Implication of Histidine at the Active Site of Hemerythrin

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Received June 6, 1969

Summary

Results presented here indicate that four of the seven histidine residues in hemerythrin are coordinated to the iron to form the active site. The data also suggest that the lysine groups are not involved in iron coordination.

Hemerythrin is a non-heme iron protein which serves as a reversible oxygen carrier in the red cells of the brachiopods and sipunculids and, from an evolutionary viewpoint, can be considered a primitive hemoglobin. Therefore, we have been interested in investigating the nature of the amino acids which constitute the iron binding site in hemerythrin in an attempt to understand the chemistry involved in an iron-amino acid complex which reversibly binds oxygen under conditions compatible with life. Klippenstein et al. (1968) have recently published the primary amino acid sequence for Goldfingia hemerythrin; however, no information has been published concerning the iron binding site. There are two iron molecules per hemerythrin monomer of molecular weight 13,500 (Boeri and Magaldi, 1957; Klotz and Nagy, 1963). Our approach to the elucidation of the structure of the active site is based on the premise that the amino acids which are bound at the iron site will be either blocked from reacting with, or will form atypical products with reagents which are "specific" for the given amino acid residues. This basic tenet has been substantiated in the case of copper peptide complexes by Bradshaw et al. (1968) who have shown that the copper bound

histidine residues cannot be alkylated.

MATERIALS AND METHODS

Preparation of Hemerythrin and Derivatives: The method of purification of hemerythrin was essentially that previously described (Florkin, 1933 and Klotz, 1957). The material prepared by these procedures had an iron content of 0.81% and an extinction coefficient of 3400 cm-liter mole^{-1} at 330 mμ. These values are identical with those reported by others (Klotz et al., 1957; Nagy and Klotz, 1965). The 280/330 ratio was 5.64 as previously reported (Love, 1957). Apohemerythrin was prepared by the procedure of Groskopf et al. (1966).

Preparation of Iron-Histidine Complex. Histidine HCl, 6 mmoles, was dissolved in 20 ml of water. To this solution was added 2 mmoles of ferric ammonium sulfate. Fifty ml of ethanol was added to this mixture with a resulting separation of a red oil which crystallized on standing overnight at 4°. The solid was recrystallized from water-ethanol and dried under vacuum at 60°. For $Fe(Hist)_2SO_4$ (5 H_2O)

	% C	%N	% H	% Fe
Cal:	26.29	15.33	4.38	10.19
Obs:	26.28	15.00	4.29	9.81

Reaction with 5-Diazo-1-H-Tetrazole. The reagent for assay of free histidine residues, 5-diazo-1-H-tetrazole (DHT), was prepared from 5-amino-1 H-tetrazole according to the method described by Horinishi et al., (1964). The solution of DHT was added to the protein in a 200 fold excess. The reaction was carried out in 1.0 M bicarbonate buffer pH 8.8. The exact conditions are indicated in the figure legends. The protein-DHT complex was freed of excess DHT by dialysis against 0.1 M bicarbonate buffer. Excess DHT was freed from the histidine-iron-DHT complex by chromatography on DEAE cellulose. Separation was achieved by elution with a saturated sodium chloride solution. The number of moles of DHT-histidine complex formed was calculated from the extinction coefficient of 1.74×10^4 cm liter moles^{-1} at 480 mμ (Horinishi et al., (1964).

Reaction with Trinitrobenzenesulfonic Acid. The number of free lysine groups was determined by reaction of the protein with trinitrobenzenesulfonic acid (TNBS). Routinely, the reaction was performed so that the final concentration was 1×10^{-5} M in protein and 5×10^{-4} M in TNBS in 0.1 M phosphate buffer at 0°. Excess TNBS was removed by dialysis before the spectra were recorded. An extinction coefficient of 1.2×10^4 cm liter mole^{-1} at 345 mμ was used in the calculation of the number of groups reacting (Habeeb, 1966).

Protein Measurement. The hemerythrin concentration was determined from the extinction coefficient of 3400 cm-liter mole^{-1} at 330 mμ (Nagy and Klotz, 1965). Apoprotein concentration was determined by a microbiuret assay (Goa, 1953). All spectral measurements were recorded on a Cary 14 spectrophotometer.

RESULTS AND DISCUSSION

Reaction with TNBS. Trinitrobenzenesulfonic acid has been reported to react with all amino groups with the exception of the ring nitrogens of histidine and proline, the phenol of tyrosine (Habeeb, 1966), and the guanidinium group (Horinski, 1964). Reaction of TNBS with oxyhemerythrin and apohemerythrin at 25° as well as 0° revealed no difference in the number of groups reacting. In each case eleven residues reacted. We conclude from these studies that the amino terminal residue and the ε amino group of lysine are not involved in coordination with the iron. Iron was not released from hemerythrin upon formation of the TNBS derivative. Furthermore, a difference spectrum of TNBS apohemerythrin vs TNBS hemerythrin revealed the 330 mμ absorption which is characteristic of hemerythrin. These results indicate that TNBS reaction with hemerythrin does not lead to demetalation and intermediate formation of apohemerythrin or apohemerythrin-TNBS derivative which would result in a spurious conclusion.

