the published procedure.[14] Turnover number for the homoserine formation is 45 μmol min^{-1} (mg of enzyme)$^{-1}$.

Standard Assay Procedure. One milliliter of aqueous buffer mixture containing 5 mM L-aspartate semialdehyde, 10 mM NADH, and 100 mM potassium phosphate, at pH 6.6, was incubated at room temperature. Reaction was initiated by addition of 0.1 unit [μmol min^{-1} (mg of enzyme)$^{-1}$] of enzyme. L-Homoserine formation was monitored as disappearance of NADH absorbance at 340 nm.

β-Cystathionase. Isolated from *E. coli*, this enzyme was purified to homogeneity by Dr. J. S. Hong of Brandeis University.[13] The turnover number for L-cysteine formation is about 50 μmol min^{-1} (mg of enzyme)$^{-1}$.

Standard Assay Condition. One milliliter of aqueous buffer mixture containing 160 mM potassium phosphate (pH 7.4), 1 mM $MgSO_4$, 2 mL L-cystathionine, and 0.1 mg of Ellman's reagent was incubated at 37 °C. Enzyme β-cystathionase was added at time zero, and the disappearance of chromophore at 412 was monitored.

For NMR studies, all the deuterated amino acid samples were dissolved in 99.8% ^2H_2O (p^{2}H 7.2). NMR spectra were obtained on Dr. A. Redfield's 270-MHz NMR spectrometer (Brandeis University). Temperature was fixed at 29.6 °C, and dioxane was used as internal standard.

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Temperature-Dependent Spin-State Equilibrium in Aquo and Hydroxo Ferric Heme Octapeptide Complexes. Model Systems for the Spin Equilibrium of Ferric Hemoproteins

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Abstract: The temperature dependencies of the magnetic susceptibilities of aquo and hydroxo ferric heme octapeptide complexes were investigated as models for the temperature-dependent spin-state equilibria of ferric hemoproteins. The shifts in proton NMR signal of the sodium 4,4-dimethyl-4-silapentanesulfonate (DSS) caused by the heme octapeptide were measured from below room temperature to well above room temperature in aqueous and aqueous–ethylene glycol solutions. Magnetic susceptibilities calculated from the shifts do not exhibit simple Curie behavior as would be expected for pure high-spin or low-spin systems. The temperature dependencies of the susceptibilities are consistent with thermal spin-state equilibria: high spin ($S = {}^5/_2$) ⇄ low spin ($S = {}^1/_2$). The equilibrium constants at 25 °C are 2.37 for aquoheme octapeptide in aqueous solution, 2.04 for (aquo/ethylene glycol)heme octapeptide in D_2O–ethylene glycol solution, 17.5 for hydroxoheme octapeptide in aqueous solution, and 1.79 for (hydroxo/alkoxo)heme octapeptide in D_2O–ethylene glycol solution. Thermodynamic values determined from plots of ln k vs. $1/T$ are $\Delta H° = -9400 \pm 400$ J/mol and $\Delta S° = -24.4 \pm 1.3$ J/(mol·K) for aquoheme octapeptide in aqueous solution; $\Delta H° = -8700 \pm 200$ J/mol and $\Delta S° = -23.4 \pm 0.7$ J/(mol·K) for aquo/ethylene glycol ferric heme octapeptide in 50% (v/v) ethylene glycol–D_2O solution; $\Delta H° = -35\,000 \pm 200$ J/mol and $\Delta S° -94 \pm 6$ J/(mol·K) for hydroxo ferric heme octapeptide in aqueous solution; $\Delta H° = -15\,900 \pm 1200$ J/mol and $\Delta S° = -48.5 \pm 3.8$ J/(mol·K) for hydroxo/alkoxo ferric heme octapeptide in 50% (v/v) ethylene glycol–D_2O solution. The thermodynamic data for these model systems are compared with those of hemoproteins. The results indicate that while suitable axial ligands to the heme iron give rise to spin-state equilibria in model systems, the thermodynamic values for hemoproteins are determined by interactions of the coordination center with the protein.

A number or reports have described model systems for the active sites of hemoproteins.[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 intrnisic properties of the coordination center. Several studies[9-24] have described the thermal spin-state equilibria of many ferric hemoproteins and their ligand substitution derivatives in an attempt to provide information relevant to their fucntion in biological systems. Many of these proteins appear to be characterized by coordination of the heme iron to an imidazole group of a histidyl residue at the fifth coordination position and the 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 ac-

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companied by changes in magnetic properties. Weak-field ligands such as F^- form high-spin hexacoordinate ferric hemoprotein complexes, and strong-field ligands such as CN^- form low-spin hexacoordinate ferric hemoprotein complexes. Ligands such as azide having intermediate field strength form complexes which exhibit temperature-dependent spin-state equilibria. Previous studies[11,25-27] have suggested the importance of the protein structure in determining the spin-state equilibrium.

