Temperature-Dependent Spin-State Equilibrium in an Azide-Ferric Heme Octapeptide Complex. A Model System for the Spin Equilibria of Ferric Heme Proteins

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Abstract: The temperature dependence of the magnetic susceptibility of an azide-ferric heme octapeptide was investigated as a model for the temperature-dependent spin-state equilibria of ferric heme proteins. The shift in the ^1H NMR signal of sodium 4,4-dimethyl-4-silapentanesulfonate (DSS) caused by the heme octapeptide was measured from 12 to 73 °C in an aqueous-ethylene glycol solution. Magnetic susceptibilities calculated from the shifts do not exhibit a simple Curie behavior as would be expected for a pure high-spin or low-spin system. The temperature dependence of the susceptibility is consistent with a thermal spin-state equilibrium: high spin ($S = 5/2$) ⇌ low spin ($S = 1/2$). The equilibrium constant is 9.4 at 25 °C. Thermodynamic values determined from a plot of ln K vs. $1/T$ are $\Delta H° = -16\,300$ J/mol (-3890 cal/mol) and $\Delta S° = -36.0$ J/(mol·K) (-8.6 cal/(mol·K)). The results indicate that suitable axial ligands to the heme iron are sufficient to provide a model for heme proteins that exhibit thermal spin equilibria. Equilibrium and thermodynamic values for the heme peptide are compared to values for heme proteins to determine the effect of protein structure on the spin-state equilibrium.

Introduction

A number of reports have described model systems for the active sites of heme proteins.[1-8] A comparison of the properties of such models to those of the proteins can be used to determine the effect that protein structure has on the intrinsic properties of the coordination center. Several studies[9-24] have described the thermal spin-state equilibria of many ferric heme proteins and their ligand substitution derivatives in an attempt to provide information relevant to their function in biological systems. Many of these proteins appear to be characterized by coordination of the heme iron to an imidazole group of a histidine residue at the fifth coordination position and to $H_2O(OH^-)$ or another amino acid side chain at the sixth coordination position. Substitution of the ligand at the sixth coordination position by added ligands is accompanied by changes in magnetic properties. 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. Ligands such as azide having intermediate field strength form complexes which exhibit temperature-dependent spin-state equilibria. Studies to date[11,25-27] have suggested the importance of the protein structure in determining the spin-state equilibrium. However, spin-state equilibria for model complexes whose axial ligation corresponds to that found in these proteins have not been investigated. A methionine-heme octapeptide complex has been reported to exhibit a thermal spin-state equilibrium,[28] although the corresponding complex in cytochrome *c* is low spin. Mössbauer spectra of the lyophilized heme undecapeptide[29] at different temperatures have suggested that an equilibrium exists between two spin states, although the axial coordination of the two spin states was not determined. In the present study the azide complex of the ferric heme *c* octapeptide has been investigated as a model for the active sites of ferric heme protein derivatives. The heme octapeptide has been characterized[30] by iron coordination to the histidyl imidazole group at one axial position and to H_2O at the second axial position. Equilibrium constants have recently been measured for the binding of azide to the heme octapeptide.[31] The temperature dependence of the magnetic susceptibility of the azide ferric heme octapeptide is now reported.

Experimental Section

Reagents. The ferric heme octapeptide, HP_{pt}, was prepared from horse heart cytochrome *c* (Sigma, type II-A) according to a modification[32] of the procedures described by Harbury and Loach.[30] Certified ethylene glycol and purified sodium azide were obtained from Fisher Scientific and used without further purification. Sodium 4,4-dimethyl-4-silapentanesulfonate (DSS) was obtained from Wilmad Glass Co. and used without further purification.

Methods. The total heme concentration was determined from the absorption spectrum of the pyridine hemochrome[33] measured on a Varian-Cary 14R spectrophotometer using a $\Delta\epsilon = 19.1$ mM^{-1}.[34] The optical absorbance of heme octapeptide in 50:50 (v/v) ethylene glycol-water was measured over the concentration range of approximately 2 mM to 1 μM. The extinction coefficient was constant, indicating that the heme octapeptide was monomeric consistent with previously observed effects of alcohol on the state of aggregation of heme compounds.[35,36] The NMR measurements were made with a Varian A 60-MHz spectrometer equipped with Varian V-6040 NMR variable temperature controller. The temperature was determined from the separation in hertz of the methyl and hydroxyl signals of methanol at low temperature[37] and from the separation of methylene and hydroxyl signals of ethylene glycol at high temperature.[38]

