated five distinct conformations of the propionic acids: (i) both propionic acid groups pointing toward the ligated histidine, (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, and (v) only PRD pointing toward the ligated histidine. The corresponding

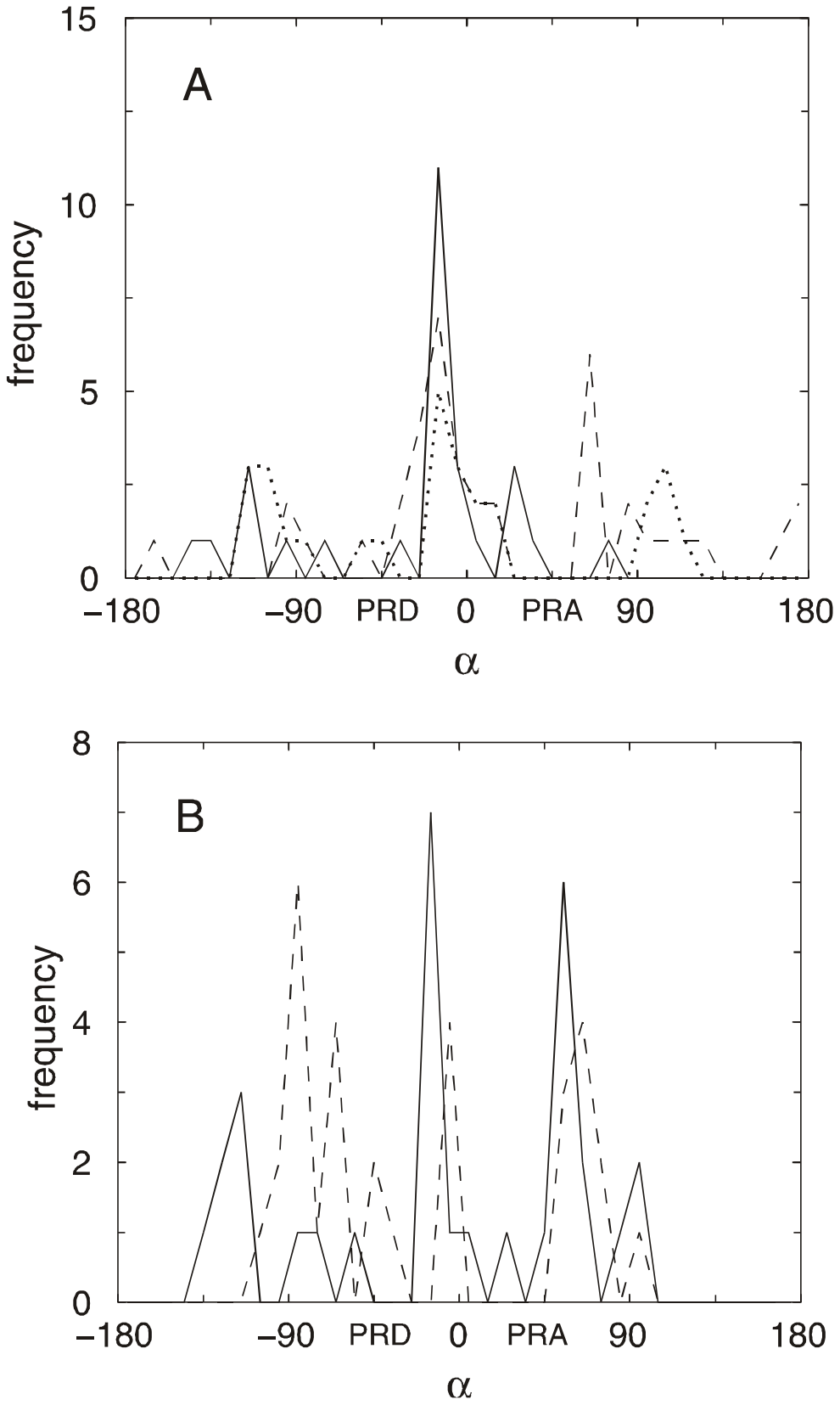

Figure 4.4. Distribution of the torsion angle α of the imidazole heme conformation for all bis-Cc and Cb heme-proteins. **Part A:** solid line: both propionic acid groups point toward imidazole; dashed line: both propionic acid groups are in the heme plane; dotted line: both propionic acid groups turn away from the imidazole. **Part B:** solid line: PRA points toward, PRD away from the imidazole; dashed line: vice versa. The location of the propionic acid groups is marked on the x-axis.

distributions of the torsion angle α for all bis-histidine heme-proteins are displayed in figure 4.4A (conformations i – iii) and in figure 4.4B (conformations iv and v). If both propionic groups point toward the ligated imidazole (figure 4.4A), the imidazole Nδ*H* group is most likely oriented between the propionic acid groups of the heme. Even if the propionic groups are in the heme plane or turned away (type ii and iii), still there is a preference for this orientation, but not so clear as for conformation (i). Beside that, these two conformations (type ii and iii) exhibit similar dependence of the angular distributions. If the propionic 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 (figure 4.4B). But here, there are a number of exceptions, where the pseudo-torsion angle α adopts values larger than $+90^0$ or smaller than -90^0. The majority of the corresponding exceptions belong to the subgroup of cytochrome b5, where the backbone orientation relative to the heme, as well as some steric hindrances caused by side chains of neighboring amino acid residues, enforce these imidazole-heme conformations and prevent the imidazole being oriented toward the propionates.