A recent study[28] of the spin-state properties of the azido ferric heme octapeptide complex indicated for the first time that suitable axial ligands to the heme iron are sufficient to provide a model for the thermal spin equilibria of hemoproteins. Ferric heme *c* octapeptide shares the same axial ligand environment as methemoglobin and metmyoglobin. It is thus of great interest to study the spin-state properties of heme octapeptide complexes in order to determine the effect of protein structure on the magnetic properties of iron–porphyrin systems. In the present study the magnetic properties of aquo and hydroxo complexes of ferric heme *c* octapeptide in aqueous and aqueous–ethylene glycol solutions are examined. The temperature dependencies of the magnetic susceptibilities of these complexes are now reported.

Experimental Section

Reagents. The ferric hemeoctapeptide, HP_{pt}, was prepared from horse heart cytochrome *c* (Sigma, type II-A) according to a modification[29] of the procedures described by Harbury and Loach.[30] Certified ethylene glycol was obtained from Fisher Scientific. Sodium 4,4-dimethyl-4-silapentanesulfonate (DSS) was obtained from Wilmad Glass Co. Deuterium oxide (99.8% D) was obtained from Stohler Isotope Chemicals. All reagents were used without further purification.

Methods. The total heme concentration was determined from the absorption spectrum of the pyridine hemochrome[31] measured on a Varian Cary 14R spectrophotometer using a $\Delta\epsilon = 19.1\ mM^{-1}$.[32] The optical absorbance of the hemeoctapeptide 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.[29,33] 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[34] and from the separation of methylene and hydroxyl signals of ethylene glycol at high temperature.[35]

Magnetic moment measurements were performed essentially as described by Evans,[36] using Wilmad (517) special NMR coaxial cell sample units. A solution of 3–5 mM heme octapeptide in aqueous or 50% (v/v) ethylene glycol–aqueous solution containing 2% DSS, 40 mM phosphate buffer 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 heme octapeptide was measured at various temperatures. The molar paramagnetic susceptibility was calculated by Evan's equation:

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

where χ_M is the molar paramagnetic susceptibility of the ferric heme octapeptide, $\Delta\nu$ 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, M is the molecular weight of the heme octapeptide, and χ_D is the diamagnetic susceptibility of the heme octapeptide calculated from Pascal's constants.[37] The value of χ_0 for a solvent mixture is calculated by the Wiedemann's additivity law:[38]

$$\chi_0 = W_1\chi_{0,1} + W_2\chi_{0,2}$$

where $\chi_{0,1}$ and $\chi_{0,2}$ are the mass susceptibilities of solvent components 1 and 2, and W_1 and W_2 are the weight fractions of solvents 1 and 2, respectively. In cgs units $\chi_0 = -0.631 \times 10^{-6}$ for 50% ethylene glycol–deuterium oxide, $\chi_0 = -0.670 \times 10^{-6}$ for 50% ethylene glycol–water, and $\chi_0 = -0.625 \times 10^{-6}$ for deuterium oxide and -0.720×10^{-6} for water.

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

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

Table I. Temperature Dependence of Effective Magnetic Moments of Aquo Ferric Heme Octapeptide in Aqueous Solution[a]

T, °C	$\Delta\nu$,[b] Hz	χ_M,[c] cgs·emu/mol	μ_{eff},[d] μ_B
−5.6	1.93	5.44×10^{-3}	3.41
1.8	2.01	5.68×10^{-3}	3.53
10.0	1.95	5.50×10^{-3}	3.53
25.5	2.04	5.78×10^{-3}	3.72
31.1	2.07	5.89×10^{-3}	3.79
37.9	2.07	5.89×10^{-3}	3.83
42.7	2.07	5.91×10^{-3}	3.86
46.5	2.13	6.09×10^{-3}	3.95
51.5	2.16	6.21×10^{-3}	4.02
58.7	2.15	6.21×10^{-3}	4.06
66.1	2.22	6.45×10^{-3}	4.18
73.0	2.21	6.42×10^{-3}	4.22
79.3	2.24	6.55×10^{-3}	4.30

[a] Experiments carried out in 40 mM phosphate buffer, pH 7.4, 2% DSS, 2.44 mM heme octapeptide in aqueous solution. [b] $\Delta\nu$, difference in NMR resonance frequencies of the DSS (sodium 4,4-dimethyl-4-silapentanesulfonate) reference peaks in the presence and absence of heme octapeptide. [c] χ_M, the molar paramagnetic susceptibility of ferric heme octapeptide. [d] μ_{eff}, the calculated effective magnetic moment of aquo ferric heme octapeptide; μ_B, the Bohr magneton.

