Azide Binding to the Cytochrome c Ferric Heme Octapeptide

A MODEL FOR ANION BINDING TO THE ACTIVE SITE OF HIGH SPIN FERRIC HEME PROTEINS

(Received for publication, March 29, 1979)

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Equilibrium constants for the binding of azide to the ferric heme c octapeptide in 50% ethylene glycol 50% buffer were measured spectrophotometrically. The equilibrium constant for azide binding at 20 °C and pH 7.4 is 29.2, which is approximately 3 to 4 orders of magnitude lower than that observed for azide binding to various ferric heme proteins. The equilibrium constant was independent of pH in the range from 7 to 8. Equilibrium constants at several temperatures exhibited an apparent van't Hoff relationship yielding thermodynamic values of $\Delta H^0 = -26,100 \text{ J/mol} \left( -6240 \text{ cal/mol} \right)$ and $\Delta S^0 = -61.5 \text{ J/mol} \left( -14.7 \text{ e.u.} \right)$. Comparison of these values to the values for the heme proteins enables one to explain the differences in equilibrium constants in terms of differences in the polarity of the heme environments. The results are consistent with the concept that the oxygen affinity of heme complexes increases with the polarity of the heme environment. The data also suggest that an increase in the polarity of the heme environment should result in a corresponding increase in the susceptibility of ferric heme dioxygen complexes toward oxidation by the dioxygen.

There has been considerable interest in the use of model systems to facilitate studies of the active sites of heme proteins (1-8). The effect that protein structure has on the intrinsic properties of the coordination center can, in part, be determined by comparison with corresponding properties of an appropriate model system. Numerous studies (9-14) have described the anionic binding equilibria of metmyoglobin and methemoglobin in an attempt to provide information about the coordination center relevant to their role as oxygen carriers. These proteins are characterized by coordination to an imidazole group of a histidine at one axial position and by H2O or OH- at the other axial position. Ligand binding appears to be associated with the substitution of H2O or OH- by the added ligand, and is accompanied by changes in optical and magnetic properties (9-11). Weak field ligands such as F- form high spin hexacoordinate ferric heme protein complexes; strong field ligands such as CN- form low spin hexacoordinate ferric heme protein complexes. Sperm whale fluorometeroglobin is characterized by a Soret absorption at 406 nm, and the cyanomethylhemoglobin is characterized by a Soret absorption at 423 nm (15). Ligands such as azide, having intermediate field strength, form complexes characterized by absorption maxima between 406 and 423 nm (15), and exhibit temperature-dependent spin-state equilibria with corresponding changes in optical spectra. Extensive studies of azide binding to metmyoglobin and methemoglobin (9, 10, 13, 14, 16, 17) have suggested the importance of the nature of the heme environment to metal-ligand interaction. Data have not been reported for the binding of anionic ligands to a model ferric heme imidazole complex for comparison to heme proteins. In the present study the heme octapeptide of cytochrome c has been used as a model system for the active site of myoglobin and hemoglobin. The heme octapeptide has been characterized by coordination to a histidyl residue of the peptide at one axial position, and by H2O at the second axial position. Equilibrium measurements and derived thermodynamic values for the binding of azide to the heme c octapeptide are reported.

**EXPERIMENTAL PROCEDURES**

**Materials**

The cytochrome c ferric heme octapeptide (HPo),1 was prepared from horse heart cytochrome c (Sigma) according to a modification (18) of the procedure described by Habbury and Loach (19). Certified ethylene glycol and purified sodium azide were obtained from Fisher Scientific and used without further purification. Measurements of pH were made with a Radiometer PHM 64 pH meter. Constant temperatures were obtained with a Lauda circulating temperature bath. Spectroscopic measurements of the equilibrium were made with a Varian Cary 14R spectrophotometer. A copper-constantan thermocouple, Hewlett Packard D. C. microvolt-ammeter model 524A, and a Beckman 10 inch recorder were used to measure and record the temperature. Lyophilized cytochrome c heme octapeptide was dissolved in 0.223 M phosphate buffer at a particular pH and then made 50% (v/v) with ethylene glycol. The pH* values of the resultant aqueous ethylene glycol solutions at a particular temperature were derived from scales determined by Hoa and Douzou (20). The heme concentration was 6.2 × 10$^{-5}$ M. Ethylene glycol was used to minimize heme-heme aggregation, and to permit equilibrium measurements at subzero temperatures.

