Influence of Amino Acid Replacements in the Heme Pocket on the Electron Paramagnetic Resonance Spectra and Absorption Spectra of Nitrosylhemoglobins M Iwate, M Boston, and M Milwaukee†

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ABSTRACT: In order to clarify the role of amino acids in the heme pocket, the electron paramagnetic resonance (EPR) spectra and the absorption spectra of the photolyzed form were obtained for nitrosylhemoglobins M in the fully reduced state. The EPR spectrum of the abnormal α^{NO} subunit of nitrosylhemoglobin M Boston [His-E7(58)α→Tyr] exhibits readily resolved hyperfine splitting due to the ^{14}N nucleus of bound NO and further superhyperfine splitting due to a trans axial ligand with $I = 1$, which implies that His-F8 in the abnormal α subunit is coordinated to the heme iron. Addition of inositol hexaphosphate (IHP) to nitrosylhemoglobin M Boston produces strong triplet hyperfine structures at both the g_y and g_z regions of the spectrum, suggesting anomalous coordination of NO. The EPR spectra of the abnormal α^{NO} subunit of nitrosylhemoglobin M Iwate [His-F8(87)α→Tyr] exhibit strong triplet hyperfines at both pH 6.5 and 9.0 regardless of the presence of IHP. NO of the abnormal α subunit presumably binds to the heme iron from the proximal side as in the carbonmonoxy form [Peisach, J., & Gersonde, K. (1977) *Biochemistry 16*, 2539–2545] and the bond between Fe and N_ϵ (His-E7) must be weak, if present. The EPR spectrum of the abnormal β^{NO} subunit in nitrosylhemoglobin M Milwaukee [Val-E11(67)β→Tyr] becomes similar to that of the α^{NO} chain as a result of the mutation. Addition of IHP to nitrosylhemoglobin M Milwaukee produces the strong triplet hyperfine structure which is attributable to the α^{NO} subunit. The absorption spectra of the photolyzed forms in the near-infrared regions were found to be sensitive to the amino acid replacement in the heme pocket.

Hemoproteins including hemoglobin, myoglobin, cytochromes, oxygenases, and peroxidases have a common prothetic group, protoheme, or its related compounds at the active center. A variety of biological functions acquired by these proteins arise from the nature of the axial ligand(s) and the structure of the heme pocket. Nitric oxide bound to hemoproteins serves as a paramagnetic probe which can provide considerable insight regarding the nature of the trans axial ligand and the interaction of nitric oxide with surrounding amino acid residues (Kon, 1968; Kon & Kataoka, 1969; Yonetani et al., 1972; Shiga et al., 1969; Henry & Banerjee, 1973; Dickinson & Chien, 1974; Henry et al., 1976; O'Keeffe et al., 1978). The three-dimensional structures of hemoglobin and myoglobin provided by X-ray crystallographic studies allow an interpretation of the EPR spectra in terms of stereochemistry. The proximal histidine-F8 is coordinated to the heme iron and is presumed to play an important role in controlling the ligand affinity (Perutz, 1970, 1976). On the distal side of the heme, His-E7 and Val-E11 lie facing the ligand binding site. They are considered to make contact with the ligand, although the role of these residues is not fully understood (Caughey et al., 1969; Tucker et al., 1978). Mutant hemoglobins in which either His-F8, His-E7, or Val-E11 is replaced by other amino acids have been found. These hemoglobins provide an opportunity to investigate the role of these amino acids in the heme pocket.

Hemoglobins M are naturally occurring valency hybrids in which the iron in the abnormal subunit has become oxidized as a result of an amino acid replacement in the heme pocket. Thus, the iron in the abnormal subunit is no longer capable of binding ligands such as O_2 and CO. The ferric heme in the abnormal subunit can be reduced by addition of sodium dithionite and, in the fully reduced form, all four hemes are capable of binding ligands such as CO and NO. We have now studied the EPR spectra of fully nitrosyl-Hb[1] M Iwate [His-F8(87)α→Tyr], Hb M Boston [His-E7(58)α→Tyr], and Hb M Milwaukee [Val-E11(67)β→Glu] (hereafter, NO Hb M is used for Hb M in which hemes both in the normal and abnormal subunits are in the ferrous nitrosyl form).

