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## Proton Magnetic Resonance Study of the Histidines in Hemerythrin and Chemical Identification of the Nonligand Histidines<sup>†</sup>

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**ABSTRACT:** Three non-iron-liganded histidines have been studied in methemerythrin azide monomers from *Phascolopsis gouldii* by 250-MHz proton correlation nuclear magnetic resonance (NMR) spectroscopy. Four of the seven histidines in the protein are not observed because of paramagnetic broadening by the coordinated iron; neither are they observed as contact or pseudocontact shifted resonances. The NMR titration of the three free histidines establishes them as normal histidines with  $pK'$  values of  $7.00 \pm 0.03$  and Hill coefficients

of 0.90, 0.81, and  $0.81 \pm 0.03$ . The chemical shift of the protonated and neutral histidines is normal, and the bandwidth of the resonance absorption is 5 Hz. A pH-dependent reversible transition in the chemical shift of the histidine C(2)H occurs at pH 6.5; above this pH the three protons occur as a singlet but break into three singlets of different chemical shift at acid pH values. Two of the three "free" histidines have been identified by their susceptibility to photooxidation as His-82 and His-34.

**H**emerythrin is a non-heme iron protein which reversibly binds oxygen (Klotz et al., 1957; Boeri & Ghiretti-Magaldi, 1957). A number of chemical and biophysical techniques, predating recent X-ray studies, have been used to elucidate the nature of the iron binding site. Mössbauer spectroscopy has shown that two antiferromagnetically coupled iron atoms (per monomeric unit of 13 000 daltons) comprise the oxygen binding site (Okamura et al., 1969; York & Bearden, 1970; Moss et al., 1971). The chemical studies have suggested that four histidyl (Fan & York, 1969; York & Roberts, 1976) and two or three tyrosyl (Rill & Klotz, 1970, 1971; York & Fan, 1971; Fan & York, 1972) residues are iron ligands. There are a total of seven histidines and five tyrosines per monomer. X-ray analysis of the myohemerythrin from *Themiste pyroides* (Hendrickson et al., 1975) suggested that histidine-25, -54,

-73, and -106 and tyrosine-67 and -109 were iron ligands. Similar studies on the methemerythrin octamer from *Themiste dyscritum* (Stenkamp et al., 1978) suggested that a fifth histidine (His-77) and only one tyrosine (Tyr-114) are ligands to the two iron atoms at the active site. It is now agreed that the latter interpretation is correct (Hendrickson, 1978). In an attempt to resolve the apparent conflict between the crystallographic and chemical data relating to the histidine composition of the iron binding site, we have investigated hemerythrin by NMR spectroscopy and in addition have identified two of the three histidines which are readily photooxidized.

### Materials and Methods

Hemerythrin azide monomers were prepared from the crystalline hemerythrin azide octamer obtained from *Phascolopsis gouldii* by disulfide interchange with cystine as previously described (York & Roberts, 1976), but with all reagents prepared in 92% D<sub>2</sub>O. The preparation was concentrated to 2 mM under vacuum in a S and S collodion bag at 25 °C. These procedures for preparing D<sub>2</sub>O solutions of hemerythrin were necessitated by the susceptibility of heme-

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rythrin to denaturation by lyophilization. "Hemerythrin azide" is the oxidized or methemerythrin form.

Apothemerythrin monomers were prepared from hemerythrin azide monomers which had been partially succinylated (Klotz, 1967) and the iron removed by dialysis against 0.01 M HCl at 0 °C. Iron content was determined by the *o*-phenanthroline method (Fortune & Mellon, 1938) and the degree of succinylation by the disappearance of free amino groups as determined by picrylsulfonyl chloride (Ozols & Strittmatter, 1966).

pH measurements of solutions of D<sub>2</sub>O were made at 25 °C by using a Corning Digital Model 112 pH meter with a small Fisher combination electrode. The pH values reported for D<sub>2</sub>O solutions are the actual meter readings, and the symbol pH\* is used to denote that fact. It has been found that the p*K'* of histidine determined in this manner in D<sub>2</sub>O is not significantly different from that determined in H<sub>2</sub>O (Roberts et al., 1969; Li et al., 1961). Adjustment of the pH\* of the hemerythrin solutions was made by adding 1 M NaOD or 1 M DCl by means of a microsyringe (Gilmont) through a fine Teflon needle (Hamilton) to a rapidly stirred solution. No denaturation of the protein was observed.