**Methods**

Three milliliters of heme octapeptide solution were added to a standard rectangular cuvette. A Teflon stopper fitted with an epoxy-covered copper-constantan thermocouple was inserted into the cuvette, which was then placed in a thermostated cuvette holder connected to the circulating temperature bath. Initial additions of a 4.62 M aqueous solution of NaN3 were followed by additions of solid NaN3 to prevent significant changes in heme concentrations. Azide was introduced by removing the cuvette from the cell compartment, adding the azide, and mixing thoroughly. The pH of the solution remained constant during the course of azide addition. Absorption changes measured at 410 nm were used to calculate equilibrium constants. Ligand stoichiometry was determined from the slope of a plot of log (A-Ao)/(A-1-Ao) versus log [NaJ, where Ao and A, are absorption at pH x and pH, respectively. The equilibrium constants at each temperature were calculated

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1 The abbreviations used are: HPo, cytochrome c ferric heme octapeptide; Mb, myoglobin; MetHb, methemoglobin; MetMb, metmyoglobin.
using the Clark et al. (21) modification of the Reed Berkson method of rectification of spectrophotometric curves.

RESULTS

Spectrophotometric titrations of the cytochrome c heme octapeptide with $N_3^-$ were performed at $35^\circ C$, $25^\circ C$, $20^\circ C$, $15^\circ C$, $5^\circ C$, $0^\circ C$, and $-10^\circ C$ at pH* 8. Fig. 1 illustrates the absorption spectra resulting from a titration at $-10^\circ C$, and is typical of titrations made at the other experimental temperatures. Ligand binding is associated with a change in absorption maxima from 399 to 410.5 nm. The solution exhibited an isosbestic point in each of the titrations. A plot of $\log (A-A_0)/(A_{00}-A)$ versus $\log [N_3^-]$, shown in Fig. 2, yields a straight line with a slope of 1.03, consistent with the binding of one ligand according to the equilibrium expression:

$$\text{HP}_{tr} + L \rightleftharpoons \text{HP}_{tr} - L$$

![Absorption Spectra](image)

**Fig. 1.** Spectrophotometric titration of the ferric heme c octapeptide with sodium azide in 50% (v/v) ethylene glycol at pH* 8.0 and $-10^\circ C$. Sodium azide concentrations are indicated in the figure.

Equilibrium constants determined at various temperatures are shown in Table I. The dependence of $K$ on temperature was examined according to the van't Hoff equation:

$$\ln K = -\frac{\Delta H^0}{R}\left(\frac{1}{T}\right) + \frac{\Delta S^0}{R}$$

Fig. 3 shows a plot of $\ln K$ versus $1/T$. The linear dependence of the plot may be fortuitous because the observed equilibrium constant may actually encompass several equilibria, each of which could be characterized by different thermodynamic values. These equilibria, involving the high and low spin forms of the heme octapeptide and its azide complex, have been observed with the heme proteins (22). Thermodynamic values obtained from the least square line are:

$$\Delta H^0 = -26,100 \pm 460 \text{ J/mol (} -6240 \pm 110 \text{ cal/mol)}$$

$$\Delta S^0 = -61.1 \pm 1.7 \text{ J/K mol (} -14.6 \pm 0.4 \text{ e.u.)}$$

The effect of proton activity on the thermodynamic values

<table>
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<th>Temperature ($^\circ C$)</th>
<th>$K_{association}$</th>
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<tr>
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<td>5</td>
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<td>0</td>
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![Effect of Temperature on $K$](image)

**Fig. 3.** Effect of temperature on the binding of azide to the ferric heme c octapeptide in 50% (v/v) ethylene glycol at pH* 8.0.

![Effect of pH* on $K$](image)

**Fig. 4.** Effect of pH* on the binding of azide to the ferric heme c octapeptide in 50% (v/v) ethylene glycol at $20^\circ C$. 

![Effect of pH* on $K$](image)
was examined by measuring the equilibrium constants at different pH* values. Fig. 4 shows a plot of the equilibrium constants at 20°C and pH* values from 6.5 to 9.0.