Force field computations on interactions of imidazole with propionic acids of heme. As, it is mentioned in the method section, intending to explain the influence of propionic groups on the imidazole ring orientation, the interaction energies between the imidazole and heme atom groups were evaluated, using specific CHARMM22 force field computations (MacKerel et al., 1998). Here, we considered the crystal structures of two cytochrome c3, PDB code 1aqe (Aubert et al., 1998), and 2cth (Matias et al., 1996). They belong to the bis-histidine ligated heme-proteins. In the structure 1aqe, the histidines His109 and His77 are axially ligated to heme, and both propionic groups are located on the same side of the heme plane as His109. The calculated interaction energies of the imidazole ring of His109 with heme are displayed in figure 4.5 as a function of the pseudo-torsion angle α describing the imidazole orientation relative to the heme. 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 Nδ*H* group of the imidazole points toward the center of the two propionic acid groups. In the considered crystal structure the corresponding value of the angle is $\alpha = 7^0$. The main part of that interaction is of electrostatic origin (dotted line in figure 4.5). 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 propionates (short dashed line in figure 4.5). The influence of the propionic groups decreases only slightly, if they are hydrogen bonded (dashed-dotted line in figure 4.5). The hydrogen bonding pattern is modeled by considering the corresponding structure (1aqe), where one crystal water (HOH209) 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(Oη). The conformation of the two water molecules was subsequently energy minimized. The influence of the propionic acids on the orientation of a ligated imidazole becomes small, if they are neutralized by protonation, which is, however, unlikely for these acidic groups (long dashed line in figure 4.5). The electrostatic potential of the propionic groups may also be shielded by salt bridges, which can be formed with arginine or lysine. In that case, we expect that the electrostatic interactions with the imidazole ring are reduced, but less than for the protonated propionic groups.

Propionic groups in cytochrome c3 (PDB code 1aqe) (Aubert et al., 1998), are on the opposite side of the heme plane for His77. Therefore, the electrostatic interactions with the imidazole ring are much weaker and consequently the relevant interval of the angle α is extended on the broad range between -90^0 to $+90^0$. Nevertheless, in the crystal structure, the corresponding value of the angle is $\alpha = 13^0$. The total interaction energy is displayed as solid

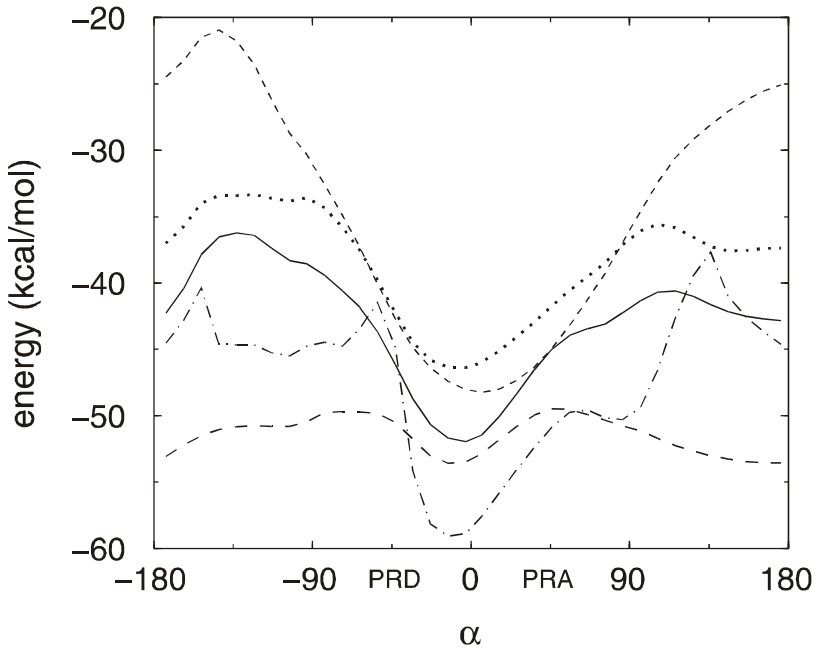

Figure 4.5. Calculated interaction energies of imidazole with heme for the torsion angle α. Both propionic groups are unprotonated (if not otherwise stated) and point toward the imidazole. The interactions were calculated with the CHARMM22 force field. The heme coordinates were taken from the crystal structure of cytochrome *c* (PDB code 1aqe) of the group bis-Cc. The imidazole of His109 was considered. Solid line: total interaction energy between imidazole and heme; dotted line: electrostatic interaction only; short-dashed line: interaction of the polar groups Nδ1-Hδ1 and Cε1-Hε1 of imidazole with the carboxyl groups of the two propionic acid groups of heme; dashed-dotted line: interaction between imidazole and heme with hydrogen bonded propionic acid groups; long-dashed line: interaction between imidazole and heme with both propionic groups protonated.

