Published in final edited form as:

Biochem Biophys Res Commun. 2012 August 10; 424(4): 771–776. doi:10.1016/j.bbrc.2012.07.032.

Tyrosine B10 triggers a heme propionate hydrogen bonding network loop with Glutamine E7 moiety

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Abstract

Propionates, as peripheral groups of the heme active center in hemeproteins have been described to contribute in the modulation of heme reactivity and ligand selection. These electronic characteristics prompted the question of whether the presence of hydrogen bonding networks between propionates and distal amino acids present in the heme ligand moiety can modulate physiological relevant events, like ligand binding association and dissociation activities. Here, the role of these networks was evaluated by NMR spectroscopy using the hemoglobin I PheB10Tyr mutant from *Lucina pectinata* as model for TyrB10 and GlnE7 hemeproteins. ¹H-NMR results for the rHbICN PheB10Tyr derivative showed chemical shifts of TyrB10 OH η at 31.00ppm, GlnE7 N ϵ_1 H/N ϵ_2 H at 10.66ppm/−3.27ppm, and PheE11 C δ H at 11.75ppm, indicating the presence of a crowded, collapsed, and constrained distal pocket. Strong dipolar contacts and inter-residues crosspeaks between Gln E7/6-propionate group, GlnE7/TyrB10 and TyrB10/CN suggest that this hydrogen bonding network loop between GlnE7, TyrB10, 6-propionate group, and the heme ligand contribute significantly to the modulation of the heme iron electron density as well as the ligand stabilization mechanism. Therefore, the network loop presented here support the fact that the electron withdrawing character of the hydrogen bonding is controlled by the interaction of the propionates and the nearby electronic environments contributing to the modulation of the heme electron density state. Thus, we hypothesize that in hemeproteins with similar electrostatic environment the flexibility of the heme-6-propionate promotes a hydrogen bonding network loop between the 6-propionate, the heme ligand and nearby amino acids, tailoring in this way the electron density in the hemeligand moiety.

Keywords

Lucina pectinata (*L. pectinata*); Hemoglobin I (HbI); cyanide-ligated ferric hemoglobin I (HbICN); Tyrosine (Tyr); Glutamine (Gln); nuclear Overhauser effect (NOE); two-dimensional nuclear Overhauser effect spectroscopy (NOESY)

Introduction

Heme groups characterize the active center of proteins such as the globins, cytochromes, and peroxidase [1]. In these proteins the heme iron serves as a source or sink of electrons during

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electron transfer or redox chemistry [2], oxygen (O₂) transport or storage, and as a gas sensor [2; 3; 4]. In essence, peripheral side groups of the heme, such as vinyl and propionate side chains have simply been considered as anchors for connecting the heme prosthetic group to the protein matrix [5]. It has been reported that in cytochrome P450 the heme-6 and 7-propionate side chains form hydrogen bonding interactions with nearby amino acids [6]. Nevertheless, the role of the hydrogen bonding network between the 6 and 7- propionate heme groups, and the distal residues in hemeprotein ligand binding, dissociation and stabilization mechanisms remains unclear. Here, we have investigated the possible inherent role of heme propionate groups in the active center of the rHbICN PheB10Tyr mutant from the clam *Lucina pectinata* using NMR spectroscopy.