Magnetic moment measurements were performed essentially as described by Evans[39] using Wilmad (517) special NMR coaxial cell sample units. A solution of 3.30 mM heme octapeptide in a 50:50 (v/v) ethylene glycol-water solution containing 40 mM phosphate buffer corresponding to a proton concentration equivalent to pH 7.60 in aqueous solution was placed in the outer tube, and a similar solution without heme octapeptide was placed in the coaxial inner tube. The frequency difference, $\Delta\nu$, of the methyl signal of DSS in the presence and absence of the heme octapeptide was measured at various temperatures. The molar paramagnetic susceptibility was calculated by Evans' equation:

$$\chi_M = \frac{3}{2\pi}\frac{\Delta\nu}{\nu}\frac{1000}{C} + \chi_0 M - \chi_D$$

where χ_M is the molar paramagnetic susceptibility of the ferric heme octapeptide (including the aquo and azide complexes), $\Delta\nu$ is the frequency separation of DSS lines in hertz, ν is the frequency (60 MHz) of the proton resonance, χ_0 is the mass susceptibility of the solvent (-0.697×10^{-6} cgs), M is the molecular weight of the heme octapeptide (1546), and χ_D is the diamagnetic susceptibility of the heme octapeptide ($\chi_D = -723.6 \times 10^{-6}$ cgs as calculated from Pascal's constants[40]).

The effective magnetic moment of iron in the heme octapeptide was calculated by the equation:

$$\mu_{eff} = \sqrt{\frac{3\chi_M kT}{N\mu_B{}^2}} = 2.84\sqrt{\chi_M T}$$

Since the binding of azide was not complete over the entire temperature range studied for the azide concentration used,[31] a correction for the fraction of magnetic moment due to the aquo-ferric heme octapeptide was made.

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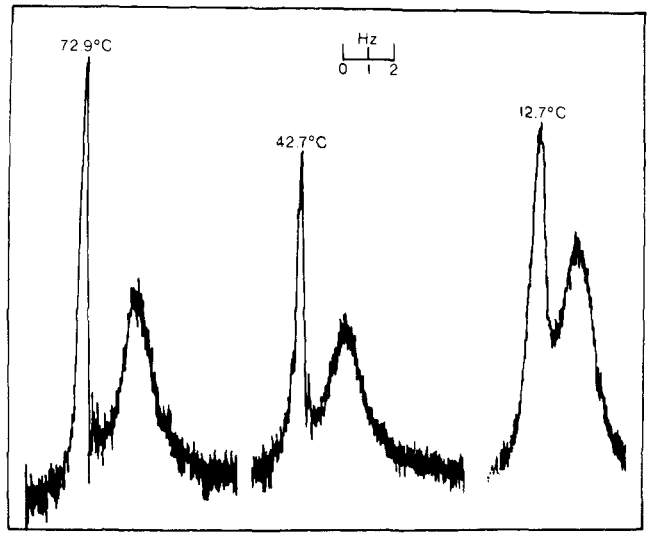

Figure 1. NMR spectra (60 MHz) showing the difference in chemical shifts of the DSS reference peaks at different temperatures due to the effect of the azide-ferric heme octapeptide at a concentration of 3.30 mM.

Table I. Temperature Dependence of Effective Magnetic Moments of Azide-Ferric Heme *c* Octapeptide

T, °C	$\Delta\nu^a$ (Hz) ± σ^b	χ_M, cgs·emu/mol[c]	μ_{eff,N_3^-}, μ_B[d]
12.7	1.46 ± 0.03	3.18×10^{-3}	2.66
13.5	1.53 ± 0.04	3.35×10^{-3}	2.74
25.5	1.52 ± 0.04	3.34×10^{-3}	2.77
31.2	1.59 ± 0.04	3.50×10^{-3}	2.85
40.2	1.65 ± 0.04	3.65×10^{-3}	2.95
42.7	1.76 ± 0.03	3.91×10^{-3}	3.06
50.3	1.74 ± 0.06	3.88×10^{-3}	3.06
57.5	1.83 ± 0.04	4.08×10^{-3}	3.16
58.8	1.82 ± 0.03	4.07×10^{-3}	3.16
66.1	1.95 ± 0.03	4.36×10^{-3}	3.31
73.3	1.95 ± 0.03	4.38×10^{-3}	3.32

[a] $\Delta\nu$, difference in NMR resonance frequencies of the DSS (sodium 4,4-dimethyl-4-silapentanesulfonate) reference peaks in the presence and absence of heme *c* octapeptide. [b] σ, standard deviation of eight or more measurements. [c] χ_M, the molar paramagnetic susceptibility of ferric heme *c* octapeptide (including the aquo and azide complexes). [d] μ_{eff,N_3^-}, the calculated effective magnetic moment for azide-ferric heme *c* octapeptide.