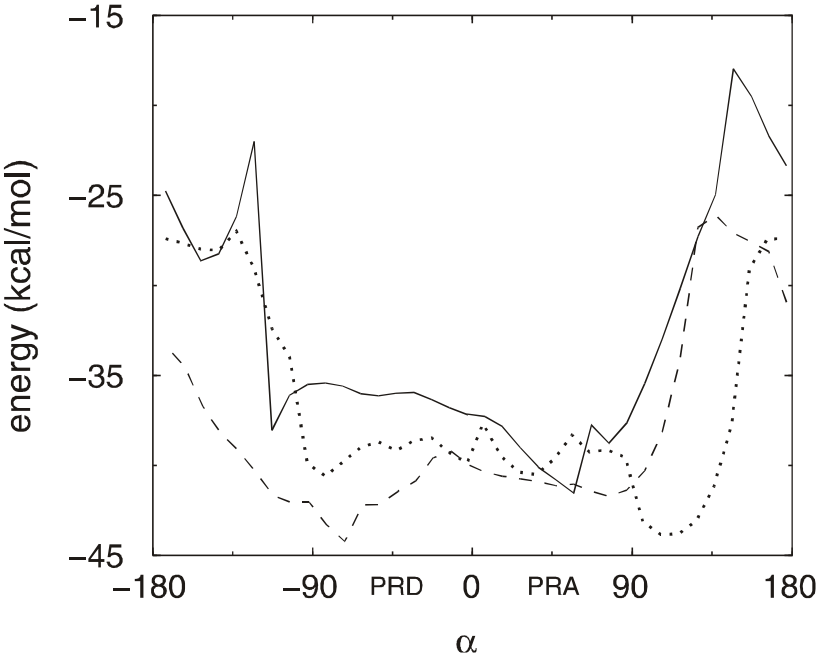

Figure 4.6. Calculated interaction of imidazole with heme as a function of torsion angle α. Both propionic groups are unprotonated and point away from the imidazole. The solid line shows results from His77 ligated to heme of cytochrome c3 structure 1aqe, where the imidazole of His77 is on the opposite side of the heme plane as the two propionic acid groups. The dashed line shows results from His35 ligated to heme of the cytochrome c3 structure 2cth, where PRD points toward the imidazole of His35 and PRA points away. The dotted line exhibits also results from the cytochrome c3 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.

line in fig. 4.6. The case, where one propionic acid group is located above and one below the heme plane, can be studied by considering the cytochrome c3 structure with PDB code 2cth (Matias et al., 1996), where PRD points toward His35 (dashed line in figure 4.6) and PRA points toward His52 (dotted line in figure 4.6). Due to the attractive interactions of the imidazole with the propionic groups the energy minimum at negative (positive) values of the angle α is deeper for His35 (His52). This partially agrees with the corresponding values of the angle α for the two histidines in the crystal structure, which are $\alpha = -63^0$ for His35 and $\alpha = -14^0$ for His52.

4.3.4 Influence of histidine backbone

Analyzing data from the PDB. The imidazole ring of the histidine coordinated to heme is at the same time covalently bound to the protein backbone. The protein backbone has a specific orientation relative to the heme. It suggests that the histidine backbone may impose steric constraints on the orientation of the imidazole ring. Figure 4.7 shows the distribution of the histidine backbone angle β(Cα-Cβ-Cγ-Nδ1) for all non-coordinated histidines (dashed line, scaled down by a factor 6) and for all histidines coordinated to heme with exception of the Mb and Hb groups (solid line). The distribution of the torsion angle β of all non-coordinated histidines exhibits two maxima, which are situated at about $+90^0$ and -90^0. In addition, the distribution of the backbone angle of coordinated histidines shows a maximum at about $\beta = \pm 180^0$, which mostly arises from the CcPo group. Namely, in the CcPo proteins, the Nδ–H group of coordinated histidine is strongly hydrogen bonded with an negatively charged Asp. It determines the orientation of the imidazole ring relative to the heme and enforces the angle β to assume such unusual values (see also section 4.3.5). The heme-proteins of the Mb (Hb) group, dotted (dash-dotted) line in figure 4.7 contribute only to β angles close to $+60^0$ ($+90^0$) but not to -90^0. This is not surprising, since in all these structures the protein backbone carrying the ligated histidine always comes from the same side of the heme plane, and the angle $\alpha = -90^0$ would mean that the imidazole ring is oriented in direction opposite to the propionates.

Force field computations of imidazole with backbone interactions. Using the molecular force field of CHARMM22, we evaluated the interactions of the imidazole ring with the histidine backbone as a function of the torsion angle β. The energy profile of the backbone angle β is displayed in figure 4.8. The total energy (solid line) exhibits two minima at β values of about -90^0 and $+90^0$, which have a well depth of 5.5 kcal/mol and 2.5 kcal/mol, respectively and two maxima at about 0^0 and $\pm 180^0$. The position of these minima and maxima is in agreement with the distribution of the backbone imidazole angle β, derived from the non-coordinated histidines in the PDB (figure 4.7). Surprisingly, steric effects have only a minor importance. The dominant part of the interaction of the imidazole ring with its histidine backbone is of electrostatic nature (figure 4.8, dashed line). However, the van der Waals interactions make the minimum at -90^0 shallower and simultaneously the minimum at $+90^0$ deeper (dotted line). The torsion angle energy is slightly enhancing the difference between the minima and maxima (dashed-dotted line).