**NMR Spectra.** The 250-MHz <sup>1</sup>H NMR spectra were obtained by using a superconducting correlation spectrometer (Dadok & Sprecher, 1974) at the NMR Facility for Biomedical Studies, Carnegie-Mellon University, Pittsburgh, PA, with a sample temperature of 30.5 °C. The spectra reported are averages of 200 1.5-s scans of a 900-Hz region at an attenuation of 40/6 dB and a time constant of 0.28 Hz. Resolution of some spectra was enhanced by processing data in the time domain by the optimum filtering function (Ernst, 1966) prior to Fourier transformation into the frequency domain. The spectrometer was locked on <sup>1</sup>H in the HDO, and the spectra were obtained by sweeping upfield or downfield with reference to this lock signal. Chemical shifts are reported relative to DDS, taking a value of 4.83 ppm for water protons relative to DDS. Additional spectra were taken at 90 MHz with a Bucker instrument coupled to a Nicolet 1080 computer programmed for correlation. Titration curves for the C(2)H histidine resonances were obtained by plotting the chemical shifts of these resonances against pH\*. The observed histidine chemical shifts ( $\delta_{\text{obsd}}$ ) are weighted averages of the protonated ( $\delta_{\text{H}^+}$ ) and neutral ( $\delta_{\text{H}^0}$ ) forms. Thus, the fraction of the protonated form of histidine is given by  $(\delta_{\text{H}^+} - \delta_{\text{obsd}})/(\delta_{\text{H}^+} - \delta_{\text{H}^0})$ , and the fraction of conjugate base is  $1 - (\delta_{\text{H}^+} - \delta_{\text{obsd}})/(\delta_{\text{H}^+} - \delta_{\text{H}^0})$ . The NMR titration data were fitted by a least-squares program to the equation  $\log ([\text{neutral His}]/[\text{protonated His}]) = npK' + npH^*$ , where p*K'* is the p*K* apparent of the histidine and *n* is the Hill coefficient. For the hemerythrin data, three parameter fits (p*K'*, *n*, and  $\delta_{\text{H}^+}$ ) were used in determining the best equation to fit the data.

**Analysis of Histidines Photooxidized.** Photooxidation of hemerythrin azide monomers mediated by methylene blue was performed as previously described (York & Roberts, 1976). The photooxidized hemerythrin was dialyzed against 0.01 M HCl at 0 °C to remove the iron and then against water to remove the HCl. The pH was then adjusted to 8.2 with ammonium carbonate. Chymotrypsin (Sigma, 3× crystallized) was then added at a ratio of 1:100 (w/w), and digestion was allowed to proceed for 4 h at 37 °C. The pH was maintained at 8.2. The digest was then lyophilized to remove water and ammonium carbonate. For preparative peptide mapping, 1 mg of the digest was spotted on 18 × 18 cm cellulose plates which were 0.7 mm thick. Electrophoresis was carried out in a pyridine-acetate buffer, pH 6.5 (pyridine-acetic acid-water,

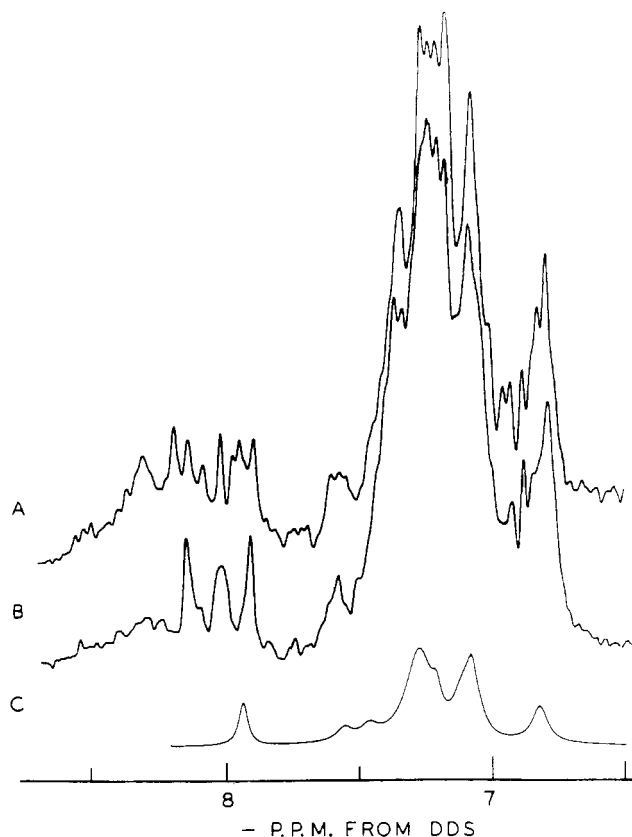


FIGURE 1: 250-MHz <sup>1</sup>H NMR spectra of the aromatic region of succinylapothemerythrin and the effect of preexchange (A) apoprotein at pH 7.5 after standing 3 days in 75% D<sub>2</sub>O at room temperature and (B) apoprotein at pH 7.5 after standing 2 h at pH 11.3 in 75% D<sub>2</sub>O and the pH\* adjusted back to 7.5. (C) Computer-simulated spectrum based on spectra of component amino acids at neutral pH.