This invertebrate clam contains three hemoglobins, HbI, HbII and HbIII, each one with diverse function. HbI is responsible for the delivery of H₂S to intracellular symbiotic bacteria that comprises about one third of the volume of *L. pectinata* gill [7]. HbI is considered a sulfide-reactive monomeric protein of 142 amino acid residues with an active site composition that involves three Phe's at B10, CD1 and E11 positions and a Gln at the E7 position [8; 9]. The natural replacement of HisE7, common in globins, by GlnE7 and the exclusive aromatic arrangement of phenylalanine residues illustrate how the unique distal environment in HbI might influence ligand interactions with the heme. For instance, the fast association of H₂S for HbI, $k_{on} = 2.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, and its slow dissociation, $k_{off} = 0.22 \times 10^{-3} \text{ s}^{-1}$, are responsible for the high affinity of ferric HbI for H₂S, which is 4000-fold greater than those of ferric HbII, HbIII and myoglobin [10]. HbI also exhibits one of the fastest known O₂ association ($k_{on} = 100\text{--}200 \mu\text{M}^{-1} \text{ s}^{-1}$) and dissociation ($k_{off} = 61 \text{ s}^{-1}$) rates [10]. HbI differ from HbII in that the former has a Phe instead of a Tyr at the B10 position. Site-directed mutagenesis studies suggested that the HbI PheB10Tyr mutant exhibits kinetics and structural properties similar to HbII [11]. Interestingly, the TyrB10 and the GlnE7 residues appear in the truncated hemoglobins (trHbs) [12]. Like HbII, most of the examined trHbs exhibit very high O₂ affinities due to unusual slow dissociation rates. This slow oxygen off rates has been suggested to be a consequence of a strong hydrogen bonding between the GlnE7 and TyrB10 with the bound ligand [13]. A hydrogen bonding network has also been invoked for the stabilization of O₂ in HbII [14]. The crystal structure of HbIIIO₂ shows a dimeric structure and a small heme pocket cavity inducing the formation of strong H-bond between the iron and the oxygen molecule, where oxygen is anchored to the heme through hydrogen bonds with TyrB10 and GlnE7 stabilizing in turn the heme (Fe^{II}) oxidation state [15]. It has been proposed that this network is responsible for the slow oxygen off rate in HbII, similar to the *Ascaris suum* hemoglobin that also has a GlnE7 and TyrB10 on the distal heme pocket [14]. Furthermore, the crystal structure of HbIIIO₂ shows a hydrogen bonding network in the heme proximal site between the HisF8 nitrogen proton, and a water molecule, which forms a Hbond with the heme 7-propionate group [15]. It has been suggested that this proximal network modulate the His98 trans-effect, affecting the oxygen dissociation rate. Similarly, NMR studies of the proximal site of deoxygenated horse heart myoglobin suggests that replacement of the heme-7-propionate with a methyl group slightly weakens the His-93-heme iron bond [16].

Herein, the CN binding and stability in the heme pocket of the rHbI PheB10Tyr mutant allowed the identification of three side chain labile protons (OH_η, N_{ε1}H, N_{ε2}H) for the distal residues TyrB10 and GlnE7 by isotopic behavior at 31.00 ppm, 10.66 ppm, and -3.27 ppm, respectively. Their relaxation time, T₁, (OH_η at 14 ms, N_{ε1}H at 25 ms), dipolar shift, and NOE effects to adjacent residues were used to place the distal residues in the heme moiety. The large NOE from the GlnE7 N_{ε2}H and dipolar contact with the 6H_β's propionate group at 0.20 and -1.40ppm indicate that this residue is very close to the heme iron and the bound ligand. Saturation of the B10OH_η peak results in a strong NOE to a proton near 6.46 ppm which is assigned to GlnE7C_{γ1}H. In addition, the results show that TyrB10 adopts a proper

orientation to allow a strong H-bond with the bound cyanide. The results also indicate that: (a) the PheB10Tyr mutation promotes an H-bonding network loop through conformational changes of heme pocket, (b) the propionate group H-bonding network restricted the flexibility of prosthetic group, and (c) the hydrogen bonding interactions between distal amino acids, ligand, and propionate groups modulate the electron density of the iron and facilitates heme-ligand stabilization. Similar interaction can be suggested for oxy-heme complexes with analogous heme distal center, like HbII and HbIII from *L. pectinata* or trHbs[12], where the hydrogen bonding network can modulate heme-O₂ affinity and chemistry.

Materials and methods

Expression and culture growth of the rHbI PheB10Tyr mutant was prepared and purified as previously reported [17]. Ferric rHbI PheB10Tyr mutant was prepared by a 1:1 oxidation reaction as described previously [18]. Cyanide rHbI PheB10Tyr complex was obtained by titration of the ferric rHbI PheB10Tyr mutant with a 200mM NaCl/20mM NaCN solution prepared with ¹H₂O(90%)/²H₂O(10%) or ²H₂O (100%). The formation of the cyanide derivative was monitored by its characteristic optical spectra [19]. 1D and 2D ¹H-NMR spectra were collected on Bruker spectrometers operating at 600MHz with pulse sequences and acquisition parameters previously described in the literature [20; 21].