The effective magnetic moment corresponds to:

$$\mu^2_{eff} = \mu^2_{H_2O} + \mu^2_{N_3^-}$$

$$\mu^2_{eff} = X_{H_2O}(\mu^2_{eff,H_2O}) + X_{N_3^-}(\mu^2_{eff,N_3^-})$$

where μ_{eff} is the effective magnetic moment of the ferric heme octapeptide, μ_{eff,H_2O} is the effective magnetic moment of the pure aquo complex, μ_{eff,N_3^-} is the effective magnetic moment of the pure azide complex, and X_{H_2O} and $X_{N_3^-}$ are the mole fractions of aquo and azide complexes, respectively. X_{H_2O} and $X_{N_3^-}$ were calculated from the thermodynamic values for azide binding to the ferric heme *c* octapeptide.[31] μ_{eff,H_2O} was determined from measurements on the 100% aquo complex.

Results

The difference in NMR resonance frequencies of the DSS peaks in the presence and absence of the azide-ferric heme octapeptide was measured from 12.7 to 73.3 °C. Figure 1 illustrates the NMR spectra at three different temperatures. The methyl signal of DSS in the solution containing the paramagnetic heme octapeptide corresponds to the resonance at lower frequency (upfield). The line width of this signal was broadened relative to the DSS signals in the absence of heme.

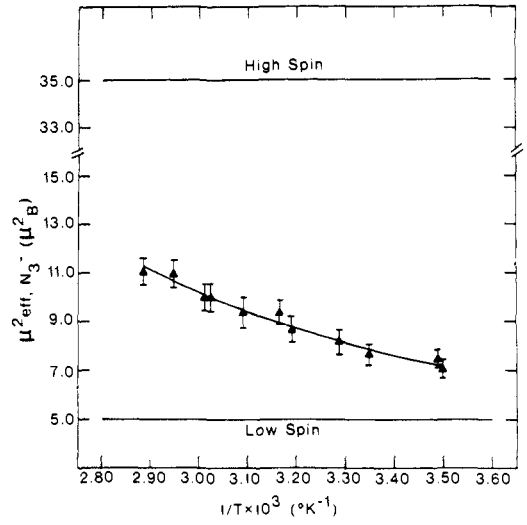

Figure 2. Effect of temperature on the effective magnetic moment of the azide-ferric heme octapeptide.

The separation between the two peaks increased with temperature, such that the resolution improved at higher temperature.

Table I lists the calculated values for the magnetic susceptibilities of the ferric heme octapeptide and the effective magnetic moments of iron(III) in the azide-heme octapeptide at various temperatures. For heme compounds which are either pure high spin or pure low spin, the temperature dependence of the susceptibility is given by Curie's law:

$$\chi = \frac{C}{T} = \frac{\mu^2_{eff} N\beta^2}{3kT}$$

Since C is a constant, the value of μ^2_{eff} should be independent of temperature if Curie's law were followed. Figure 2 shows that μ^2_{eff,N_3^-} deviates from Curie's law. All the values of μ^2_{eff,N_3^-} fall within the range between 35 and 5, where 35, μ^2_{HS}, is the theoretical value of μ^2_{eff} for high-spin ferric heme compounds, and 5, μ^2_{LS}, is the μ^2_{eff} value observed for low-spin ferric heme compounds.[10] The variation of μ^2_{eff,N_3^-} with temperature strongly suggested that the complex exhibits a thermal spin-state equilibrium: high spin $\rightleftharpoons$ low spin. An equilibrium constant at each temperature was calculated from the following relationship.

$$K = \frac{\mu^2_{HS} - \mu^2_{eff}}{\mu^2_{eff} - \mu^2_{LS}}$$

The dependence of K on temperature was examined according to the van't Hoff equation:

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

Figure 3 shows a plot of ln K vs. $1/T$. Thermodynamic values obtained from the least-squares line are:

$$\Delta H^\circ = -16\,300 \pm 840 \text{ J/mol} \quad (-3890 \pm 200 \text{ cal/mol})$$

and

$$\Delta S^\circ = -36.0 \pm 2.5 \text{ J/(mol·K)} \quad (-8,6 \pm 0.6 \text{ cal/mol})$$

By using the values of ΔH° and ΔS° obtained, the values of K at each temperature can be calculated. The theoretical value of μ^2_{eff} can be calculated by the formula:

$$\mu^2_{eff} = \frac{35 + 5K}{1 + K}$$

The smooth line shown in Figure 2 corresponds to the theoretical curve for μ^2_{eff} vs. $1/T$.