Table II. Temperature Dependence of Effective Magnetic Moments of Aquo/Ethylene Glycol Ferric Heme Octapeptide in D_2O-Ethylene Glycol Solution[a,b]

T, °C	$\Delta\nu$, Hz	χ_M, cgs·emu/mol	μ_{eff}, μ_B
−10.9	2.78	5.62×10^{-3}	3.43
−7.2	2.89	5.87×10^{-3}	3.53
1.8	2.90	5.92×10^{-3}	3.61
8.9	2.87	5.89×10^{-3}	3.65
15.7	2.95	6.08×10^{-3}	3.75
18.6	3.01	6.22×10^{-3}	3.81
25.2	3.01	6.26×10^{-3}	3.87
31.1	3.00	6.38×10^{-3}	3.95
40.2	3.05	6.40×10^{-3}	4.00
45.4	3.03	6.39×10^{-3}	4.04
52.4	3.05	6.46×10^{-3}	4.10
59.7	3.11	6.62×10^{-3}	4.20
68.7	3.06	6.64×10^{-3}	4.23

[a] Experiments carried out in 40 mM phosphate buffer, pH 7.6, 2% DSS, 3.70 mM heme in 50% v/v D_2O–ethylene glycol solution. [b] See Table I for definition of terms.

Results

The difference in NMR resonance frequencies of the DSS peaks in the presence and absence of the ferric heme octapeptide was measured from −11 to 79 °C. The methyl signal of DSS in the solution containing the paramagnetic heme octapeptide corre-

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Table III. Temperature Dependence of Effective Magnetic Moments of Hydroxo Ferric Heme Octapeptide in Aqueous Solution[a,b]

T, °C	$\Delta\nu$, Hz	χ_M, cgs·emu/mol	μ_{eff}, μ_B
−5.6	1.02	2.50×10^{-3}	2.31
1.8	1.03	2.52×10^{-3}	2.35
8.9	1.02	2.49×10^{-3}	2.37
15.7	1.05	2.57×10^{-3}	2.43
25.5	1.16	2.87×10^{-3}	2.62
30.3	1.28	3.22×10^{-3}	2.79
40.2	1.42	3.64×10^{-3}	3.02
46.1	1.46	3.75×10^{-3}	3.10
50.3	1.54	3.98×10^{-3}	3.21
53.2	1.43	3.68×10^{-3}	3.10
57.1	1.65	4.31×10^{-3}	3.37
60.3	1.50	3.88×10^{-3}	3.22
66.1	1.69	4.45×10^{-3}	3.48
73.0	1.68	4.44×10^{-3}	3.51

[a] Experiments carried out in pH 12.01 phosphate buffer, with 2% DSS, 2.85 mM heme octapeptide in aqueous buffer. [b] See Table I for definition of terms.

Table IV. Temperature Dependence of Effective Magnetic Moments of Hydroxo/Alkoxo Ferric Heme Octapeptide in D_2O-Ethylene Glycol Solution[a,b]

T, °C	$\Delta\nu$, Hz	χ_M, cgs·emu/mol	μ_{eff}, μ_B
18.4	1.90	6.46×10^{-3}	3.88
25.2	1.94	6.61×10^{-3}	3.97
32.9	1.99	6.83×10^{-3}	4.09
39.7	2.03	7.02×10^{-3}	4.19
45.6	2.14	7.42×10^{-3}	4.35
52.4	2.18	7.60×10^{-3}	4.45
59.6	2.24	7.86×10^{-3}	4.57
68.1	2.14	7.52×10^{-3}	4.53
77.3	2.37	8.40×10^{-3}	4.85

[a] Experiments carried out in pH 12.0, 40 mM phosphate buffer, with 2% DSS, 2.26 mM heme octapeptide in 50% v/v D_2O-ethylene glycol solution. [b] See Table I for definition of terms.

sponds to the resonance at lower frequency (upfield). The line width of this signal was broadened relative to the DSS signal in the absence of heme. The separation between the two peaks increased with temperature such that the precision of measurements improved at higher temperature.