Results

Figure 1 compares the ¹H-NMR spectra of the rHbICN PheB10Tyr variant in ¹H₂O (1A) and ²H₂O (1B). Comparison of these spectra reveals the presence of three strongly relaxed labile protons at 14.86ppm, 31.00ppm and -3.27ppm assigned to the axial proximal residue HisF8N_{δ1}H, and distal residues TyrB10OH_η and GlnE7N_{ε2}H, respectively. Figure 1C shows the 1D NOE spectrum upon saturation of the TyrB10OH_η peak at 31.00ppm. It is characterized by a short longitudinal-relaxation time (T₁) at approximately 14.0ms with diverse NOE to TyrB10 ring protons (C_εH and C_δH), GlnE7 side chain (C_{β1}H, C_{β2}H, C_{γ1}H, C_{γ2}H), and PheE11 ring protons (N_pH, C_{β1}H and C_{β2}H). Significantly, no NOE to GlnE7N_{ε1}H and GlnE7N_{ε2}H was observed, showing strong decoupling among these protons. The assignments were based on the presence or absence of the isotopic proton and by comparing the values of the axial conserved residue and the heme peripheral groups including methyl, vinyl and propionate protons as reported in the literature [1; 21]. For example, the pyrrole 1-CH₃ and 5-CH₃ methyl groups have been observed in the ¹H-NMR spectra of hemoglobins and myoglobins in the 30.00-17.00ppm downfield region. On the other hand, the 19.90ppm chemical shift has been assigned to the 6-H_α propionate group, while the chemical shifts at 19.40ppm, -6.10ppm and -7.99ppm have been designated to a 2-H_α/H_{β1}/H_{βc} vinyl group, respectively. These heme hyperfine shifts patterns have been reported for hemoproteins active in both diamagnetic and paramagnetic region, specifically in six coordinated heme complexes with low spin, Fe(III) oxidation, and ½ spin state configuration [1]. The pattern of heme chemical shifts observed here is similar to that observed for wild-type *Lucina pectinata* HbICN with some significant differences in the propionate and vinyl regions [21; 22]. The main difference between the rHbICN PheB10Tyr and the wtHbICN (not shown) spectra is that for the former the 6-H_α propionate peak appears as a downfield peak at 19.90ppm, while for the latter this is an upfield peak present at 17.25ppm. Likewise, the 2-H_α vinyl peak for rHbICN PheB10Tyr appears very downfield shifted at 19.40ppm while for wtHbICN this is an upfield peak present at 17.07ppm.

Figure 2 shows the results of a variable temperature study that facilitated the assignment of the hyperfine shifted resonances, as illustrated on the insets under the diamagnetic envelope. As observed here, the His/CN⁻ axial-ligand interaction in the heme pocket reflects narrowest

lines and large magnetic anisotropy in the NMR spectra, indicating that the quality of the ^1H -NMR spectra of low-spin ferrihemoproteins strongly depends on the axial ligands [1]. In the wtHbICN complex from *Lucina pectinata* the 6-propionate protons were assigned as follow: C_αH at 5.70ppm, C_αH at 17.25ppm, C_βH at 0.13ppm and C_βH at -1.20ppm. Also, the 7-propionate protons were identified as C_αH at 0.94ppm, C_αH at 2.08ppm, C_βH at -1.65ppm and C_βH at 0.80ppm [23]. These data together with our results from the temperature dependent studies and the inversion-recovery experiments (not shown) of rHbICN PheB10Tyr, allow distinguishing between the 6-H_α at 7.23ppm, $6\text{-H}_\alpha'$ at 19.90ppm, 6-H_β at 0.20ppm, $6\text{-H}_\beta'$ at -1.40ppm and 7-H_β at -1.30ppm propionate protons as shown in Figure 2. In addition, the major NOEs upon saturating the γ -meso shift at 5.15ppm in the inversion-recovery spectrum of the rHbICN PheB10Tyr, suggest strong connections with the α and β protons of the 6-propionate group as predicted for the mean pyrrole H/CH_3 and meso-H shifts [1; 24]. Heme-assignments deduced herein are compared with the paramagnetic systems of several hemoproteins with the standard Mb fold [20] and confirm the hyperfine shifts assignments for the rHbICN PheB10Tyr variant derivative. Figure 3A shows a NOESY cross peaks map that reveals dipolar connectivities for the key distal residues TyrB10, GlnE7, and PheE11 present in the active center of the rHbICN PheB10Tyr mutant. These inter-residues patterns reveal strong coupling when the PheB10Tyr single point mutation is introduced into the rHbICN active site. The intra-residue NOESY cross peak for the GlnE7 is clearly detected in the upper-field portion of the diagonal. Resonances for the GlnE7 side chain were located and assigned together with the characteristic pattern of TOCSY contacts and major NOEs connections with GlnE7 $\text{N}_{\text{e}2}\text{H}$ in a diamagnetic window ranging from 13.00 to -4.00ppm (Figure 3, B-C). The 1D portion of one slice NOESY (Figure 3C) at -3.27ppm locates the strong to moderate dipolar contact between the GlnE7 $\text{N}_{\text{e}2}\text{H}$ with the 6-H_β at 0.20ppm and the $6\text{-H}_\beta'$ at -1.40ppm.