4.3.5 Imidazole-heme conformations for different groups of heme-proteins

Most of the observed histidine orientations in the heme-proteins can be explained by considering the influence of the propionic groups and/or the histidine backbone on the imidazole ring. The pseudo-torsion angle γ characterizes the orientation of the histidine

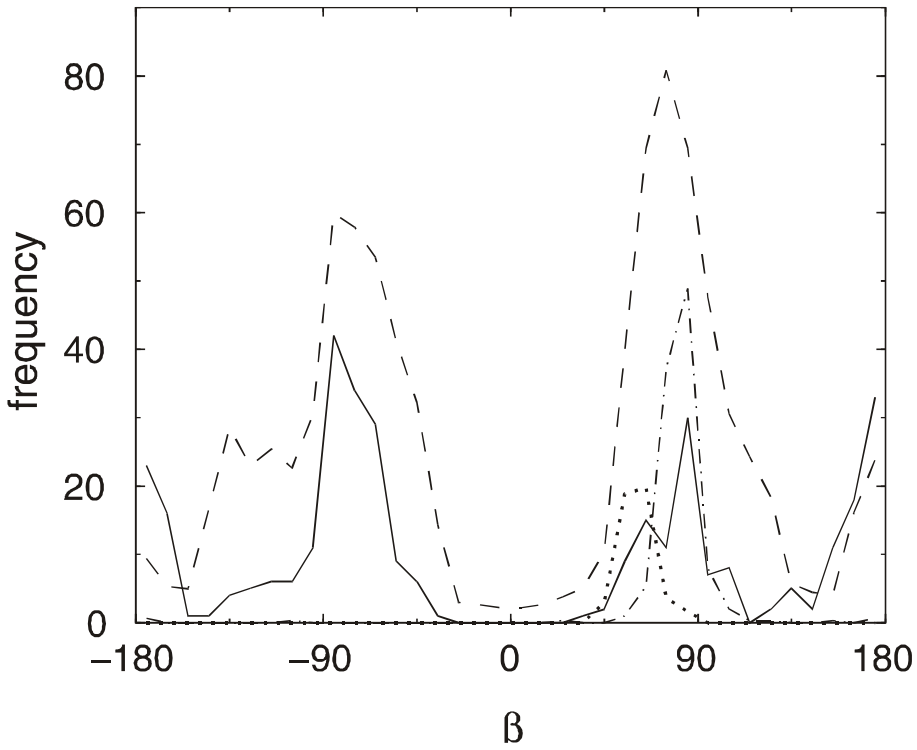

Figure 4.7. Overview of imidazole histidine backbone angles β derived from the PDB. Dashed line: Data from all non-coordinated histidines of the PDB scaled down by the factor 6. Solid line: Data from all heme-proteins possessing hemes with axially coordinated histidines with exception of the Mb and Hb groups. Dotted (dashed-dotted) line: Data from the Mb (Hb) group scaled down by the factor 4.

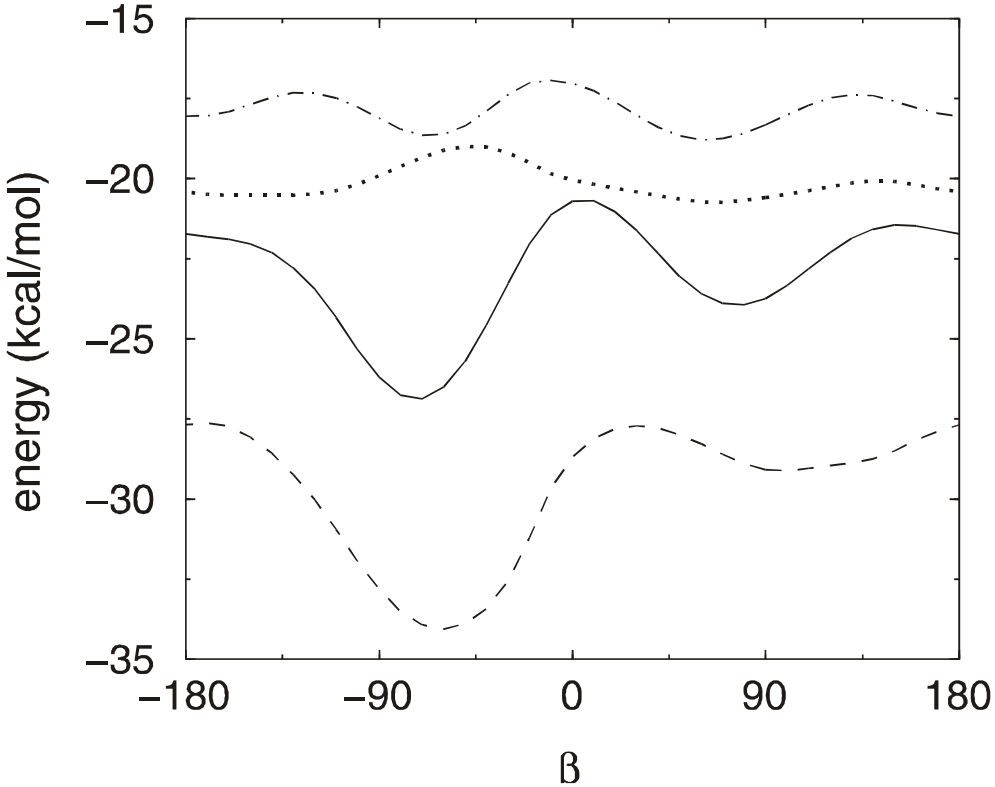

Figure 4.8. Calculated interaction energies between imidazole and its histidine backbone. The interaction energy is calculated with the CHARMM22 force field and displayed as function of the torsion angle β. Solid line: total energy, dashed line: electrostatic energy, dotted line: van der Waals (Lennart-Jones) energy and dashed-dotted line: torsion energy term.

