Characterization of N-Acetylated Heme Undecapeptide and Some of Its Derivatives in Aqueous Media: Monomeric Model Systems for Hemoproteins[§]

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The heme undecapeptide of cytochrome *c* has been converted to a bis(N-acetylated) derivative by reaction with acetic anhydride. The structure of the product has been confirmed by liquid secondary-ion mass spectrometry. As anticipated, the N-acetylated molecule exhibits much less tendency to aggregate in aqueous solution than its heme undecapeptide precursor. Around neutral pH, one axial ligand on the heme iron is provided by the same histidine residue as in the native cytochrome. The other axial ligand can be varied by the addition of exogenous donor species to produce a range of hemoprotein model compounds exhibiting mixed axial ligation. Contrary to the findings of Othman *et al.* [*Biochemistry* **1994**, *33*, 15437−15448] concerning heme octapeptide, the N-acetylated undecapeptide showed no tendency to bind more than one exogenous ligand per heme. At concentrations approaching millimolar and in the absence of exogenous ligands, the N-acetylated molecule may either be monodispersed, exhibiting a characteristic high-spin ($S = {}^5/_2$) ferric heme electron paramagnetic resonance (EPR) signal, or exist in an EPR-silent and presumably aggregated form. Interestingly, the system displays a novel dependence on the buffer with regard to which of these two forms is present in a given sample. There is no evidence in any of the spectra for the existence of an intermediate-spin ($S = {}^3/_2$) ferric heme as suggested by Wang and Van Wart [*J. Phys. Chem.* **1989**, *93*, 7925−7931] to be present in aqueous solutions of N-acetylated heme octapeptide. Also, in contrast to another earlier report concerning the underivatized undecapeptide [Clore *et al. Inorg. Chim. Acta* **1981**, *56*, 143−148], the N-acetylated molecule showed no evidence of catalase activity. In fact, the heme chromophore was surprisingly unstable in the presence of hydrogen peroxide.

Introduction

Heme peptides are obtained by proteolytic digestion of cytochrome *c*. In addition to the covalently bound heme, they are known to retain the fifth ligand on the heme iron in the native cytochrome, *i.e.* histidine 18.[1] Given that the sixth ligand is variable,[2] their potential usefulness as hemoprotein models exhibiting mixed axial coordination is obvious. However, spectroscopic studies employing heme peptide model compounds to probe axial ligation in poorly characterized hemoproteins are very scarce. Undoubtedly, the most significant reason for this is the tendency of one heme peptide to fill its sixth coordination position on the heme iron with a primary amine donor of another heme peptide molecule.[2−4] This intermolecular coordinate bond formation leads to oligomerization and the presence of multiple derivatives even when exogenous ligands are also present.[4] Preparing derivatives of these molecules suitable for experimental use as model compounds in aqueous media, that is, in monomeric form with known axial ligands, has often required the addition of organic solvents[3,4] or detergents.[3−6]

It would be exceedingly useful to have a heme peptide system which can be solubilzed in aqueous solution and maintained in monomeric form over a wide range of pH and concentration, without the necessity of adding detergents or other solvents to prevent aggregation. Derivatization of the primary amines in order to suppress intermolecular coordinate bond formation, first reported by Wang and van Wart for the heme octapeptide,[7] appears to be a viable strategy. With this objective in mind, we report the preparation of bis(*N*-acetyl) heme undecapeptide (*N*-Ac-HUP), together with spectroscopic characterization of some of its ligand adducts in aqueous solution around neutral pH. These *N*-Ac-HUP derivatives appear to represent a relatively simple and well-behaved class of hemoprotein model compounds compared to their unacetylated analogues.