Overall, Table I summarizes the ^1H -NMR results obtained for rHbICN PheB10Tyr showing the distal residues rearrangement when compared to the wtHbICN complex. The significant changes in hyperfine chemical shifts, such as the 10.66ppm for GlnE7 $\text{N}_{\text{e}1}\text{H}$ and -3.27ppm for GlnE7 $\text{N}_{\text{e}2}\text{H}$ in the rHbICN PheB10Tyr mutant compared to the 14.05ppm for GlnE7 $\text{N}_{\text{e}1}\text{H}$ and -1.39ppm for GlnE7 $\text{N}_{\text{e}2}\text{H}$ in the wtHbICN complex, reveal that a conformational effect results when Phe is substituted by Tyr in the B10 position. For example, the side chain GlnE7 $\text{NH}_2\text{-CO}$ is shifted ($\Delta\delta = \delta_{\text{wild type}} - \delta_{\text{mutant}}$) upfield with $\Delta\delta = 3.39\text{ppm}$ and $\Delta\delta = 1.88\text{ppm}$ near to the 6-propionate from its native structural position. In addition, comparison of ^1H -NMR of wtHbI [21; 25] and the recombinant PheB10Tyr mutant cyanomet complexes reveals rotation about the 6-propionate $\text{CH}_\alpha\text{-C}_{\text{pyrrole}}$ bond of the heme in the rHbICN PheB10Tyr heme pocket. Thus, differences in the chemical shifts corresponding to the alpha protons ($\text{H}_\alpha/\text{H}_{\alpha'}$) and beta protons ($\text{H}_\beta/\text{H}_{\beta'}$) of the 6-propionate group are expected.

Discussion

The ^1H -NMR spectrum of rHbICN PheB10Tyr confirms a strong downfield shift for the $6\text{-H}_\alpha/\text{H}_{\alpha'}$ propionate with peaks at 7.23ppm and 19.90ppm. Saturation transfer between amide, $\text{N}_{\text{e}2}\text{H}$, in the GlnE7 distal residue in rHbICN PheB10Tyr reveals strong and moderate NOEs connections with the 6-propionate $\text{C}_\beta\text{H}/\text{C}_\beta'\text{H}$, respectively. The structural behavior of the distal residues and the heme group in the active center was established by the NOESY cross-peak and NOE patterns including the paramagnetic relaxation properties, distinctive of the heme iron with 6th coordinate cyanide axial ligand [1]. The interactions that involve the peripheral groups in the heme via NOE showed a stable contribution in the rHbICN PheB10Tyr moiety. Regarding this, structural changes are revealed throughout major NOEs connections of the TyrB10 O_ηH at 31.00ppm with GlnE7 $\text{C}_{\gamma 1}\text{H}/\text{C}_{\gamma 2}\text{H}$ at 6.46/4.31ppm and PheE11 $\text{C}_{\beta 1}\text{H}/\text{C}_{\beta 2}\text{H}$ at 5.33/7.36ppm. Figure 4 shows a schematic that describes the heme

cavity in *Lucina pectinata* rHbICN PheB10Tyr mutant with the distal residues in contact with the heme, using iron-centered reference coordinate system (x' , y' , z') for a porphyrin and defines the orientation of the axial His plane by the position of helices relative to the x' -axis [1]. As illustrated, the single PheB10Tyr mutation promotes a constrained heme pocket through structural conformational changes that include significant movements of the key distal side chains residues GlnE7 and PheE11 in the active center. A change in orientation is observed for GlnE7 as well as the 6- propionate group, which allows dipole interactions. Rotation of the heme 6-propionate group has also been observed in the photosynthetic cytochrome c_6 Q51V variant from *Phormidium laminosum*, which showed that the axial Met ligand was stabilized through polar interactions with the imidazole and heme 6-propionate. The latter was possible owing to a 180° rotation of both heme propionates upon imidazole binding [26].