The EPR spectrum of nitrosylhemoglobin as well as the optical absorption spectrum and infrared stretching frequencies due to bound nitric oxide are considerably influenced by the quaternary structure (Rein et al., 1972; Cassoly, 1974; Taketa et al., 1975; Maxwell & Caughey, 1976; Perutz et al., 1976). In the T state, the EPR spectrum of nitrosylhemoglobin exhibits a strong triplet hyperfine due to the ^{14}N nucleus of bound nitric oxide. Experiments with nitrosyl hybrid hemoglobins demonstrated that the α^{NO} subunit is predominantly responsible for the strong triplet hyperfine and that the EPR spectrum of the β^{NO} subunit is only slightly affected by the switch of the quaternary structure (Henry & Banerjee, 1973; Nagai et al., 1978). When the infrared stretching frequencies and the EPR spectra of nitrosylhemoglobin are compared with those of model heme complexes, it is proposed that the Fe-N_ϵ (His-F8) bond in the α^{NO} subunit is either broken or severely stretched in the T state (Maxwell & Caughey, 1976; Perutz et al., 1976; Szabo & Perutz, 1976; Nagai et al., 1978).

Nitrosylhemoglobin can be photolyzed at liquid helium temperature (Iizuka et al., 1974). The photolyzed form of nitrosylhemoglobin in the T state exhibits anomalous peaks

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[1] Abbreviations used: Hb, hemoglobin; EPR, electron paramagnetic resonance; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; Tris, tris(hydroxymethyl)aminomethane; IHP, inositol hexaphosphate.

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of absorption at 774, 791, and 822 nm which are predominantly associated with the α subunit (Nagai et al., 1978). We suggested that the anomalous photolyzed form may correspond to the four-coordinated ferrous heme which is the photolyzed product of five-coordinated nitrosylheme. In the present work we have also investigated the photolysis of nitrosylhemoglobins M Iwate, M Boston, and M Milwaukee at liquid helium temperature to determine whether the spectrum of the photolyzed form is sensitive to the nature of the amino acid substitutions within the heme pocket.

Materials and Methods

Hemoglobins M Milwaukee, M Iwate, and M Boston were purified in the carbonmonoxy form by ion-exchange chromatography on a column of Amberlite IRC-50 equilibrated with 0.1 M sodium phosphate buffer, pH 7.0 (5.32 g/L Na_2HPO_4, 3.9 g/L $NaH_2PO_4 \cdot 2H_2O$). After the pale pink band of hemoglobin A was eluted with the same buffer, the molarity of the buffer was raised to 0.5 M to elute hemoglobin M and hemoglobin A_2. The eluted hemoglobin solution was gel filtered against Tris-Cl buffer (6.4 g/L tris(hydroxymethyl)aminomethane, 2.54 mL/L concentrated HCl) and applied to a column of DE-52 (Whatman) equilibrated with the same buffer. After hemoglobin A_2 was eluted with the same buffer, hemoglobin M was eluted with 0.06 M potassium phosphate buffer (16.2 g of KOH, 40.8 g of KH_2PO_4 in 5 L). The purity of hemoglobin M was checked by isoelectric focusing of the completely oxidized form (Righetti & Drysdale, 1971). Hemoglobin solution was gel filtered against 1 mM Na_2HPO_4 and then stripped of phosphate by passage through a Dintzis column (Nozaki & Tanford, 1967).

A solution of 1 mM hemoglobin M or hemoglobin A (heme basis) was freed from oxygen in a tonometer by repeated evacuation and flushing with nitrogen and finally with carbon monoxide. The abnormal ferric subunit of hemoglobin M was reduced under an atmosphere of carbon monoxide by addition of 0.05 volume of 100 mM solution of sodium dithionite. Hemoglobin M Milwaukee was reduced within an hour even at 4 °C but hemoglobin M Boston and hemoglobin M Iwate had to be left to stand overnight at room temperature to achieve complete reduction. After complete reduction, free carbon monoxide was removed by repeated evacuation and flushing with nitrogen and nitric oxide gas washed with 0.1 M NaOH solution was injected into the tonometer with an air-tight syringe. The tonometer was gently shaken under strong light to replace CO with NO.

Nitrosylhemoglobin thus obtained was anaerobically transfered to an optical cuvette or an EPR sample tube which had been flushed with nitrogen gas. The EPR spectra of nitrosylhemoglobin were measured at liquid helium temperature with an X-band spectrometer (type JEOL ME-2X). The instrument settings were as follows: microwave power, 1 mW; field modulation width, 5 G; field modulation frequency, 100 kHz. The optical absorption spectra were recorded at 4.2 K on Shimadzu D-40 DF.S spectrophotometer. The absorption spectra of the photolyzed form were obtained as previously reported (Nagai et al., 1978).