25:1:225), and chromatography was carried out in a 1-butanol-pyridine-acetic acid-water (90:60:18:72) system. Diagnostic survey maps were developed with 0.2% ninhydrin in acetone.

Histidine-containing spots were identified by the Pauly spray (Mann & Leone, 1953) and tyrosine-containing spots by the  $\alpha$ -naphtholresorcinol spray (Easley, 1965). For isolation of peptides from TLC plates, the plates were developed with 0.02% ninhydrin in 1-butanol-acetone-acetic acid (7:3:1). For amino acid analysis, the cellulose containing the peptide in question was scraped off the thin-layer plate and the peptide was extracted from the cellulose with three extractions of 6 M HCl. The peptide was hydrolyzed at 110 °C in a sealed tube which had been flushed with nitrogen and evacuated. Amino acid analysis was performed on a Beckman 120C amino acid analyzer.

## Results

**NMR of the Aromatic Region of Apothemerythrin.** Spectra of the alkyl region of apothemerythrin and hemerythrin azide monomer were unremarkable and are not shown. The observed spectra were very similar to the spectra calculated from the amino acid composition.

Representative proton correlation spectra of the aromatic region of apothemerythrin are shown in Figure 1 at pH 7.5 along with the spectrum calculated from the amino acid composition. The assignment of specific protons to resonances in the spectrum is based on the chemical shift and the pH dependence of the chemical shift. The resonances at -6.8 and -7.1 ppm arise from the tyrosine ortho and meta protons, respectively, while the pH-independent resonance at -7.3 ppm

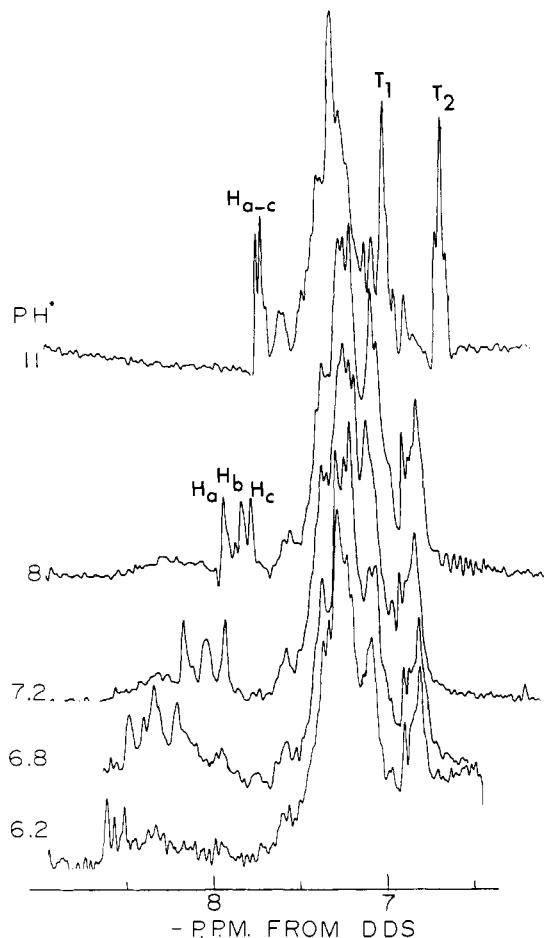


FIGURE 2:  $^1\text{H}$  NMR spectra of the aromatic region of preexchanged succinylapohemerythrin at different  $\text{pH}^*$  values in 0.1 M phosphate buffer in 75%  $\text{D}_2\text{O}$ . Histidine C(2) protons appear between  $-7.6$  and  $-8.6$  ppm, and each resonance labeled contains two protons. Resonance at  $T_1$  is the 3,5 protons of tyrosine, and that at  $T_2$  is the 2,6 protons of tyrosine. Spectra are resolution enhanced by the factors 5.5 and 10.

is due to the phenylalanine protons. In the histidine C(2)H region a large number of resonances are observed (Figure 1, spectrum A). This spectrum was further clarified (spectrum B) by adjusting the  $\text{pH}^*$  to 11 for 2 h to allow deuterium exchange to occur with easily exchangeable amide protons, after which the  $\text{pH}^*$  was returned to 7.5.