The detection of NOESY cross peaks between the C₆H and the adjacent C₈H on the PheE11 aromatic ring at 11.75ppm and 9.26ppm reveals strong displacement of the ring with respect to its native structure that have ring signals at 7.47ppm and 7.53ppm [21]. Based on this, we suggest that the side chain of PheE11 swing 3.91Å closed to the ligand in the heme pocket compared to the native HbICN complex as consequence of PheB10 substitution. Conserved residues such as PheCD1, AlaE8, LeuE14, AlaG8 and SerE10 are shown as probe for the sequence specific assignment through the NOE connectivities. These dipolar interactions in the distal cavity determine the geometry and properties of the active heme center for the rHbICN PheB10Tyr variant. Moreover, the x-ray crystal structure of the HbII-HbIII cyanide complex from *Lucina pectinata*, which has the same active center as rHbICN PheB10Tyr, shows that the 6-propionate and the GlnE7 residue have H-bonding orientation with distances between the 6- propionate C_β and the GlnE7N_{e2} of 4.37Å in HbII chain A and 4.32Å in HbIII chain B and between the 6-propionate C_β and GlnE7O_{e1} of 3.49Å in HbII chain A and 3.58Å in HbIII chain B (data unpublished). Taken together, the results suggest that the dipolar contacts between the GlnE7 distal residue and the 6-propionate group are due to the tyrosine at the B10 position and that this residue is responsible for the new electrostatic interactions. Thus, a hydrogen bonding network is established in the distal region between the GlnE7 and 6-propionate group in rHbICN PheB10Tyr relative to the axial His plane, due to the electron withdrawing character of the hydrogen bonding, which contribute significantly to the modulation the heme iron electron density. This hydrogen bonding network loop between propionate, heme ligand and nearby amino acids support the fact that the heme reduction potentials are in part controlled by the interaction of the propionates and the nearby electronic environments [27].

Analyses of the resonance Raman spectra of deoxy, metcyano, carbonmonoxy, and oxy HbI derivatives have suggested the presence of moderate hydrogen bonding between Arg-99 and the heme-7-propionate. However, ¹H-NMR indicated that hydrogen bonding between the heme-6- propionate and amino acid residues is absent [28]. The flexibility of the heme-6-propionate observed in the rHbICN PheB10Tyr variant suggest that hemeproteins with the same electrostatic environment have the ability to induce a hydrogen bonding network loop between GlnE7, TyrB10, 6-propionate group, and the heme ligand. We suggest that for these systems the hydrogen bonding network may help to create a driving force that may modify the oxygen-heme reactivity, activate the heme chemical environment, and regulates the iron electron density of the heme, facilitating in turn oxygen metabolism. Hence, for *Lucina pectinata* HbII and HbIII as well as other trHbs this network might activate O₂ delivery and chemistry. Indeed, the X-ray crystal structure of the trHb from the green algae *Chlamydomonas eugametos* with cyanide as ligand (trHbC-CN⁻) (PDB: 1DLY) strongly suggest H-bonding networks between GlnE7, TyrB10, 6-propionate group, and the bound ligand, which can modulate O₂ metabolism [29]. Analysis of the structure indicates distances for polar contacts between the 6-propionate C_β and the GlnE7N_{e2} at 4.87Å, the 6-

propionate C_β and GlnE7O_{e1} at 4.15 Å, TyrB10O_η and GlnE7C_γ at 3.44 Å, and TyrB10O_η and CN at 2.36 Å [30]. Similarly, a sperm whale myoglobin mutant having GlnE7 and TyrB10 (PDB: 1F63) and the hemoglobin from *Ascaris suum* (PDB: 1ASH), which naturally bears these two residues at the distal site show distances between the heme 6- propionate and nearby residues that can induce the H-bonding network mentioned above [31]. For instance, in the Mb mutant, designated to mimic the distal pocket of oxy-*Ascaris suum* Hb, possible hydrogen bonding interactions between 6-propionate C_β and the GlnE7N_{e2}, 6-propionate C_β and GlnE7O_{e1}, and TyrB10O_η and GlnE7C_γ are observed with x-ray distance of 4.54 Å, 3.63 Å, and 4.10 Å, respectively [32; 33]. It is important to mention that the reactivity and affinity of these proteins for O₂ have been attributed to the H-bonding network between the TyrB10/GlnE7 pair. However, as we have shown interactions of this pair with flexible propionates groups should also be taken into account when analyzing the metabolism of O₂ with these proteins.