Reaction with DHT. DHT is reported to react with tyrosine and the ring nitrogens of histidine (Horinishi et al., 1964). In the case of histidine a di-DHT derivative is obtained with an absorbance maximum at 480 mμ when DHT is present in excess. When stoichiometric concentrations are used, a mono-DHT derivative is formed with a maximum at 360 mμ. In the reaction of the iron-histidine complex with DHT, the data presented in Figure 1 indicate that even with a 200 fold excess DHT the dominant product is a mono-DHT derivative. These results suggest that one of the histidine ring nitrogens is complexed with iron and is not free to react with DHT. Some di-DHT histidine complex was formed which we attribute to decomposition of the iron complex during the reaction.

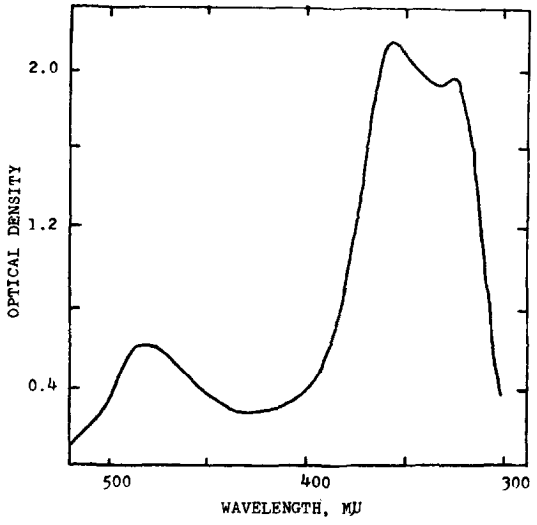

Figure 1. The absorption spectrum of the DHT-iron-dihistidine complex. The derivative was made by treating, in 1 M bicarbonate pH 8.8, the ferric dihistidine complex with DHT and removing excess DHT by DEAE chromatography. The final concentration of reactants were 1 x 10^{-4}M in ferric dihistidine and 1 x 10^{-2}M in DHT. The reaction was carried out at 25° and was complete in 30 minutes.

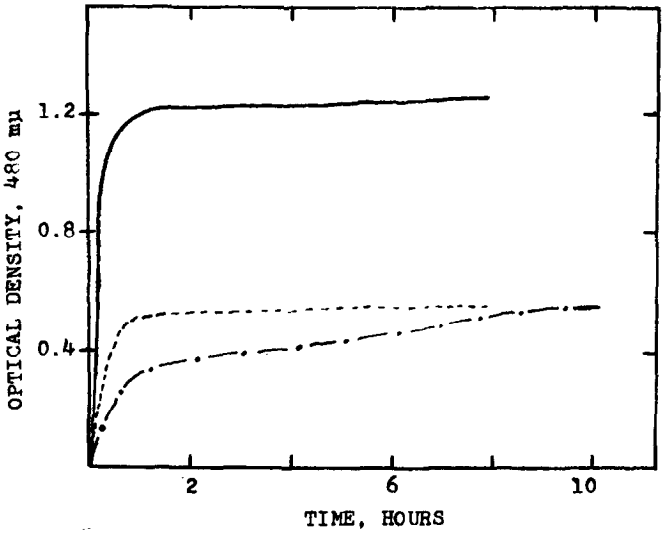

Figure 2. Rates of reaction of oxyhemerythrin and its apoprotein and subunits with DHT. _____, apoprotein; - - - - - -, hemerythrin subunits; - . - . - ., oxyhemerythrin. Reactions were carried out at final concentrations of 1 x 10^{-5}M protein and 1x 10^{-2}M DHT in 1 M bicarbonate buffer at pH 8.8 and at 25°C. The rates were followed by the increase in the 480 mμ absorption which measures the formation of the di-DHT derivative.