The NMR chemical shifts of ferric heme octapeptide were measured in aqueous phosphate buffer. Measurements were made in solutions with proton activities equivalent to those of pH 7.4 and 12.0 aqueous solution.

It has been shown that for heme octapeptide there is only one hydrogen ion equilibrium at alkaline pH. This equilibrium is associated with the dissociation of the ligated water with a pK_a value of 10.0.[39] Therefore, in aqueous solution the heme octapeptide is characterized by coordination of a water molecule at the sixth position at pH 7.4 and by hydroxide ion at pH 12.0. In aqueous–ethylene glycol solution the sixth ligand will be water or ethylene glycol at pH 7.6 and hydroxide or alkoxide at pH 12.0.

Tables I–IV list the calculated values for the magnetic susceptibilities of the ferric heme octapeptide and the corresponding effective magnetic moments of iron(III) at various temperatures in aqueous solution (aquoheme octapeptide), in ethylene glycol–D_2O solution [(aquo/ethylene glycol)heme octapeptide], in basic aqueous solution (hydroxoheme octapeptide), in basic ethylene glycol–D_2O [(hydroxo/alkoxo)heme octapeptide].

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:

$$\chi = C/T = \mu_{eff}^2 N\mu_B^2/3kT$$

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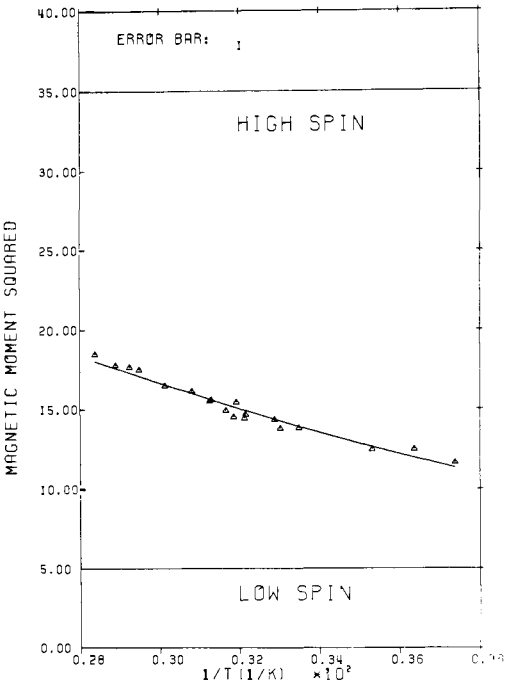

Figure 1. Effect of temperature on the effective magnetic moment of the aquo ferric heme *c* octapeptide in aqueous buffer solution. The high-spin and low-spin lines represent the theoretical values for high-spin and low-spin ferric heme compounds.

Table V. Thermodynamic Values for the Spin-State Equilibria of Ferric Heme Octapeptide Ligand Complexes

ligand	$\Delta H°$, J/mol	$\Delta S°$, J/(mol·K)
H_2O	−9400 ± 400	−24.4 ± 1.3
D_2O/ethylene glycol	−8700 ± 200	−23.4 ± 0.7
hydroxide	−35000 ± 200	−94 ± 6
hydroxide/alkoxide	−15900 ± 1200	−48.5 ± 3.8

Since C is a constant, the value of μ_{eff}^2 should be independent of temperature if Curie's law is followed. Figure 1 shows a representative plot of the temperature dependence of μ_{eff}^2 which indicates that μ_{eff}^2 for these heme compounds all deviate from Curie's law. All the values of μ_{eff}^2 fall within the range between 35 and 5, where 35, μ_{HS}^2, is the theoretical value of μ_{eff}^2 for high-spin ferric heme compounds, and 5, μ_{LS}^2, is the μ_{eff}^2 value observed for low-spin ferric heme compounds.[10] The variation of μ_{eff}^2 with temperature strongly suggested that each of these heme complexes exhibits a thermal spin-state equilibrium: high spin ⇄ low spin.

An equilibrium constant at each temperature was calculated for each complex by using the following relationship:

$$K = (\mu_{HS}^2 - \mu_{eff}^2)/(\mu_{eff}^2 - \mu_{LS}^2)$$

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

$$\ln K = -\Delta H°/RT + \Delta S°/R$$

Figure 2 shows a representative plot of ln K vs. $1/T$ for these heme compounds. Thermodynamic values obtained from the least-square lines are shown in Table V.