Acknowledgments

This work was supported by funds from the NSF (Grant 0843608) and NIH-NIGMS/MBRSCORE 5 S06GM008103-36. Special thanks to Dr. Gerd N. La Mar for his motivation on NMR of hemoproteins. We also thank Dr. Jeffrey S. De Ropp for the NMR facilities in the University of California at Davis, Dr. Vasyl Bondarenko for assistance with NMR experiments, Dr. Ruth Pietri for helpful suggestions and Dr. Carmen Cadilla for the construction of the rHbI mutant.

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Highlights

- H-bonding network loop by PheB10Tyr mutation is proposed.
- The propionate group H-bonding network restricted the flexibility of the heme.
- The hydrogen bonding interaction modulates the electron density of the iron.
- Propionate H-bonding network loop explains the heme-ligand stabilization.

Figure 1.

600 MHz ^1H -NMR spectra of rHbICN PheB10Tyr, pH 7.0, 25°C. (A) $^1\text{H}_2\text{O}$ with saturation frequency at 31.00ppm in the inset. (B) $^2\text{H}_2\text{O}$ solvent. (C) 1D NOE spectrum upon saturation of the TyrB10OH $_{\eta}$ peak in $^1\text{H}_2\text{O}$. Labeled signals include heme signals in Fisher Notation (i.e. 1-CH $_3$, 6H $_{\alpha'}$) and amino acid signals in the helix position (i.e. B10 $_{\eta}$ = Tyr29 ring OH $_{\eta}$; B10 $_{\epsilon}$ = Tyr29 ring C $_{\epsilon}$ H; E7 $_{\epsilon 2}$ = Gln64 N $_{\epsilon 2}$ H; F8 $_{\delta 1}$ = His96 imidazole N $_{\delta 1}$ H). Labile protons signals are identified with an asterisk(*).

Figure 2.

Temperature dependence of the 600-MHz ^1H -NMR spectrum of rHbICN PheB10Tyr at pH 7.0 in $^1\text{H}_2\text{O}$. The increasing hyperfine shift resolution for the propionate protons signals are illustrated in the up-field region, 0.00 to -4.00ppm , at the temperature increase due to Curie relaxation. The variable temperature spectrum was observed in the range of $15\text{--}30^\circ\text{C}$ with increments of 5°C between each one.

Figure 3.

A 2D ^1H - ^1H NOESY (80ms mixing time) at pH 7.0, 25°C in $^1\text{H}_2\text{O}$ showing the (A) coupling between the TyrB10, GlnE7, and PheE11 spin systems; (B) NOEs observed in the GlnE7 side chain; (C) Majors NOEs detected in the 1D slice of NOESY corresponding to coupling with GlnE7N $_{\epsilon 2}$ H.

Figure 4.

Schematic representation of the distal heme pocket of rHbICN PheB10Tyr mutant showing major NOE dipolar contacts between distal residues (circles) and heme group. The NOEs connectivities are illustrated using double arrows where dashed (---) and solid (-) lines enclose the moderate and strong dipolar contacts, respectively. The heme group is located face on view from the proximal side HisF8.

Table 1

Comparison of ^1H -NMR chemical shifts of the B10, E7 and E11 distal amino acid residues in rHbICN PheB10Tyr mutant and wtHbICN from *Lucina pectinata*.

Residues	Protons	Chemical Shifts (ppm) ^(a)	
		rHbICN PheB10Tyr	wtHbICN ^{b, (c)}
Tyr ²⁹ (B10)	N _p H	8.54	–
	C _α H	4.30	–
	C _{β1} H	3.43	–
	C _{β2} H	3.63	–
	C _δ Hs	8.22	8.70
	C _ε Hs	11.60	13.07
	C _ζ H	–	18.77
	O _η H	31.00	–
Gln ⁶⁴ (E7)	N _p H	8.59	8.93
	C _α H	3.98	4.62
	C _{β1} H	2.90	3.03
	C _{β2} H	2.60	3.29
	C _{γ1} H	6.46	7.63 (7.47)
	C _{γ2} H	4.31	4.97 (4.90)
	N _{ε1} H	10.66	14.05(13.80)
	N _{ε2} H	–3.27	–1.39 (–1.40)
Phe ⁶⁸ (E11)	N _p H	9.09	9.89
	C _α H	3.18	3.81
	C _{β1} H	5.33	9.05
	C _{β2} H	7.36	6.40
	C _δ Hs	11.75	7.47
	C _ε Hs	9.26	7.53
	C _ζ H	7.53	7.87

(–) not observed.

^aChemical shifts in ppm at 25°C, pH 7.0 (rHbICN PheB10Tyr) and pH 7.2 (wtHbICN).

^b[21].

^(c)[25].