In the case of oxygenated hemerythrin, which is an octomer, Figure 2 shows an initial rapid reaction with DHT followed by a slow reaction. First order plots (Figure 3) of the data indicate that the initial fast reaction is pseudo first-order with a rate constant of 0.15 min^{-1} and corresponds to the reaction of two moles of histidine per mole of hemerythrin. The slow phase is also pseudo first-order, 0.4 hr^{-1}, and corresponds to the reaction of one additional histidine per mole. When the octomeric oxyhemerythrin is converted to monomeric subunits by reaction with cyanogen bromide (Nagy, 1965) then all three histidines react at the fast rate (Fig. 2). The third residue is therefore most likely near the subunit binding site and reacts very slowly in the polymer but upon monomer formation is free to react at the same rate as the other two free histidines. The apoprotein reacts with DHT very rapidly and shows the formation of 7 moles of di-DHT derivative per

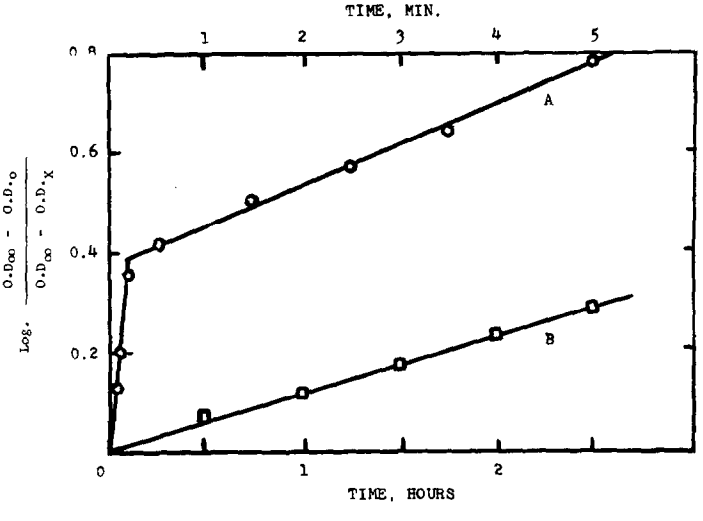

Figure 3. First-order plots of rate of reaction of DHT with oxyhemerythrin. —□— , time scale in hours; —○— , time scale in minutes. First order plots were made according to Frost and Pearson (1961). Conditions were identical to those in the legend of Figure 2. Curve B is an expanded view of the fast reaction rate of curve A.

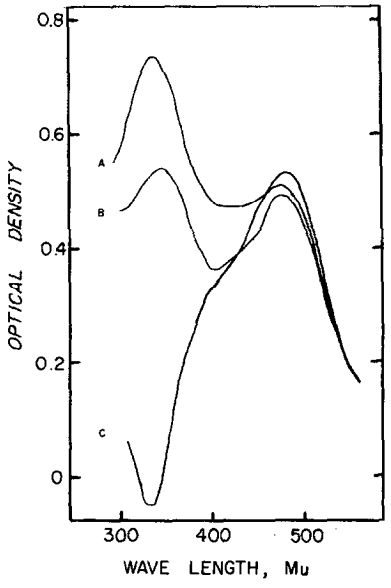

Figure 4. Spectral characteristics of DHT hemerythrin derivatives. Final concentrations were apoprotein (curve C), 0.43 x 10^{-5}M, oxyhemerythrin (curve A) and hemerythrin subunits (curve B) were 1.0 x 10^{-5}M. Other parameters were as stated in the text.

mole protein (Fig. 2 and 4), i.e. only a 480 mμ band and none of the 360 mμ band which is characteristic of the mono-DHT derivatives; however, the 330 mμ monoderivative spectra cannot be quantitated because of strong background absorption by undialyzable DHT side products. The background DHT absorption was removed by dialysis against 8 M urea and the 360 mμ absorption was used to calculate that four mono-DHT derivatives were formed per mole of hemerythrin. The inference is that these four histidine residues are bound to the two iron atoms and therefore yield the mono- rather than the di-DHT derivatives. Reaction of oxyhemerythrin with DHT does not significancly denature the protein since we find the iron remains non-dialyzable. Unfortunately we cannot determine if the spectral characteristic of the iron protein complex is present after reaction with DHT because of the high extinction of the DHT-histidine absorption. Work is in

progress to determine which histidines in the primary sequence are coordinated to the iron. It appears that all the histidine residues are near the surface of the hemerythrin molecule since all react with the polar DHT electrophile.

The absorption maximum of the monohistidine derivatives is shifted from the characteristic value of 360 mμ in the octomeric oxyhemerythrin. This absorption is shifted to 340 mμ in the monomeric methemerythrin (Figure 4) and to the characteristic 360 mμ in the presence of 8 M urea. The urea effect is reversible, i.e., upon removal of urea by dialysis the spectra shifts back to 340 mμ. These results indicate a change in the ionic and/or hydrophobic environment of the histidine-iron binding site as a function of the quaternary and tertiary structure of the hemerythrin. From the direction of the spectral shift we suggest that the iron binding site is hydrophilic compared to the urea solution. Classically, a red shift has been observed for the absorption of tyrosine and tryptophan in water vs urea solution (Wetlaufer et al., 1958; Bovey and Yanari, 1960). The model histidine-mono-DHT spectrum also shows the characteristic red shift in less polar solvents. These observations suggest that the polar iron site is permeated by the 8 M urea with the resultant red shift in the absorption spectrum of the mono-DHT-hemerythrin.

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