**DISCUSSION**

The red shift in the position of the Soret absorption band upon addition of azide to the heme octapeptide is also characteristic of the absorption changes associated with the binding of azide to methemoglobin and metmyoglobin. The absorption maximum at 410.5 nm for the heme octapeptide-azide complex may be compared to values of 417 nm and 420 nm for the azide complexes of horse metmyoglobin and horse methemoglobin (23), respectively. The relative positions of the absorption maxima reflect in part differences between porphyrin side chains in the c-type heme of the model, and the b-type heme of the proteins. The binding of 1 azide ion/heme peptide is also characteristic of the ligand-binding equilibrium of the methemeproteins. This stoichiometry is in contrast to the ligand-binding properties observed for model heme complexes which do not have a covalently attached axial ligand. The absence of a significant dependence of the equilibrium constant on pH* in the range 7 to 8 is consistent with the observed absence of proton ionization of the HP, in this pH range (24). The pH dependence of the equilibrium constants for azide binding to methemeproteins and the ferric heme octapeptide at 20°C and pH 7.4 together with other available constants for comparison. The affinity of the heme octapeptide for azide is approximately 2 to 4 orders of magnitude less than that observed for the methemeproteins. Differences between the affinities of the proteins and of the model for azide are related to differences in the thermodynamic values for complex formation. The thermodynamic values shown in Table II indicate that the ΔH° value for the octapeptide is approximately 4 to 11 kcal/mol less than those of the heme proteins at pH 7.4. The significantly smaller ΔH° for the heme octapeptide model may be explained in terms of differences in the type and differences in the heme environment, i.e., nonbonded and bonded interactions.

**Heme Type**—It has been observed previously by Sonn and Akasura (17) that azide binding to different porphyrin-substituted metmyoglobins is characterized by equilibrium constants that increase with an increase in the electron-withdrawing power of the porphyrin sidechain. The affinity constant for the protoheme protein is 1.5-fold greater than that for the mesoheme protein. Similar differences in affinity have been observed for azide binding to porphyrin-substituted porphyrines (16). The electron withdrawing power of porphyrin c may be expected to be intermediate between proto- and mesoporphyrins, such that a somewhat greater equilibrium constant would be expected for a protoheme model complex than that observed for the heme c octapeptide. Thus, differences in porphyrin type would appear to make a very small contribution to differences in enthalpy observed for the ferric heme octapeptide and methemeproteins.

**Nonbonded Interactions**—The proteins are thought to provide a relatively hydrophobic environment for the heme. This is in contrast to the polar environment of the heme octapeptide in the aqueous ethylene glycol solution. The heme environment in the protein would then be equivalent to a medium of lower dielectric constant, which should result in a more negative ΔH° for the electrostatic interaction of the positively charged heme and the negatively charged azide. The protein environment should thus stabilize the heme-azide complex to a much greater extent than the aqueous environment of the heme octapeptide relative to the dissociated species in each system. The much less negative ΔH° value for the *Aplysia* metmyoglobin albeit a lower pH suggests that its heme environment is more polar than that of the other methemeproteins. This is consistent with the observation that the *Aplysia* absorption maxima of 400 nm for *Aplysia* acid metmyoglobin (29) is blue-shifted relative to those of leghemoglobin and myoglobin (30).

**Bonded Interactions**—The greater negative value for the ΔH° of the proteins as compared to the ΔH° of the heme octapeptide may be associated with additional bonding interactions between the amino acid sidechains of the protein and the bound azide. A hydrogen bond has been postulated (31, 32) between the Nα of the azide and the Nε of the distal histidine. Alternatively, it has been suggested (33) that the iron-bound nitrogen of the azide serves as an acceptor for the electron pair on an unprotonated nitrogen of the distal histidine. The contribution of the distal histidine to the stabilization of the heme-azide complex can be evaluated by comparing the anionic ligand-binding properties of abnormal hemoglobins and myoglobins, where the distal histidine has been substituted by another amino acid. The pK' of the acid-alkaline transition,

\[
\text{Fe}^{2+} (\text{OH})_2 = \text{Fe}^{2+} (\text{OH}) + \text{H}^+ 
\]

is lower for *Chironomus* hemoglobin than for horse hemoglobin and horse myoglobin (34). The *Chironomus* hemoglobin is characterized by the substitution of isoleucine for the distal histidine (35). This suggests that a nonpolar amino acid provides greater stability than that of the histidine in anionic binding to the heme.