Values of K at each temperature were calculated from the determined values of $\Delta H°$ and $\Delta S°$. The theoretical values of the effective magnetic moment were calculated by using the formula:

$$\mu_{eff}^2 = (35 + 5K)/(1 + K)$$

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

Discussion

Thermal Spin-State Equilibria. The high-spin–low-spin thermal equilibria observed for many ferric hemoproteins and their de-

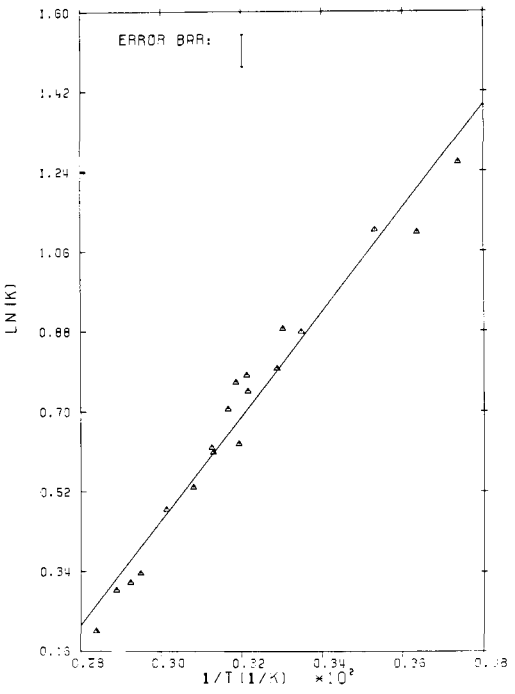

Figure 2. Effect of temperature on the equilibrium constant for the high-spin to low-spin transition of the aquo ferric heme *c* octapeptide in aqueous buffer solution.

rivatives have previously been considered to be a unique characteristic of proteins.[11] Recent studies have also emphasized the influence of protein structure in determining the spin equilibria through a control of axial ligation.[25-27] A recent study of the magnetic properties of azido ferric heme octapeptide[28] has demonstrated however 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.[40-42] Recently, high-spin hexacoordinate ferric heme complexes containing two weak field ligands have been characterized.[25,43,44] Low-spin ferric heme complexes have been shown to be hexacoordinate containing moderate to strong field anions such as perchlorate[48,49] or tricyanomethanide $[C(CN)_3^-]$,[50] and the resulting ferric porphyrin derivatives are characterized by intermediate spin states ($S = {}^3/_2$) or a quantum mechanical mixture of high and intermediate spins.[51] Both five-coordinate and 6-coordinate complexes with intermediate-spin ground state have been structurally characterized. These studies indicate that the nature of the axial ligand is an important factor in determining the spin state of the heme iron.

Table VI. Thermodynamic Values[a] for the Spin-State Equilibria of Aquo Ferric Heme Octapeptide and Methemoproteins

	ΔH°, cal/mol	ΔS°, cal mol^{-1} deg^{-1}	T_c,[b] K	K^{25} [c]	ref
CCP	3510	12.8	274	1.67	11
Mb	3640	9.39	388	0.24	52
meso-Mb	2910	5.98	487	0.15	16
Hb	−608	0.765		1.90	13
HRP	3395	11.3	300	0.95	53
H8PT/H_2O	−2240	−5.8	386	2.37	this work
H8PT/D_2O–EG	−2090	−5.6	373	2.04	this work

[a] All the measurements were made in solution. [b] T_c is the temperature at which $K = 1$. [c] K^{25}, equilibrium constant for HS $\rightleftarrows$ LS equilibrium at 25 °C. [d] Abbreviations: CCP, cytochrome *c* peroxidase; Mb, myoglobin; meso-Mb, mesomyoglobin; Hb, hemoglobin; HRP, horseradish peroxidase; H8PT, horse heart heme *c* octapeptide.

Table VII. Thermodynamic Values[a] for the Spin-State Equilibria of Hydroxo Ferric Heme Octapeptide and Methemoproteins

	ΔH°, cal/mol	ΔS°, cal mol^{-1} deg^{-1}	T_c,[b] K	K^{25} [c]	ref
CCP	−5220	−22.5	232	0.08	11
Hb	−1886	−5.07	372	1.88	13
Mb	−1230	−5.8	212	0.43	54
HRP	−3631	−11.7	315	1.28	53
H8PT/H_2O	−8400	−22.5	373	17.5	this work
H8PT/D_2O–EG	−3800	−11.6	328	1.79	this work

[a] All measurements were made in solution. [b] T_c is the temperature at which $K = 1$. [c] K^{25}, equilibrium constant for HS $\rightleftarrows$ LS at 25 °C. [d] Abbreviations: CCP, cytochrome *c* peroxidase; Mb, myoglobin; Hb, hemoglobin; HRP, horseradish peroxidase; H8PT, horse heart heme *c* octapeptide.