Results

EPR Spectra of Fully Nitrosylhemoglobins M. In the absence of IHP, NO Hb A shows rhombic EPR spectra without any readily observable hyperfine structure at both pH 6.5 and 9.0 (Figure 1b,c). As first demonstrated by Rein et al. (1972), addition of IHP to NO Hb A produces a strong triplet hyperfine centered at $g = 2.009$ with a hyperfine coupling constant of 16.5 G (Figure 1a).

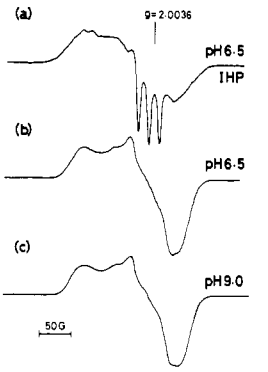

FIGURE 1: EPR spectra of 1 mM (heme) NO Hb A. (a) In 0.1 M Bistris, 2 mM IHP, pH 6.5; (b) in 0.1 M Bistris, pH 6.5; (c) in 0.1 M Tris, pH 9.0

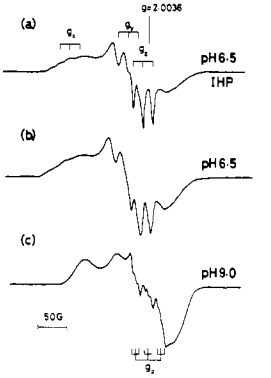

FIGURE 2: EPR spectra of 1 mM (heme) NO Hb M Boston in the fully reduced state. (a) In 0.1 M Bistris, 2 mM IHP, pH 6.5; (b) in 0.1 M Bistris, pH 6.5; (c) in 0.1 M Tris, pH 9.0.

The EPR spectrum of NO Hb M Boston measured at pH 9.0 shows a readily resolved triplet hyperfine due to the ^{14}N nucleus of bound nitric oxide and a further triplet superhyperfine due to a nucleus with $I = 1$ (Figure 2c). The readily resolved hyperfine structure of NO Hb M Boston at pH 9.0 is attributable to the abnormal α subunit since the EPR spectrum of NO Hb A does not exhibit such a structure at pH 9.0 and the EPR spectrum of the β^{NO} subunit is unaffected by the state of the α subunit (Henry & Banerjee, 1973; Nagai et al., 1978). The only possible candidate with $I = 1$ in the abnormal α subunit is the proximal histidine-F8. Therefore, it is concluded that, in the fully nitrosyl form, N_ϵ of proximal histidine (F8) is coordinated to the heme iron, whereas in the ferric form the iron is five-coordinated with a phenolate oxygen of tyrosine-E7α as the only axial ligand (Pulsinelli et al., 1973). Addition of IHP to NO Hb M Boston causes a development of strong triplet hyperfines both at g_y and g_z regions (Figure 2a). The EPR spectrum of NO Hb M Boston measured at pH 6.5 exhibits strong triplet hyperfine even in the absence of IHP, implying that NO Hb M Boston is predominantly in the T state under this condition (Figure 2b). After the measurement of the EPR spectrum, the pH of the sample was adjusted to pH 9.0 with 3 M Tris solution. This sample exhibited an EPR spectrum identical with that shown in Figure 2c, indicating that the changes in the EPR spectra observed here are reversible.

Throughout the experimental conditions examined here, NO Hb M Iwate shows an EPR spectrum composed of two distinct paramagnetic species: the five- and six-coordinated NO–heme complexes (Figure 3a–c). Since Hb M Iwate has proximal histidine (F8) in the α subunit replaced by tyrosine, it is concluded that NO–heme is five-coordinated in the abnormal α subunit and six-coordinated in the β subunit.

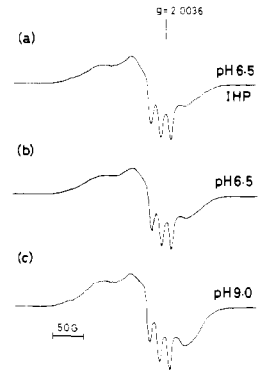

FIGURE 3: EPR spectra of 1 mM (heme) NO Hb M Iwate in the fully reduced state. (a) In 0.1 M Bistris, 2 mM IHP, pH 6.5; (b) in 0.1 M Bistris, pH 6.5; (c) in 0.1 M Tris, pH 9.0.