The  $\text{pH}^*$  dependence of the aromatic region is shown in Figure 2 for the preexchanged apohemerythrin. The titration could not be performed below  $\text{pH}^*$  6.7 because the apohemerythrin became too viscous. At  $\text{pH}^*$  11.3 the resolution of the histidine C(2)H and the tyrosine ortho and meta protons from other resonances is optimal. On the basis of the presence of ten 2,6 tyrosine hydrogens in the area of the ortho  $^1\text{H}$  resonance at 6.6 ppm, seven histidine C(2)H's are calculated to be present in the resonance at  $-7.64$  ppm, as seen in Figure 2 at  $\text{pH}^*$  11.0. The latter protons appear as two groups of three protons plus one proton shifted to slightly higher field. As the pH is lowered these histidine protons break up into three groups of unique histidines with two histidines in each group ( $\text{H}_a$ ,  $\text{H}_b$ , and  $\text{H}_c$ ). The third histidine is difficult to follow and is not included in the titration curve of the histidine C(2) protons shown in Figure 3. The best theoretical fit to the data is shown by the solid lines. The calculated  $\text{pK}'$  values and Hill coefficients are shown in Table I. Since it was not technically possible to experimentally determine the chemical shift of the completely protonated histidine, the lines were calculated based on a three parameter fit where  $\text{pK}'$ ,  $n$ , and  $\delta_{\text{H}^+}$  were free. The

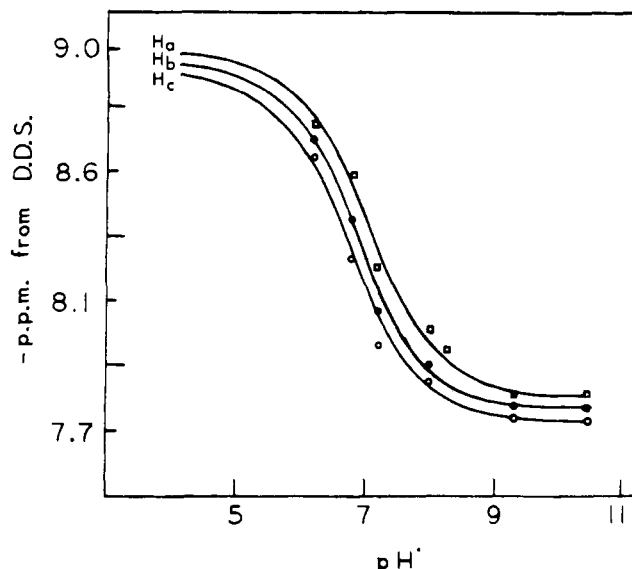


FIGURE 3: Plot of the  $^1\text{H}$  NMR titration data for the histidine protons shown in Figure 2. The solid line represents the least-squares fit to the data. The calculated  $\text{pK}'$  values and Hill coefficients are shown in Table I.  $r = 0.99, 0.99$ , and  $0.95$  for  $\text{H}_a$ ,  $\text{H}_b$ , and  $\text{H}_c$ , respectively, which are the three sets of histidine protons.

Table I: Least-Squares Analysis of NMR Titration Data for the Histidine Residues in Hemerythrin

	NMR peak	$\text{pK}'$	Hill coeff
apohemerythrin	$\text{H}_a$	$7.04 \pm 0.07$	$0.77 \pm 0.04$
	$\text{H}_b$	$6.86 \pm 0.07$	$0.81 \pm 0.04$
	$\text{H}_c$	$6.70 \pm 0.05$	$0.79 \pm 0.03$
hemerythrin azide monomer	$\text{H}_1$	$7.00 \pm 0.07$	$0.90 \pm 0.03$
	$\text{H}_2$	$7.00 \pm 0.07$	$0.81 \pm 0.04$
	$\text{H}_3$	$7.00 \pm 0.07$	$0.81 \pm 0.04$

histidine proton resonances below  $\text{pH}^*$  8 were broader (7–10 Hz) than normally observed:  $\sim 5$  Hz (Markley & Ibanez, 1978) for a molecule of 14 000 daltons.