Many ferric hemoproteins 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^-, or azide. The axial ligation of the iron in the heme octapeptide corresponds to that in these proteins. The present and previously[28] observed thermal spin-state equilibria of ferric heme octapeptide complexes indicate that the particular axial ligands are sufficient to yield a system which exhibits a spin-state equilibrium.

Equilibrium and thermodynamic values for the ferric heme octapeptide complexes are compared with those of ferric hemoglobin, myoglobin, cytochrome *c* peroxidase, and catalase in Tables VI and VII. These hemoproteins all contain protoheme, the *b*-type heme, as their prosthetic groups. The fifth ligand in ferric hemoglobin and myoglobin is known to be the imidazole group of a histidyl residue. The fifth ligand in catalase and peroxidase is also known to be the imidazole group of a histidyl residue although the ionization state of the imidazole group is not yet clear. It has been speculated to be the imidazolate anion[55] based on the red-shifted Soret band and lowered CO affinities of these hemoproteins and model studies of imidazolate complexes of ferrous porphyrins.[55] Equilibrium and thermodynamic data for the thermal spin-state equilibrium of mesoheme substituted myoglobin is also included in Table VI for comparison.

Thermal Spin Equilibria of (Aquo/Ethylene Glycol)- and Aquoheme Octapeptide Complexes. In aqueous–ethylene glycol

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solutions, ethylene glycol as well as water may be expected to bind to the heme iron since alcohols are known to complex with metal ions[56] and displace water in coordination compounds.[57] The sixth ligand coordination of the heme octapeptide in the mixed solvent is therefore probably best represented by the equilibria shown in eq 1, where ROH = ethylene glycol.

$$\begin{array}{ccc} \text{heme-OH}_2 & + \text{ROH} \overset{K_1}{\rightleftharpoons} & \text{heme-OHR} + \text{H}_2\text{O} \\ \text{HS} & & \text{HS} \\ \Big\updownarrow K_2 & & \Big\updownarrow K_4 \\ \text{heme-OH}_2 & + \text{ROH} \overset{K_3}{\rightleftharpoons} & \text{heme-OHR} + \text{H}_2\text{O} \\ \text{LS} & & \text{LS} \end{array} \quad (1)$$

The study on nickel halide complexes of ethylene glycol[58] suggested that ethylene glycol is slightly better than water as a ligand. Since their ligand field strengths are only slightly different, one may consider that the stabilities of the aquo and ethylene glycol complexes are approximately equal such that the values of the equilibrium constants between aquo and ethylene glycol complexes, namely K_1 and K_3, are likely to be close to 1.

The partition between aquo and ethylene glycol complexes therefore depends upon the mole fraction of D_2O and ethylene glycol in the solvent, $X_{D_2O,solv}$ and $X_{EG,solv}$, respectively.

The equilibrium between high-spin and low-spin complexes depends on the energy difference between these two states. Since the energy separation is a function of the difference in crystal field stabilization energies of these two states, which in turn is proportional to the ligand field strength for a particular type of complex, the thermodynamic values for spin-state equilibria of two very similar ligands should be approximately equal, such that $K_2 \cong K_4$. The thermodynamic values measured for the D_2O–ethylene glycol system should be, therefore, approximately equal to those of the aquo system. Thus, even if K_1 and K_3 are not equal to 1 as previously considered but the values of K_2 and K_4 are approximately equal, the observed effective magnetic moments should be close to that of the aquo system such that the D_2O–EG system should represent a good approximation for the monomeric aquo system.

The magnetic properties of the heme octapeptide in water were studied to explore the effect of solvent on the spin-state equilibrium. The temperature dependence of the magnetic susceptibility of the heme octapeptide in aqueous buffer at pH 7.4 also appears to be consistent with a spin-state equilibrium. However, aqueous solution studies of porphyrins and metalloporphyrins are often complicated by various dimerization processess including the formation of covalent μ-oxo-bridged dimer,[59] dihydroxo-bridged dimer,[60] or van der Waals π donor-acceptor interactions,[60] as represented by the equilibrium: 2 monomers $\rightleftarrows$ dimer.