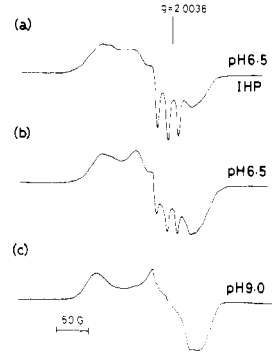

FIGURE 4: EPR spectra of 1 mM (heme) NO Hb M Milwaukee in the fully reduced state. (a) In 0.1 M Bistris, 2 mM IHP, pH 6.5; (b) in 0.1 M Bistris, pH 6.5; (c) in 0.1 M Tris, pH 9.0.

The EPR spectrum of NO Hb M Milwaukee measured at pH 9.0 is similar to that of the isolated α^{NO} chain, whereas the EPR spectrum of NO Hb A measured at pH 9.0 is an arithmetic mean of the spectra of the α^{NO} and β^{NO} chains (Figure 4a–c). This result suggests that the EPR spectrum of the abnormal β^{NO} subunit has become similar to that of the isolated α^{NO} chain as a result of the mutation. Addition of IHP to NO Hb M Milwaukee at pH 6.5 causes a switch of the quaternary structure which is accompanied by a development of the strong triplet hyperfine structure (Figure 4a). Since the EPR spectrum of the β^{NO} subunit is insensitive to the switch of the quaternary structure (Nagai et al., 1978), the triplet hyperfine is attributable to the α^{NO} subunit. The strong triplet hyperfine partly appeared at pH 6.5 even in the absence of IHP implying that some fraction of the molecule has undergone the R→T transition under this condition.

Absorption Spectrum of the Photolyzed Nitrosylhemoglobin. The absorption spectra of photolyzed form of nitrosylhemoglobin were obtained at 4.2 K. The photolyzed form of NO Hb A in the presence of IHP shows anomalous peaks of absorption of nearly equal intensity at 774, 791, and 822 nm, whereas in the absence of IHP only a broad absorption maximum is noted at 765 nm (Figures 5A and 5C). The experiment with nitrosyl-hybrid hemoglobins showed that the α^{NO} subunit within the T state hemoglobin is predominantly responsible for the anomalous photolyzed form (Nagai et al., 1978). The photolyzed form of NO Hb M Boston measured at pH 6.5 in the presence of IHP shows anomalous absorption maxima at 771, 794, and 823 nm (Figure 6A). The absorption maxima at 794 and 823 nm are weaker than the corresponding maxima of photolyzed NO Hb A. NO Hb M Boston at pH 9.0 exhibits neither the strong EPR triplet hyperfine nor the anomalous photolyzed form in the absorption

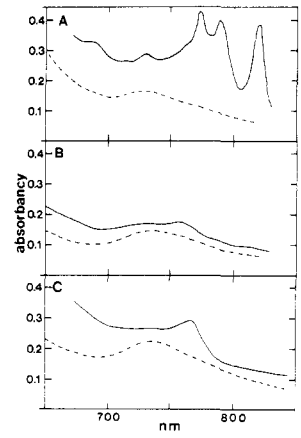

FIGURE 5: Absorption spectra of 1 mM (heme) NO Hb A (---) and its photolyzed form (—) measured at 4.2 K. (A) In 0.1 M Bistris, 2 mM IHP, pH 6.5; (B) in 0.1 M Bistris, pH 6.5; (C) in 0.1 M Tris, pH 9.0. Light path length: 3 mm.

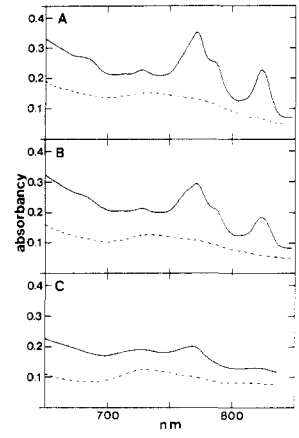

FIGURE 6: Absorption spectra of 1 mM (heme) NO Hb M Boston in the fully reduced state (---) and its photolyzed form (—) measured at 4.2 K. (A) In 0.1 M Bistris, 2 mM IHP, pH 6.5; (B) in 0.1 M Bistris, pH 6.5; (C) in 0.1 M Tris, pH 9.0. Light path length: 3 mm.