**NMR of the Aromatic Region of Hemerythrin.** As expected, the NMR spectrum of the hemerythrin azide is not as well resolved as the apoprotein spectra because of the dipolar paramagnetic relaxation of the protons by the iron-iron active center (parts c–g of Figure 4). The spectrum of the oxyhemerythrin octamer is shown in Figure 4b and that of deoxyhemerythrin octamer produced by the addition, under nitrogen, of just enough sodium dithionite to render the solution colorless is shown in Figure 4a. In the latter case the two iron centers are both high spin with four unpaired electrons (York & Bearden, 1970), which obliterates all fine structure in the spectrum.

The tyrosine ring protons are not as evident in the hemerythrin azide spectra (parts c–g of Figure 4) as they were in the apoprotein at alkaline  $\text{pH}^*$ . However, the ortho protons appear as a distinct peak below  $\text{pH}^*$  5.5 at  $-6.6$  ppm.

As anticipated, those histidines which are not ligands to the iron appear as well resolved resonances between  $-7.7$  and  $-8.9$  ppm. The chemical shifts of these protons ( $\text{H}_1$ ,  $\text{H}_2$ , and  $\text{H}_3$ ) are dependent on the  $\text{pH}^*$  as is shown in Figure 5. At  $\text{pH}^*$  values above 6.5 only one histidine resonance is observed. At  $\text{pH}^*$  6.0 one histidine ( $\text{H}_1$ ) is shifted further downfield (0.03 ppm) than the other two ( $\text{H}_2$  and  $\text{H}_3$ ), and at  $\text{pH}^*$  5.0 all three histidine C(2) hydrogens appear further downfield and are well resolved. No further shift in these protons occurs below  $\text{pH}^*$  3.8. Figure 5 shows the calculated titration curves for the three nonligand histidine C(2) protons compared to the experimental

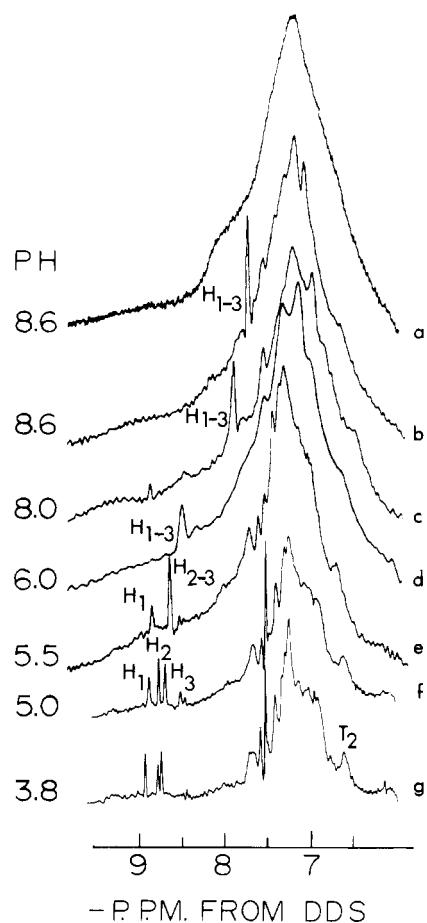


FIGURE 4:  $^1\text{H}$  NMR spectra of the aromatic region of oxy- and deoxyhemerythrin and hemerythrin azide at different  $\text{pH}^*$  values in 0.1 M phosphate buffer in 75%  $\text{D}_2\text{O}$ . Topmost curve (a) is oxyhemerythrin treated with sodium hydrosulfite to generate deoxyhemerythrin. Spectra of the oxyhemerythrin octamer are shown in (b) and (d). Remaining spectra are of monomeric hemerythrin azide. Spectra f and g are resolution enhanced by the factors 5.5 and 10.  $\text{T}_2$  indicates the tyrosine 2,6 hydrogens.

data. The  $\text{H}_1$  data gave the best fit to the calculated line where  $n = 0.90 \pm 0.03$  and the  $\text{pK}'_a = 7.00$ . The data for  $\text{H}_2$  and  $\text{H}_3$  also fit a dissociation with a  $\text{pK}'_a = 7.00$  but an  $n$  of 0.81.

A search was made 40 ppm downfield and 10 ppm upfield for contact shifted (Fermi, 1930) protons of those histidines which are iron ligands, but none were found.

The hemerythrin azide monomers were prepared in  $\text{D}_2\text{O}$  3 weeks prior to taking the spectra; consequently, adequate exchange had occurred in amide protons and it was not necessary to subject the samples to high  $\text{pH}^*$  for exchange. The UV spectrum of the hemerythrin azide active site does not change when the  $\text{pH}^*$  is lowered to 3.8; consequently, the resolution of the histidines into three separate resonances at acid  $\text{pH}^*$  is not due to denaturation of the active site. The titration was reversible.