The ΔS° value for the formation of the heme octapeptide-azide complex is less negative than that observed for most of the other methemeproteins, as shown in Table II. A less negative value for the model would be expected if less reorganization of the immediate surrounding occurs with the model than with the protein. A less negative ΔS° value would also be expected from the lack of apparent steric restrictions to the formation of the azide complex with the model system, since x-ray structures indicate that azide binding to the protein is restricted to a particular orientation by steric interactions (31). The less negative ΔS° value observed for the binding of azide to the heme octapeptide should contribute to a larger equilibrium constant than that of the protein. The greater affinity of the proteins for azide is thus due to a more negative

**TABLE II**

<table>
<thead>
<tr>
<th>Type</th>
<th>K&lt;sub&gt;app&lt;/sub&gt;</th>
<th>ΔH°</th>
<th>ΔS°</th>
<th>Ref.</th>
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<tr>
<td>Human A MetHb</td>
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<td>-17,400</td>
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<td>10, 12</td>
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<td>Horse MetHb</td>
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<td>-12,900</td>
<td>-18.4</td>
<td>25</td>
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<tr>
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<td>-10,800</td>
<td>-14.5</td>
<td>26</td>
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<tr>
<td>Sperm whale MetMb</td>
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<td>-11,550</td>
<td>-18.4</td>
<td>27</td>
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<tr>
<td>Horse heart MetMb</td>
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<td>-14.7</td>
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</table>

<sup>a</sup> Calorimetric.

<sup>b</sup> Spectrophotometric.

<sup>c</sup> Estimated from Fig. 1, Ref. 10.

<sup>d</sup> Estimated from Fig. 1, Ref. 10, and Fig. 4, Ref. 12.

<sup>e</sup> pH 6.5.

<sup>f</sup> pH 7.0.

<sup>g</sup> 21°C.

<sup>h</sup> At acid pH. ΔS° value calculated.
Azide Binding to Ferric Heme Octapeptide

Δ\text{H}^0 than that of the characteristic model.

Conclusions—A comparison of the thermodynamic values for azide binding to the ferric heme octapeptide and the methemoproteins suggests that the dominant factor contributing to the association constant is the polarity of the heme environment, which is reflected in the Δ\text{H}^0. To the extent that Δ\text{H}^0 for azide binding provides information about the polarity of the heme environment, the experimental results have relevance to the effect of environment on the oxygen affinity of heme complexes, and the reversibility of dioxygen heme complex formation. Heme dioxygen complexes have been formulated as superoxide ferric heme iron bonds (36, 37). A more polar environment should then favor the formation of dioxygen heme complexes in proteins, as indicated by studies of model heme complexes (38-40). Oxygen affinity values for the heme proteins listed in Table II correlate with the polarity of the heme environment. This observation implies that the heme environment in Aplysia metmyoglobin may be significantly different from that of the Aplysia deoxymyoglobin. This possibility is further reinforced by the observation that the Soret band of deoxymyoglobin (29) is red-shifted relative to the other proteins, consistent with a more nonpolar heme environment.

The results in Table II suggest that anion binding decreases as the polarity of the heme environment increases. A more polar heme environment should also favor the dissociation of the dioxygen heme complex to ferric heme and superoxide anion, thus contributing to the irreversible binding of oxygen to heme proteins and model systems as previously postulated (44, 45). This conclusion is supported by the fact that leg-hemoglobin, which is particularly susceptible to auto-oxidation (46), has been characterized by a more polar heme environment than that of sperm whale myoglobin (47). Thus the results suggest that a more polar heme environment confers both an increased oxygen affinity and increased susceptibility to irreversible oxygenation.

Acknowledgments—We wish to acknowledge Dr. Gerald Wagner, whose initial experiments involving the heme octapeptide and azide suggested the need for an investigation of azide-binding equilibria. We wish to thank Ya-Ping Huang for a sample of the HPpt and Dr. Pierre LeBreton for helpful discussions.

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Azide binding to the cytochrome c ferric heme octapeptide. A model for anion binding to the active site of high spin ferric heme proteins.
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