The formation of μ-oxo-bridged Fe(III) porphyrin dimers is always accompanied by a significantly diminished magnetic moment[61] often less than 2.0 μ_B for high-spin Fe(III) iron, due to antiferromagnetic coupling through the μ-oxo bridge. On the other hand, in dihydroxo-bridged dimers or dimers formed by π donor–acceptor interactions, the magnetic moment is only slightly decreased.[61] Since the effective magnetic moments of heme octapeptide in aqueous solution are only slightly lower than those measured in ethylene glycol–D_2O solution, it may be concluded that the differences are due to the differences between water and ethylene glycol as the sixth ligand. The results are also consistent with a system composed of dimers or aggregates for which the heme iron has magnetic properties similar to the monomer. In this case the formation of the π-type dimer would be most likely. This latter analysis is in agreement with optical and CD studies[62] of the ferric heme octapeptide in aqueous solution at pH 7.0, which indicated the formation of π-type aggregates.

Table VIII. Effect of pH on the Effective Magnetic Moment of Heme Compounds[a]

	neutral		alkaline		
	μ_{eff}, μ_B	pH	μ_{eff}, μ_B	pH	ref
hemin *c* (aqueous)	5.5	7.1	5.3	12.7	61
H8PT (aqueous)	3.72	7.4	2.62	12.0	this work
H8PT (50% D_2O–EG)	3.87	7.6	3.97	12.0	this work
HP_p (aqueous detergent)	2.3	7	2.3	11	65

[a] μ_{eff}, effective magnetic moment; μ_B, Bohr magneton; H8PT, horse heart heme *c* octapeptide; HP_p, horse heart heme *c* undecapeptide.

Equilibrium and thermodynamic values for the ferric heme octapeptide in aqueous and mixed aqueous–ethylene glycol solutions provide an interesting comparison to aquo ferric hemoproteins. At 25 °C, the equilibrium constants for the hemoproteins exhibit almost a tenfold difference. Thus, while aquometmyoglobin is primarily high spin at room temperature, aquomethemoglobin is reported to be primarily low spin. Except for hemoglobin, the $\Delta H°$ and $\Delta S°$ values for the protohemoproteins are quite similar. Substitution of the heme prosthetic group has a significant effect on both the $\Delta H°$ and $\Delta S°$ values as indicated for proto- and mesomyoglobin. The equilibrium constants for the aquo and aquo/ethylene glycol heme octapeptide complexes are most similar to methemoglobin although the thermodynamic values are much lower than those of all the hemoproteins. On the basis of a comparison of the thermodynamic values for the aquoheme octapeptide and aquohemoproteins, it is apparent that factors other than the axial ligands and the particular porphyrin group are involved in determining the position of the spin-state equilibrium.

Thermal Spin-State Equilibria of (Hydroxo/Alkoxo)- and HydroxoHeme Octapeptide Complexes. Table VII compares the equilibrium and thermodynamic values for the spin-state equilibria of the ferric heme octapeptide in alkaline aqueous and mixed aqueous solutions to hydroxo ferric hemoproteins. The $\Delta H°$ and $\Delta S°$ values for hemoglobin and myoglobin are quite close when compared to other classes of heme compounds. On the other hand, the hydroxo ferric heme octapeptide in 50% v/v D_2O–ethylene glycol solution has spin-equilibrium data similar to those of horseradish peroxidase, while the values in aqueous alkaline solution are much more negative.

Table VIII indicates the effect of pH on the effective magnetic moments of various porphyrin compounds at 25 °C. The magnetic moment for hemin *c* is relatively constant with a slight decrease in value with increasing pH. This was explained in terms of the formation of dihydroxo-bridged or π-complex dimer.

Heme octapeptide in 50% v/v D_2O–ethylene glycol shows a slight increase in magnetic moment as pH increases. This is consistent with previous observations that hydroxide has a somewhat weaker ligand field than water. In aqueous alkaline solution the magnetic moment of the heme octapeptide is appreciably lower than that at neutral pH. This can be explained by formation of dimers. But instead of hydroxo-bridged or π-complex dimers, the results seem to be more consistent with the formation of μ-oxo-bridged dimers. Therefore, only the values of (hydroxo/alkoxo)heme octapeptide in D_2O–ethylene glycol should be compared to those of the hemoproteins.