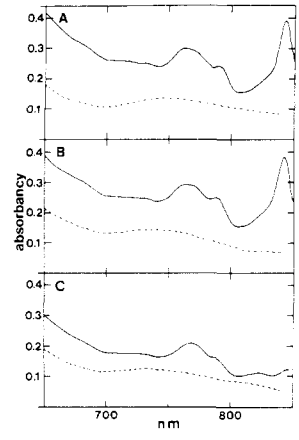

FIGURE 7: Absorption spectra of 1 mM (heme) NO Hb M Iwate in the fully reduced state (---) and its photolyzed form (—) measured at 4.2 K. (A) In 0.1 M Bistris, 2 mM IHP, pH 6.5; (B) in 0.1 M Bistris, pH 6.5; (C) in 0.1 M Tris, pH 9.0. Light path length: 3 mm.

spectrum (Figure 6C). The photolyzed form of NO Hb M Iwate at pH 6.5 both in the presence and absence of IHP exhibits broad absorption maxima at 763 and 790 nm and a sharp maximum at 844 nm (Figures 7A and 7B). The absorption maximum at 844 nm is weakened at pH 9.0, whereas others remain unchanged (Figure 7C). NO Hb M

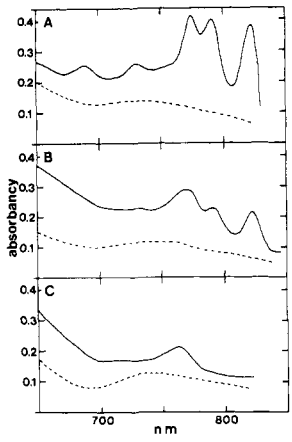

FIGURE 8: Absorption spectra of 1 mM (heme) NO Hb M Milwaukee in the fully reduced state (---) and its photolyzed form (—) measured at 4.2 K. (A) In 0.1 M Bistris, 2 mM IHP, pH 6.5; (B) in 0.1 M Bistris, pH, 6.5; (C) in 0.1 M Tris, pH 9.0. Light path length: 3 mm.

Milwaukee shows the photolyzed form similar to that of NO Hb A except that the anomalous absorption spectrum is observed at pH 6.5 even in the absence of IHP (Figures 8A–8C).

Discussion

Hb M Boston [His-E7(58)α→Tyr] shows low oxygen affinity with no heme–heme interaction indicating that the molecule is frozen in the T quaternary structure (Suzuki et al., 1966). An X-ray crystallographic study by Pulsinelli et al. (1973) showed that the heme in the abnormal α subunit is five-coordinated and that only the phenolate oxygen of tyrosine-E7α is coordinated to the heme iron as an axial ligand. The coordination of tyrosine to the heme iron is considered to lock the α subunit in the tertiary T state and consequently the quaternary T state is stabilized. In the fully nitrosyl form, the abnormal α^{NO} subunit of Hb M Boston exhibits readily resolved nine-line hyperfine structure of the EPR spectrum which indicates that the proximal histidine is coordinated to the heme iron and that nitric oxide is coordinated from the distal side of the heme. The bulky tyrosine at E7α exerts considerable influence on the mode of NO binding to the heme so that the nine-line hyperfine structure is readily resolved. Caughey et al. (1969) studied the infrared CO stretching frequency of fully reduced Hb M Saskatoon (Emory) [His-E7(63)β→Tyr] which has the same mutation at the equivalent position in the β subunit. The infrared CO stretching frequency of the abnormal β subunit was considerably affected by the mutation. The EPR spectrum of NO Hb M Boston measured at pH 6.5 in the presence of IHP is composed of contributions from two distinct paramagnetic species: one shows a trough at $g = 1.981$ which is characteristic of the six-coordinated NO–heme complex and the other shows strong hyperfine splitting at both the g_y and g_z regions. The former is attributable to the β^{NO} subunit and the latter to the abnormal α^{NO} subunit. The strong triplet hyperfine indicates that the Fe–N_ϵ bond in the abnormal α^{NO} subunit is either broken or severely stretched as in NO Hb A in the T state (Perutz et al., 1976; Maxwell & Caughey, 1976). The additional hyperfine at the g_y region suggests that the mode of NO binding is restricted by the steric hindrance of tyrosine-E7α. The triplet hyperfine is observed even in the absence of IHP, indicating that even in the fully reduced state the T structure is stablized by the mutation.