backbone relative to the heme. We have shown that values of the angle β at about $\beta = 0^0$ and $\pm 180^0$ are prohibited or at least not very likely. Since, γ fulfills the relationship in eq. 4.1, the imidazole orientations, where $\gamma \approx \alpha$ or $\gamma \approx \alpha \pm 180^0$ become also prohibited. Consequently, if the protein backbone comes from direction between the two heme propionates ($\gamma = 0^0$), the normally favorable orientation of the imidazole pointing toward the propionates ($\alpha = 0^0$) will be prevented, due to the unfavorable interactions between imidazole ring and protein backbone. On other hand, the preferred angular regime of the torsion angle β is at about $+90^0$ and -90^0 (figure 4.7 and 4.8). Again including the relation between the angles α, β, and γ (eq. 4.1), and depending on the orientation of the protein backbone of histidine relative to the heme, the interaction of the imidazole with the propionic groups determines to which of the two allowed regimes, at $+90^0$ or at -90^0, the angle β will belong. Hence, the angle β adopts the value, which allows the imidazole to orient itself such that its NδH group points toward the heme propionates. By these kind of considerations, we were able to explain the orientations of imidazoles in all groups of heme-proteins, except CcPo, where a strong hydrogen bond determines the imidazole orientation.

Cytochrome c groups. In most of the cytochrome *c* structures the imidazole NδH group is directed in between the two propionic groups PRA and PRD (figure 4.2, dashed and dotted lines). In both cytochrome *c* groups (mono- and bis-Cc) the angle α is close to 0^0, the angle β is typically around 90^0 or -90^0 (see figure 4.10), while the angle γ assumes the values close to $+90^0$ or -90^0, respectively. In these two groups of heme-proteins, the orientation of the backbone relative to the heme and the interaction of the imidazole with its histidine backbone allows the imidazole ring to adopt orientations with the angle α around 0^0 or $\pm 180^0$. Since the angle α assumes values close to 0^0, it is obvious that the imidazole orientations are determined by interactions with the propionic acid groups.

These heme-proteins contain the c-type heme, which is covalently bound with two cysteines attached at the pyrrole rings B and C. 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 angle α characterizing the orientation of the ligated imidazole relative to the heme is displayed in figure 4.9A for the Mb and Hb proteins. It adopts the values of $\alpha \approx 45^0$ for Mb (solid line) and $\alpha \approx 65^0$ for Hb (dotted line). These two groups of heme-proteins exhibit a large sequence and structure identity and with the exception of a few mutants, the imidazole NδH group points toward the propionic group PRA, which is on the same side of the heme plane as the imidazole ring. Since the other propionic group PRD is situated on the opposite side of the heme plane, its interaction with the imidazole should be weak. In addition, the orientation of the imidazole ring is stabilized by a hydrogen bond between the imidazole NδH group and a backbone CO group (see also section 4.3.2). A close neighborhood of the histidine-heme coordination for a typical crystal structure of Hb (1aox, Kavanaugh et al., 1998) with $\alpha = 67^0$ is displayed in the Figure 4.11, top part. The backbone-heme orientation, represented by the angle γ, for the Mb and Hb proteins is also shown in figure 4.9A, dashed line. Since for the Mb/Hb groups the angle γ is around -25^0, the preferred imidazole orientation, where the NδH group is pointing toward the center of the propionic acids ($\alpha = 0^0$) is severely hampered by the interaction of the imidazole ring with its histidine backbone. Since, the distribution of the angle γ has its maximum slightly shifted toward negative values (figure 4.9A), it is not surprising to see that the preferred orientation of the imidazole–heme angle α is in the angle interval around $\alpha = +90^0$ rather than at $\alpha = -90^0$.