Table II. Thermodynamic Values[a] for the Spin-State Equilibrium of Azide-Ferric Heme Octapeptide and Metheme Proteins

	$\Delta H°$, cal/mol	$\Delta S°$, cal/(mol·deg)	K^{25}, deg[b]	T_c,[c] K	ref
horse heart heme *c* octapeptide,[d] NMR	−3890 ± 200	−8.6 ± 0.6	9.4	452	this work
horse myoglobin					
IR[e]	−5600 ± 290	−15.1 ± 1.0	7.0	375	14
magn suscept[f]	−2740 ± 400	−6.8 ± 1.4	3.3	403	9
visible absorpt spectrosc[g]	−4000	−9.5	7.2	421	24
sperm-whale myoglobin (wet crystal) magn suscept[h]	−3660	−10.2	2.84	359	10
sperm-whale myoglobin, IR[i]	−5600 ± 1700	−14.6 ± 6.0	8.8	383	14
magn suscept[h]	−3746	−9.58	4.5	391	12
horse hemoglobin, magn suscept[h]	−5094	−13.62	5.8	374	13
human hemoglobin A, IR[j]	−6700	−17	18	400	14

[a] All the measurements were made in solution unless otherwise indicated. [b] K^{25} degrees for HS ⇄ LS equilibrium. [c] T_c is the temperature at which $K = 1$. [d] From 285 to 347 K. [e] From 250 to 320 K. [f] From 274.8 to 299.7 K. [g] 285 to 318 K. [h] From liquid N_2 to room temperature. [i] 250 to 294 K. [j] At three temperatures only (including 8 and 35 °C).

Discussion

The high spin-low spin thermal equilibrium observed for many ferric heme proteins and their derivatives has been considered to be a unique characteristic of proteins.[11] Recent studies have emphasized the influence of protein structure in determining the spin equilibrium through a control of axial ligation.[25-27] The present study demonstrates for the first time that a thermal spin-state equilibrium can be observed in a model heme system whose axial ligation is characteristic of these proteins,

High-spin model ferric heme complexes have previously been shown to be five coordinate containing a weak-field ligand.[41-43] Recently, high-spin hexacoordinate ferric heme complexes containing two weak field ligands have been characterized.[25,44] Low-spin ferric heme complexes have been shown to be hexacoordinate containing moderate to strong field ligands.[43,45-57] These studies indicate that the nature of the axial ligands is an important factor in determining the high-spin or low-spin character of the heme iron. Many ferric heme proteins including hemoglobin and myoglobin, which exhibit high spin-low spin equilibria at ambient temperature, have been characterized as hexacoordinate complexes containing a moderate to strong field ligand such as imidazole and a ligand of weak to moderate field strength such as water (OH^-) and azide.

The axial ligation of the iron in the heme octapeptide corresponds to that in these proteins, which suggests that the particular axial ligands are sufficient to yield a system that exhibits a spin-state equilibrium.

Equilibrium and thermodynamic values for the azide-ferric heme octapeptide and ferric heme proteins are compared in Table II. The equilibrium constant for the azide heme octapeptide appears to be close to the values for azide metmyoglobins determined by infrared spectroscopy. However, equilibrium constants determined by infrared spectroscopy are considerably higher than those determined by the magnetic susceptibility method.[14] The equilibrium constant for the heme octapeptide may actually be more similar to that of hemoglobin if the magnetic data provide more accurate results. However, the thermodynamic values may provide a better basis for exploring the effect of protein structure on the spin-state equilibrium. The $\Delta H°$ and $\Delta S°$ values for the heme octapeptide are within experimental error very close to those of sperm whale myoglobin, but less negative than those of horse hemoglobin as determined by magnetic susceptibility.