The oxygen affinity of Hb M Iwate [His-F8(87)α→Tyr] is very low and the heme–heme interaction is completely abolished (Hayashi et al., 1966). The X-ray crystallographic study on Hb M Iwate showed that in the abnormal subunit both His-E7α and Tyr-F8α are coordinated to the heme iron giving a six-coordinated ferric heme complex (Greer, 1971). The EPR spectrum of fully nitrosylated Hb M Iwate is composed of two distinct paramagnetic species; one shows the strong triplet hyperfine which is characteristic of five-coordinated NO–heme complexes and the other shows a trough at $g = 1.981$ which is characteristic of six-coordinated NO–heme complexes. The former is attributable to the abnormal α^{NO} subunit and the latter to the normal β^{NO} subunit. The EPR spectra of NO Hb M Iwate remained almost unchanged on addition of IHP or on raising the pH to 9.0. Peisach & Gersonde (1977) studied the optical absorption spectra of CO-liganded Hb M Iwate in the fully reduced state and found that the CO-liganded form of the reduced α subunit bears strong spectral resemblance to the CO-liganded β subunit. They concluded that CO is coordinated to the heme iron from the proximal side of the heme and His-E7α is bound to its trans position. In the fully nitrosyl form, NO in the abnormal α subunit is apparently bound to the heme from the proximal side as in the CO form and the bond between Fe and N_ϵ of His-E7α would be either broken or severely stretched since the Fe–N_ϵ bound is weak when NO is bound in its trans position (Mingos, 1973). It is not clear if NO Hb M Iwate lies in the T or R state under the experimental condition. Since the Fe–His-F8 bond is no longer present in the α subunit, the structural change of globin may not be reflected on the electronic state of the heme even if the quaternary structure of the molecule is switched.

The X-ray crystallographic studies on Hb M Milwaukee by Perutz et al. (1972) showed that, in the half-ferric state, both glutamate-E11β introduced by mutation and His-F8β are coordinated to the heme iron. The absorption spectra of CO- and NO-liganded Hb M Milwaukee in the fully reduced state are identical with those of CO and NO Hb A, respectively, suggesting His-F8β is coordinated to the heme. The EPR spectrum of NO Hb M Milwaukee in the R state (pH 9.0) indicates that the EPR spectrum of β^{NO} subunit in the R state has become similar to that of the isolated α^{NO} chain. Val-E11 lies very close to the ligand binding site in the β subunit (Perutz, 1970) and the replacement of Val-E11 by Glu should change the mode of NO binding so as to make the EPR spectrum of β^{NO} subunit similar to that of the α^{NO} chain. In the T state, the EPR spectrum of NO Hb M Milwaukee shows the strong triplet hyperfine structure which is attributable to the α^{NO} subunit.

In previous work (Nagai et al., 1978), we demonstrated that the photolyzed form of the five-coordinated NO–heme within hemoglobin exhibits an anomalous absorption spectrum in the near-infrared region. If the five-coordinated NO–heme complex is photolyzed, this should leave behind the four-coordinated protoporphyrin iron(II). The anomalous absorption spectrum of the photolyzed form is in fact similar to that of tetraphenylporphyrinatoiron(II) dissolved in benzene which is thought to be a four-coordinated heme (Brault & Rougee, 1974). The absorption spectrum of the photolyzed form is found to be very sensitive to the amino acid replacement in the heme pocket. The replacement of the distal His-E7 by Tyr in NO Hb M Boston weakened the absorption maxima at 790 and 820 nm. This might be due to the changes in the dielectric constant in the heme pocket caused by the mutation. The replacement of proximal His-F8 by Tyr in NO Hb M Iwate altered both the intensity and peak position of the photolyzed form. The 820 nm band of photolyzed NO

Hb A is red shifted by 20 nm in photolyzed NO Hb M Iwate. NO Hb M Milwaukee in the T state exhibits the anomalous photolyzed form identical with that of NO Hb A since the anomalous photolyzed form is predominantly attributable to the α^{NO} subunit and both hemoglobins have normal α subunits.

In the present study, we have demonstrated that the amino acid replacement in the heme pocket considerably influences the mode of ligand binding to the heme. Changes in the EPR spectra of NO Hb and in the absorption spectra of the photolyzed derivative of NO Hb are shown to be associated with the state of heme within the quaternary structure of hemoglobin and, in addition, differences due to mode of ligand binding are clearly demonstrated. Further studies involving EPR measurements on the single crystal NO Hb should provide considerable insight into the interaction of each amino acid with the ligand.

Acknowledgments

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