**Identification of Photooxidized Histidines.** The photooxidized protein has been characterized (York & Roberts, 1976) and was found to contain only four histidines compared to seven in hemerythrin. Two tryptophans were also oxidized but no tyrosine, methionine, or cysteine. In addition, the active-site UV spectrum was not destroyed.

The schematic drawing of the peptide map of the major chymotryptic peptides of hemerythrin and photooxidized hemerythrin is shown in Figure 6. Those peptides which contain histidine are stippled. Those histidine-containing peptides which no longer stain with the Pauly histidine spray

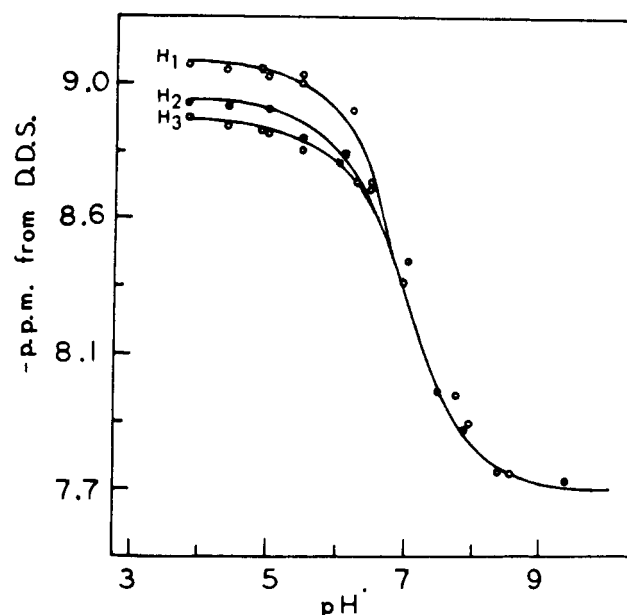


FIGURE 5: Plot of the  $^1\text{H}$  NMR titration data for the three histidine protons ( $\text{H}_1$ ,  $\text{H}_2$ , and  $\text{H}_3$ ) shown in Figure 4. The solid lines represent the least-squares fit to the data. The calculated  $\text{pK}'$  values and Hill coefficients are shown in Table I.  $r = 0.98, 0.93$ , and  $0.91$  for  $\text{H}_1$ ,  $\text{H}_2$ , and  $\text{H}_3$ , respectively.

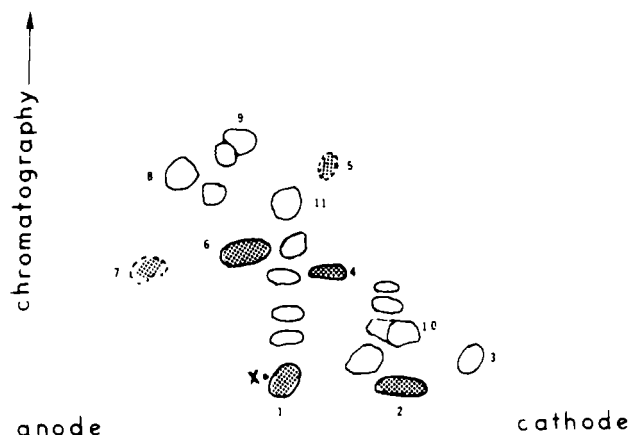


FIGURE 6: Schematic drawing of a peptide map of the major chymotryptic peptides of hemerythrin and photooxidized hemerythrin from *P. gouldii*. Histidine-containing peptides are stippled. Those that disappear on photooxidation are circled with a broken line (numbers 5 and 7). Electrophoresis was in pyridine-acetate buffer ( $\text{pH}$  6.4) for 90 min, 17 V/cm. Chromatography was in butanol-acetic acid-water (200:30:75 v/v). "X" indicates the origin.

after photooxidation are circled with a dashed line (peptides 5 and 7). The chymotryptic peptide map of carboxymethylated apohemerythrin has been published (Subramanian et al., 1968), and the composition of each peptide has been identified. The map is similar to the one we obtained (Figure 6) for the major chymotryptic peptides. From specific amino acid staining reactions and the composition of some peptides, we have been able to establish a correspondence between the peptides on our map and those on the map reported by Subramanian et al. (1968). Those "landmark" peptides which allow a correspondence to be established between our map and Subramanian's are peptides 3 and 8–11. Peptide 3 has the amino acid composition (Table II) corresponding to Subramanian's peptide C25. Peptide 8 gave a rusty rose color, and peptide 9 consistently gave an initial yellow ninhydrin stain which later turned blue as observed previously (Subramanian et al., 1968) for peptides in the same relative mapping position.