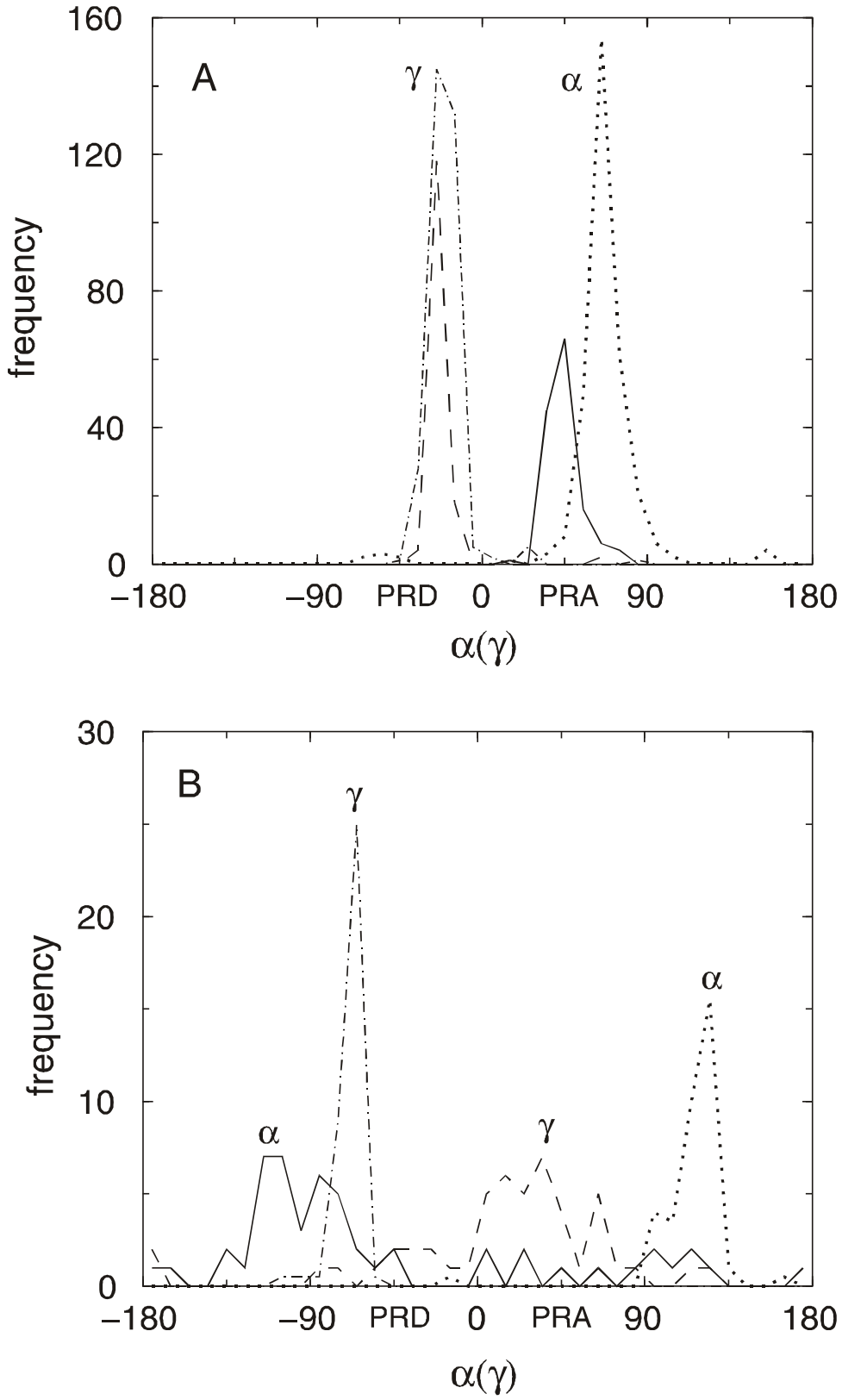

Figure 4.9. Distribution of the torsion angles α and γ for different heme-proteins. The torsion angle α characterizes the orientation of the imidazole relative to the heme represented by solid and dotted lines. The torsion angle γ characterizes the orientation of the histidine backbone relative to the heme depicted by dashed and dashed-dotted lines. **Part A:** Solid and dashed lines refer to the angle distributions of heme-proteins from the Mb group. Dotted and dashed-dotted lines refer to the Hb group of heme-proteins. **Part B:** Solid and dashed line refer to the Cb group of heme-proteins. Dotted and dashed-dotted lines refer to the CcPo group of heme-proteins.

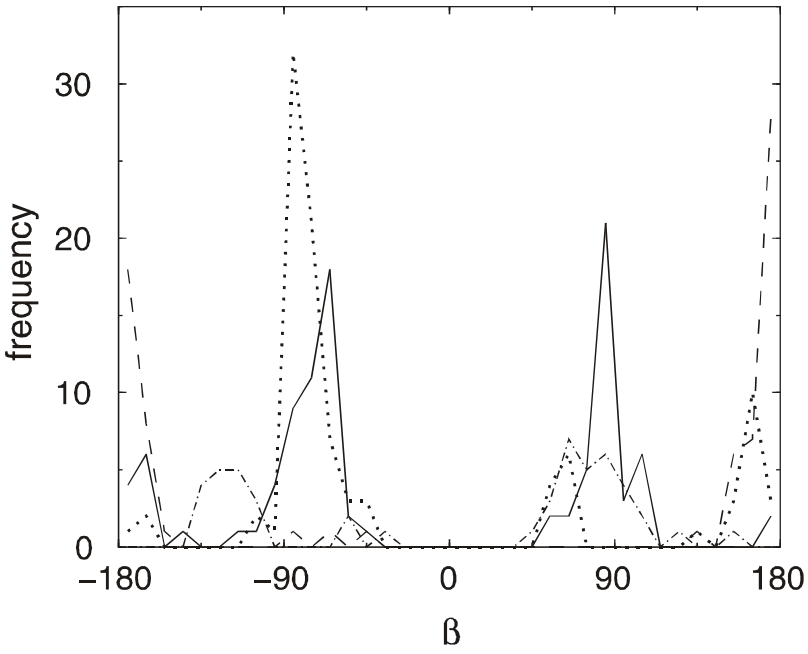

Figure 4.10. Distribution of the torsion angle β characterizing the orientation of the imidazole ring relative to its histidine backbone for different groups of heme-proteins derived from the PDB. Solid line: bis-Cc, dashed line: CcPo, dotted line: mono-Cc, dashed-dotted line: Cb.