Parameters Affecting Thermal Spin Equilibria. A number of structural parameters have been suggested to affect the spin-state equilibrium in hemoproteins, including the metal–ligand bond distance,[26] the position of the iron relative to porphyrin plane,[26] and protein–porphyrin interactions[28] associated with porphyrin core expansion.[25,44,64] From the thermodynamic data on aquo

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and azido hemoprotein derivatives it is clear that all protoheme proteins studied have $\Delta H°$ and $\Delta S°$ values which are significantly different from the corresponding mesoheme proteins[16] in which two ethyl groups replace two vinyl groups of protoheme. Perutz et al.[65] and Kendrew[66] demonstrated that the two vinyl groups of the protoheme in hemoglobin (and myoglobin) have several contacts with neighboring residues. It has earlier been suggested[16] that substitution of these vinyl groups with ethyl groups is likely to change the interaction between porphyrin and apoprotein, thereby affecting the spin state of the heme iron through a change in the position of the iron with respect to the porphyrin plane. Alternatively, it has been suggested[28] that a change in the interaction between porphyrin and protein may affect the spin state through a core-expansion mechanism. While the difference between the spin-equilibrium properties of proto- and mesoheme proteins may suggest the importance of interactions between apoprotein and the porphyrin, the much greater difference between thermodynamic data for the spin equilibria of a protein coordinated to different axial ligands at the sixth position indicates the importance of the axial ligation. Among the different ligands included in the current studies, water is regarded to have a stronger ligand field than OH^- such that in aquoheme compounds the difference in energy between low-spin and high-spin states should be greater than the corresponding hydroxo compounds. One would, therefore, expect that a low-spin ground state should be more likely in aquohemoproteins than in hydroxohemoproteins. Reported data indicate that the ground state is low spin in hydroxohemoproteins and high-spin in most aquohemoproteins. In contrast, the ground state is low spin for both aquo and hydroxo ferric heme octapeptide complexes. It is evident therefore that the interplay between axial ligand and apoprotein-porphyrin interactions determines the spin state of a particular heme compound. The present data indicate the extent to which the equilibrium thermodynamics depend on the interaction between protein and the particular heme axial ligand complex involved.

Enthalpy-Entropy Compensation. There is an interesting relation between each pair of $\Delta H°$ and $\Delta S°$ values for the spin equilibria of various hemoproteins. It can be seen that as $\Delta H°$ gets more negative or positive, $\Delta S°$ always changes in the same direction. This behavior is referenced to as enthalpy-entropy compensation. The ratio of $\Delta H°/\Delta S°$ is defined as T_c. Tasaki[67] explained the observed enthalpy-entropy relation in protein spin equilibria in terms of a linear temperature dependence of the energy barrier between high- and low-spin states and a small enthalpy difference at temperature T_c. He attributed the temperature-dependent energy barrier as due to the weak interaction between the porphyrin and its nearest polypeptide chain. As temperature increases, the number of interactions change. Therefore, the energy difference between high- and low-spin states would change with temperature accordingly and result in a linear relationship between $\Delta H°$ and $\Delta S°$. Tamura[53] suggested that the temperature T_c is characteristic of the protein and relatively independent of the axial ligand involved. However, calculated T_c values are not identical for all ligands of a particular hemoprotein. The thermodynamic data for the aquo-, hydroxo- and azidoheme octapeptide complexes observed in this and the previously reported[28] study also indicates that $\Delta H°$ changes with $\Delta S°$ although the T_c values are not equal for each complex. The results suggest then that the compensation phenomenon may be primarily associated with interactions between the porphyrin and solvent or protein environment and that the deviations in the T_c value may be associated with particular ligand-environment interactions.

Conclusion

The present and previously observed magnetic properties of the heme octapeptide complexes indicate that the spin-state equilibria are characteristic of unconstrained model complexes which have axial ligands analogous to hemoproteins and their derivatives. The equilibrium constants and thermodynamic values for these complexes provide references for assessing the affects of protein structure on the spin-state equilibria. Further studies will be required to determine what parameters contribute to the differences between model and hemoprotein complexes.

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Electronic Effects on the Binding of Dioxygen and Carbon Monoxide to Hemes

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Abstract: Chelated protoheme (protoheme having a proximal imidazole covalently attached) is compared with two analogues in which the two vinyl groups are replaced by either two electron-donating ethyl groups or two electron-withdrawing acetyl groups. In this series neither the kinetic nor equilibrium constants for CO binding vary appreciably. By contrast, the dioxygen dissociation rate increases along the series ethyl < vinyl < acetyl, with a consequent lowering of the equilibrium constant for dioxygen binding. These results are discussed in terms of the dipolar nature of the $Fe^{\delta+}$-$OO^{\delta-}$ bond.

The relationship between the structure of a heme and its ligand binding properties has been studied in a wide variety of systems. Altered ligand binding to hemoproteins reconstituted with hemes bearing differing 2- and/or 4-substituents has been interpreted as reflecting electronic factors on these affinities. However, because protein conformation is also sensitive to the nature of the heme, alternative steric explanations have been offered. The binding of ligands and the interplay of steric and electronic effects have been discussed in detail in three recent reviews.[2,3]

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