Table II: Amino Acid Composition of Selected Peptides from the Chymotryptic Peptide Map of Photooxidized Hemerythrin<sup>a</sup>

peptide	amino acid composition
2	Gly, 0.9 (1.0); Phe, 1.0 (1.0); His, 0.75 (1.0); Lys, 1.4 (1.0)
3	Gly, 1.0 (1.0); Lys, 1.62 (1.0); Ile, 1.0 (1.0)
10	Tyr, 1.3 (1.0); Lys 1.0 (1.0)
11	Tyr, 1.1 (1.0)

<sup>a</sup> Cellulose containing the peptide in question was scraped off the plate, extracted with 6 M HCl, and hydrolyzed under vacuum. The hydrolysate was analyzed on a Beckman 120C amino acid analyzer. The values in parentheses are those reported by Subramanian et al. (1968).

Peptides 10 and 11 have the composition shown in Table II and correspond to peptides C4 and C24, respectively. Having established a correspondence between our chymotryptic peptide map and that of Subramanian's, other assignments of histidine-containing peptides can be made based on mapping position. Peptide 7 moves furthest to the anode and is well resolved from neighboring peptides. It disappears on photooxidation and corresponds to C9 which contains His-34. Peptide 5 is also in a unique mapping position and is also susceptible to photooxidation; it corresponds to C10 and contains His-82. Peptide 2 has the composition shown in Table II and as expected from its mapping location is C11, containing His-54. Peptides 6 and 4 are in locations equivalent to C5/6 and C23 and contain His-25 and His-101, respectively. Peptide 1 remains near the origin, is histidine positive, and therefore corresponds to C18 which contains His-73 and His-77. On photooxidation this peptide remains Pauly positive, indicating that at least one of the histidines is not photooxidized.

## Discussion

Although the hemerythrin apoprotein is unlikely to be in a native conformation since the active site cannot be regenerated by the addition of iron to the apoprotein, the five tyrosines and seven histidines known to be present in the molecule can be accounted for in the NMR spectra. The apparent  $pK'$  values of the histidines are consistent with normal unperturbed histidines observed in nonmetalloproteins (Markley & Ibanez, 1978; Markley, 1973). In addition, the chemical shifts of the protonated and neutral forms are normal. The Hill coefficient of less than unity is to be expected if the net positive charge in the vicinity of the titrating histidine increases as the pH is lowered, which makes it energetically more difficult to add protons. This is the situation that occurs during the addition of acid to a neutral protein like hemerythrin.

In the native iron chelate of apohemerythrin, the intensity of the tyrosine resonance is markedly decreased and broadened compared to that of the iron-free protein. The intensity of the histidine-associated resonance is also decreased. The fact that the three histidine proton resonances are not broadened strongly suggests that these histidines are far enough away from the paramagnetic iron center so as not to be ligands to the iron. Their  $pK'$  values are also consistent with this conclusion, as is the fact that the chemical shifts are normal and very similar in the neutral form to those in the apoprotein. The breakup of the single histidine C(2)H resonance containing three protons into three different resonances below pH 6.5 suggests a pH-induced conformation change that places the free histidines in slightly different chemical environments.

The establishment of an appropriate base line makes the quantitation of the number of protons in the three histidine

resonances  $H_{1-3}$  difficult; however, the three resonances are equal in area and each must correspond to either one or two protons. If one bases the determination of the number of protons in  $H_{1-3}$  on the number of protons in the aromatic envelope between -6.5 and -7.8 ppm, then the calculation yields either three or four protons depending on whether one assumes that the chemical or X-ray model is correct, respectively. Theoretically, only two protons are expected if the X-ray model is correct. Therefore, quantitative analysis of the data is consistent with three free histidines but is not unequivocal. The qualitative observation that  $H_1$ ,  $H_2$ , and  $H_3$  are equal in area, titrate with the  $pK'$  expected of histidine, and are of the approximate area expected for three histidine C(2) protons strongly suggests that they are three histidine protons.

It is unlikely that  $H_1$ ,  $H_2$ , and  $H_3$  are incompletely exchanged amide protons because amide protons would not titrate in the pH range in which these protons titrate (Markley et al., 1975).