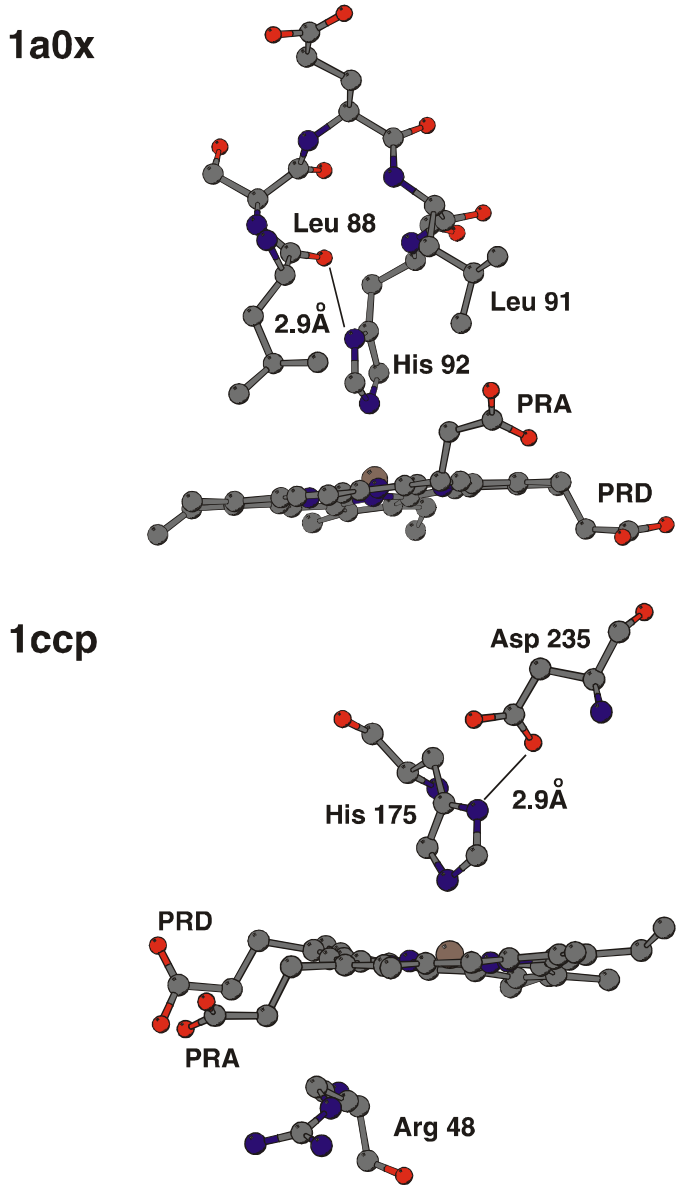

Figure 4.11. Examples of heme-proteins, where the NδH group of an imidazole ligated to heme is not oriented toward the center of the propionic acid groups. **Top:** Displays the heme neighborhood in the crystal structure of hemoglobin 1a0x, where the orientation of the His92 backbone prevents the generally preferred orientation of the imidazole of His92 toward the propionic acid groups. The hydrogen bond of the imidazole NδH group with the backbone carbonyl of Leu88 is only of secondary importance. **Bottom:** Displays the heme neighborhood in the crystal structure of cytochrome *c* peroxidase 1cpp, where the imidazole of His175 is oriented differently, because the NδH group is forming a strong hydrogen bond with the negatively charged aspartate Asp235.

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 of about $\alpha = -90^0$, i.e. the NδH group points away from both propionic acid groups, albeit it is not too far from the propionic acid group PRD (solid line in figure 4.9B). In this group of heme-proteins, the backbone angle γ of the imidazole relative to the heme is not far from $\gamma = 0^0$ (dashed line in figure 4.9B). Hence, the NδH group of the imidazole ligated to heme can not 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 orientation of the imidazole with respect to the heme is preferentially at a negative values of the angle α, close to $\alpha = -90^0$.

Cytochrome c peroxidase group. The heme-proteins of the CcPo group exhibit a very high degree of sequence identity. The backbone Cα-Cβ bond vector of ligated histidine is oriented at angle γ around -90^0 relative to the heme (figure 4.9B, dashed-dotted line), what would not prevent a favorable orientation of the imidazole ring toward the propionic groups. Nevertheless, the distribution of the torsion angle α adopts its maximum at $+120^0$ (figure 4.9B, dotted line). In this orientation the imidazole ring can no longer interact strongly with the propionic groups. But, at the same time the interaction with its histidine backbone is unfavorable, since the backbone angle β assumes almost exclusively values close to $\pm 180^0$ (figure 4.10, dashed line). In figure 4.10, the distribution of the backbone angle β for different heme-protein groups are compared. Hence, for the CcPo group, the orientation of the ligated imidazole ring can be explained neither by interactions with the propionic groups nor with the histidine backbone. Here other factors determine the orientation of the imidazole ring. We made a closer inspection of the heme surrounding to find such factors.

In heme-proteins of CcPo group an aspartate (Asp235), which is located at the heme edge opposite to the two propionic groups forms a hydrogen bond with the NδH group of the imidazole ligated to heme (figure 4.11, bottom part). Since aspartate is negatively charged, this hydrogen bond is particularly strong enforcing this orientation of the imidazole. Moreover, in CcPo proteins both propionic groups are situated on the opposite side of the heme plane from the ligated histidine. This conformation of the propionates weakens their influence on the imidazole orientation. Figure 4.11 bottom part displays a typical crystal structure of CcPo (PDB code 1ccp, Wang et al., 1990) where $\alpha = 124^0$. 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 furthermore. Hence, it can be understood that in CcPo heme-proteins the imidazole ring adopts an orientation, which does not depend on the propionic acid groups, but it is determined by specific very strong hydrogen bond.

4.3.6 Mutual orientation of two axially coordinated histidines

Finally, we investigated the mutual orientation of the two axial histidines in the groups of the bis-histidine ligated heme-proteins, by measuring the difference of the angle α ($\Delta\alpha = |\alpha_2 - \alpha_1|$) that the projection of the NδH groups of two coordinated histidines on the heme plane form. The results are displayed in figure 4.12. Two different groups of heme-proteins (Cb and bis-Cc) possess two axial histidines as ligands. For the group bis-Cc (figure 4.12, solid line), the majority of imidazole planes ligated to heme are oriented parallel to each other. That is not unusual, since for the whole group of cytochrome *c*, the preferred orientation of the imidazoles is to point toward the center of 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 $\Delta\alpha$ are preferred (dashed line in figure 4.12). We did not find for any of these proteins $\Delta\alpha \approx 180^0$, where the imidazole planes are parallel but with oppositely oriented N-H groups. Since at least one histidine is oriented toward propionates, the second one should be oriented oppositely, what is highly unlikely.