A number of structural parameters have been suggested to effect the spin-state equilibrium through an enthalpy effect. It has been observed that the metal-ligand bond distances in high-spin ferric heme compounds are longer than those of the corresponding low-spin complexes.[26] In proteins, a restricted

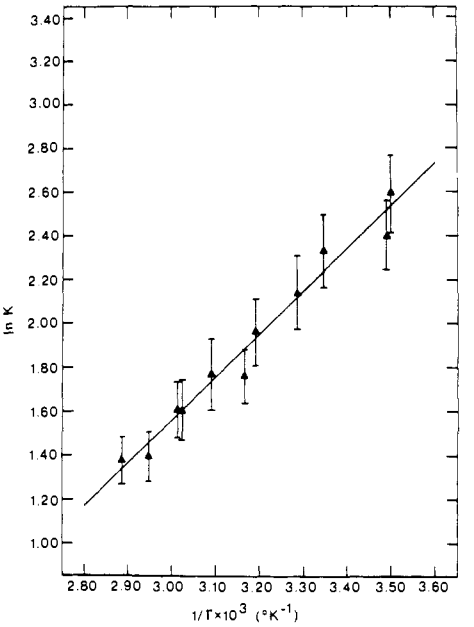

Figure 3. Effect of temperature on the equilibrium constant for the high-spin to low-spin transition of the azide-ferric heme octapeptide.

metal-ligand coordination may lead to longer bond distances that favor the high-spin state and correspond to a less negative $\Delta H°$ for the high-spin to low-spin equilibrium. It may also be possible for the protein to enforce a metal-ligand bond distance that is shorter than that of unrestricted ligation, with the effect of favoring the low-spin state and giving rise to a more negative $\Delta H°$.

Previous studies have suggested that the spin state depends on the position of iron relative to the porphyrin plane.[26] The metal ions of low-spin hexacoordinate compounds are located in the porphyrin plane, while the metal ions of high- and mixed-spin hexacoordinate hemes appear to be displaced from the mean plane of the porphyrin,[48,49] except that in $[FeL_2(TPP)]ClO_4$ (L = tetramethylene sulfoxide), with two equivalent axial ligands, the iron atom is precisely located in the plane.[25] Through the aforementioned restriction on metal-ligand coordination, the protein may influence the position of the heme irons with respect to the porphyrin plane, thereby affecting the $\Delta H°$ for the spin-state equilibrium.

A recent experiment has also shown that in a high-spin ferric heme compound the porphyrin core is expanded.[25] It is therefore possible that the porphyrin core in the high-spin configuration of the protein would be somewhat expanded relative to that of the low-spin configuration. Crystal-structure

studies of hemoglobin and myoglobin indicate that there are numerous van der Waals contacts between the heme and protein.[50,51] Repulsive interactions between the heme pocket and an expanded porphyrin core may destabilize the high-spin state and favor the transition to the low-spin state with a more negative $\Delta H°$ value.

Concurrent effects on entropy may be associated with each of the structural parameters considered. Greater freedom of bond rotation in the high-spin state may result from a longer metal-axial ligand bond associated with restricted metal-ligand coordination. This effect would result in a more negative $\Delta S°$. An expanded porphyrin core in the high-spin state may experience a more confined orientation in the heme pocket such that the $\Delta S°$ for the spin-state equilibrium may be less negative.

Some restriction on the axial ligation of the histidyl imidazole of the heme octapeptide may be possible due to the fact that the ligand and the porphyrin are covalently attached to the peptide. However, no strain on the iron-histidine bond is apparent from a consideration of a Corey-Pauling-Koltun (CPK) model of the heme octapeptide with the iron in the plane. Thus, the closeness of the thermodynamic values for the heme octapeptide and sperm whale myoglobin determined from magnetic susceptibility suggests that the protein either has no effect on the spin transition or that two opposing factors cancel each other. Therefore, either axial ligand coordination in sperm whale myoglobin is unhindered or the effect of a restricted coordination is compensated by the effect of the heme pocket on an expanded porphyrin core. On the other hand, the more negative thermodynamic values for horse hemoglobin suggest that two opposing factors may effect the spin-state equilibrium. It is possible that the protein conformation restricts axial ligation, resulting in a longer metal-ligand bond, which gives rise to a less negative $\Delta H°$ and a more negative $\Delta S°$ value, while the effect of an expanded porphyrin core inside the heme pocket leads to a more negative $\Delta H°$ and a less negative $\Delta S°$ value. The net effect of these two factors is such that the spin-state transition for horse hemoglobin exhibits more negative $\Delta H°$ and $\Delta S°$ values.

It is interesting to note that a more negative $\Delta H°$ value is accompanied by a more negative $\Delta S°$ value, such that the equilibrium constants of different proteins fall in a narrow range as previously observed.[12]

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