The model of the iron binding site which has been proposed on the basis of the X-ray diffraction data is highly asymmetric from a ligand field viewpoint. This model ignores the Mössbauer data which require identical chemical environments for both iron atoms. Both Okamura et al. (1969) and York & Bearden (1970) found identical environments for the two iron atoms based on the Mössbauer spectra in both the high-spin deoxyhemerythrin and the antiferromagnetically coupled methemerythrin. It has been shown that the quadrupole splitting of high-spin ferrous compounds is very sensitive to ligand field strengths of the coordinating ligands (Hudson & Whitfield, 1966; Takashima & Tateishi, 1965; Golding et al., 1966). For example, the quadrupole splitting for ferrous oxalate is 1.32 mm/s compared to 1.08 mm/s for ferrous gluconate. Based on this sensitivity of the quadrupole splitting, we would expect to observe two pairs of quadrupole split lines in the deoxyhemerythrin spectra rather than the one pair observed if the assignment of three histidines coordinated to one of the iron atoms and two histidines and one tyrosine coordinated to the other iron atom as suggested by Stenkamp et al. (1978) and Loehr et al. (1978) is correct. The strong electron-withdrawing phenolic group of tyrosine would certainly be expected to change the d electron density at the iron to which it is coordinated as compared to the d electron density at the iron coordinated by the three histidines. The conflicting conclusions may arise from the fact that the X-ray studies were performed on methemerythrin. It is possible that ligand exchange occurs upon oxidation of deoxyhemerythrin to methemerythrin. One would not be able to observe this in the Mössbauer spectra since the latter compound contains antiferromagnetically coupled  $Fe^{3+}$ , and the quadrupole splitting is not very sensitive to coordinating ligands. Ligand exchange is supported to some extent by the observation that deoxyhemerythrin is not antiferromagnetically coupled (York & Bearden, 1970) and, therefore, most likely does not have the bridging carboxylate ligands between the iron centers as proposed in methemerythrin (Stenkamp et al., 1978).

Analysis of the chymotryptic peptide maps of control and photooxidized hemerythrin indicates that histidine-82 and -34 are photooxidized and are therefore not likely iron ligands. These results are in agreement with the X-ray studies previously cited as well as the fact that these two residues are variable between different hemerythrin (Klippenstein, 1972; Ferrell & Kitto, 1971; Klippenstein et al., 1976; Loehr et al., 1978); yet the active-site spectrum is constant. Our results also indicate that histidine-25, -54, and -101 are definitely not

photooxidized, a conclusion consistent with their role as iron ligands as deduced from both X-ray studies. Since both histidine-77 and -73 occur in the same chymotryptic peptide, we are not able to deduce which if either is susceptible to photooxidation. The third photooxidized histidine may not be one unique histidine but a summation of partially oxidized iron-bound histidines; however, this interpretation is not consistent with the appearance of three unique histidines in the NMR.

#### Acknowledgments

We thank Dr. A. Bothner-By and the NMR Facility for Biomedical Studies at Carnegie-Mellon for making it possible to obtain the 250-MHz proton correlation spectra and Dr. Ken Seamons for generating the theoretical NMR spectra of apohemerythrin.

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## Resonance Raman Studies of Pyrocatechase-Inhibitor Complexes<sup>†</sup>

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**ABSTRACT:** The resonance Raman spectra of native pyrocatechase and its benzoate and phenolate complexes were investigated by using the available lines of an argon and a krypton laser. The data provide evidence for the presence of two distinct tyrosines coordinated to the active-site iron. The two tyrosines exhibit different  $\nu_{\text{CO}}$  values which show maxi-

mum resonance enhancements at different excitation wavelengths. Moreover, one tyrosine is more susceptible to changes in the active-site environment. Pyrocatechase is the only example thus far among iron-tyrosinate proteins where the tyrosines coordinating the iron are distinguishable.

**T**he intradiol dioxygenases, pyrocatechase (EC 1.13.1.1) and protocatechuate 3,4-dioxygenase (EC 1.13.1.3), catalyze the cleavage of catechols to *cis,cis*-muconic acids with the incorporation of the elements of molecular oxygen (Nozaki,

1974). The enzymes have a high-spin ferric center in the active site and exhibit visible spectra with absorbance maxima near 450 nm ( $\epsilon \sim 3000\text{--}4000 \text{ M}^{-1} \text{ cm}^{-1} \text{ Fe}^{-1}$ ). Recent resonance Raman studies (Tatsuno et al., 1978; Keyes et al., 1978; Felton et al., 1978; Que & Heistand, 1979; Bull et al., 1979) have assigned this visible absorption band as arising from a tyrosinate-to-iron charge-transfer interaction resulting in the enhancement of several tyrosinate vibrational modes, similar to those found for the transferrins and uteroferrin (Gaber et al.,

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