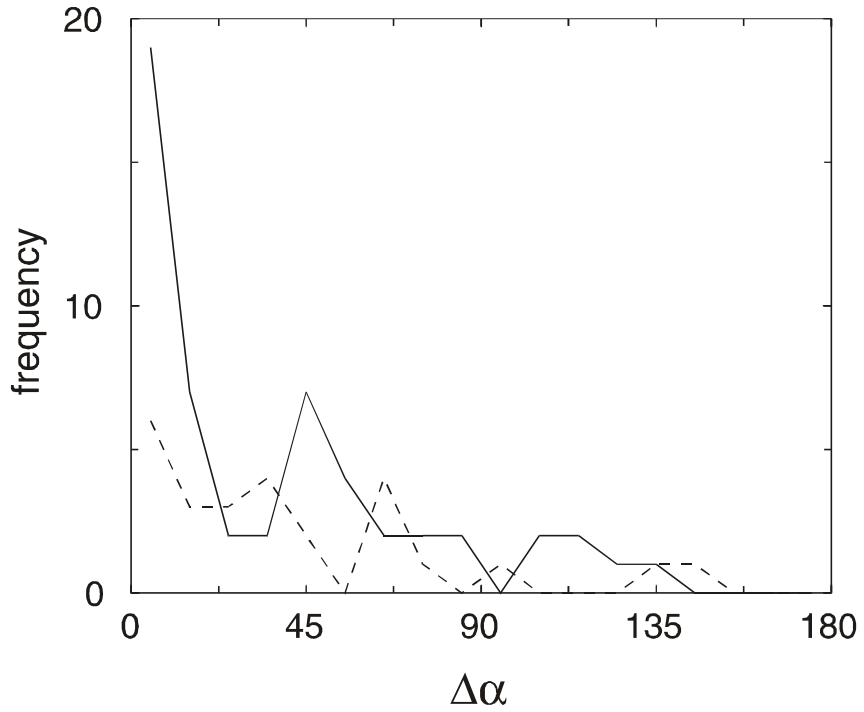

Figure 4.12. Relative orientation of the imidazole planes of histidines axially coordinated to heme, as measured by the torsion angle difference $\Delta\alpha = |\alpha_2 - \alpha_1|$. The distributions were derived from heme-proteins of the PDB, where the heme is axially coordinated by two histidines. Solid line: From the cytochrome *c* group (bis-Cc). Dashed line: From the cytochrome *b* group (Cb).

4.4 Conclusions

Factors determining conformations of imidazole axially coordinated to heme in heme-proteins were investigated by analyzing the crystal structures from the PDB. We discriminated six groups of heme-proteins: myoglobin (Mb), hemoglobin (Hb), cytochrome *c* with one (mono-Cc) or two (bis-Cc) axially coordinated histidines, cytochrome *c* peroxidase (CcPo) and bis-histidine ligated cytochrome *b* (Cb). Data from PDB structures show that the preferred orientation of the NδH group of imidazole ligated to heme is toward the propionic groups. That indicates the existence of an interaction of imidazole with the propionates. The imidazole adopts also a preferred orientation with respect to its histidine backbone. 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, determines also the orientation of the imidazole with respect to the heme. Considering these two factors that are mainly of electrostatic nature, we were able

to explain the orientation of axially coordinated imidazoles for all families of heme-proteins, except the CoPo group.

By molecular force field computations the interactions of imidazole with propionic groups, with histidine backbone and with two cysteines covalently attached to the heme were evaluated and correlated with results from searching in the PDB. Results from force field computations are in agreement with experimental data. Namely, they showed that there is an energy minimum when the Nδ*H* group of the imidazole is oriented toward the propionic groups and that there are energy minima 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.

Since the propionic acids often prefer to remain charged, the acidic groups of the propionates may often be at the protein surface. Hence, the vector Fe-CHA 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 $+90^0$ (-90^0). Due to the interactions of the imidazole ring with the backbone, the torsion angle β can be in one of the two allowed regimes around $\beta = +90^0$ or -90^0. Thus, according to the eq. 4.1, the torsion angle α can generally assume values around 0^0, in agreement with the fact that for the majority of heme-proteins the bond vector Nδ-*H* points toward the center of the propionic acids.

Analyzing the crystal structures of heme-proteins, we can conclude that the hydrogen bonding pattern does not determine the overall orientation of imidazole, although it is probably used by nature to fine tune the orientation of imidazole axially ligated to heme. Most often the H-bond acceptor is the CO group 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 ring. We found that the Nδ*H* group of imidazole ligated to heme can assume a number of hydrogen bonds that differ from the native one. Beside 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. In some cases strong hydrogen bonds of the imidazole Nδ*H* group with negatively charged acidic residues can also be important. Thus, in the group of cytochrome *c* peroxidase, the orientation of the imidazole is determined by a strong hydrogen bond of the Nδ*H* group with the aspartate residue Asp235. It enforces the imidazole ring to adopt an unusual orientation with torsion angle $\alpha = 120^0$.

One may wonder that 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 indirectly it applies a significant influence on the conformations of imidazoles axially coordinated to heme.