Experimental Section

Heme undecapeptide (HUP) was prepared by proteolytic digestion of cytochrome *c* (Sigma, beef or horse heart, 500 mg quantities) and purified as previously described.[1,8] All samples migrated as single bands upon electrophoresis at pH 4.0 on polyacrylamide gels. Purity of preparations was confirmed by amino acid analysis and liquid secondary ion mass spectrometry (LSIMS).[1] *N*-Ac-HUP was prepared from HUP by N-acetylation following a procedure described by Riordan and Vallee[9] with some minor modifications. Approximately 50 mg of HUP was dissolved in half-saturated (20 °C) aqueous sodium acetate until a concentration of 70−99 μM HUP was obtained. After cooling to 0 °C and with constant stirring, five aliquots of acetic anhydride were added at 10 min intervals to a final volume of 125 μL of acetic

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§ Abbreviations: HUP, heme undecapeptide; HEPES, *N*-2-(hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; *N*-Ac-H8P, *N*-acetyl heme octapeptide; *N*-Ac-HUP, bis(*N*-acetyl) heme undecapeptide; EPR, electron paramagnetic resonance; LSIMS, liquid secondary-ion mass spectrometry.

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anhydride per 2.0 mL of initial HUP solution. The reaction mixture was then refrigerated at 4 °C for 15 h. Completion of the N-acetylation reaction was confirmed by the absence of any absorbance increase at 420 nm in the trinitrobenzenesulfonic acid test for free (*i.e.* primary) amines.[10] Following lyophilization, the sample, redissolved in a minimum quantity (<20 mL) of 0.1 M aqueous NaOH, was applied to a 20 × 5 cm Sephadex G-10 (slurried in H_2O) column. The column was developed with a small quantity of the NaOH solution and then elution continued with H_2O. A distinct color change from scarlet to brown-red was observed as the required *N*-Ac-HUP product was separated from residual reactants and excess hydroxide. The entire band was collected and lyophilized for storage.

Prior to analysis by liquid secondary ion mass spectrometry (LSIMS), it was necessary to remove sodium ions from the *N*-Ac-HUP preparations. Samples were first prepared as aqueous solutions (10 mg of solid/mL of H_2O). After bubbling with HCl gas for 5 min, the solution was then applied to a 10 × 1 cm Sepadex LH-20 (slurried in methanol) column and eluted with methanol. Subsequently, 2 μL of this methanolic solution was mixed with 2 μL of a 2% (w/w) solution of trifluoroacetic acid in glycerol. HUP samples were prepared as previously descibed[1] for purposes of comparison. LSIMS data were collected using a VG Autospec MS with EBE-geometry, equipped with a cesium ion gun operated at 25 kV and an emission beam of 2 μA.

Electronic absorption spectra were recorded on Hewlett-Packard, Perkin-Elmer Lambda 5, Shimadzu 160 A, and Varian DMS 100 spectrophotometers. Concentrations of heme peptide solutions were determined using the value 31.2 mM^{-1} cm^{-1} for the extinction coefficient of the 550 nm band of the pyridine hemochromagen derivative of *c*-type hemes.[11]

EPR measurements were obtained using a hybrid instrument consisting of a Varian E109E console, used to provide the field modulation to a Bruker B-E 25 magnet, with an ER 082 power supply and B-H 15 field controller, plus a Varian E102 microwave bridge and V453.3 cylindrical cavity. The spectrometer was fitted with an Oxford Instruments ESR 900 liquid helium flow cryostat.

Results and Discussion

(i) Structure of *N*-Ac-HUP. The base peaks of mass spectra (LSIMS) of N-acetylated samples (not shown) are at $m/z = 617$, with further well resolved peaks at $m/z = 1944$, 1945, and 1946. The base peak corresponds to the fragment ion [protoheme + 1]$^+$ as previously obtained for various unacetylated heme peptide preparations and described elsewhere.[1] Peaks corresponding to [M − 1]$^+$, [M + 1]$^+$ and, if sodium ions are present in the matrix, [M + 23]$^+$ are routinely obtained by LSIMS.[12] Also, in the particular case of ferric hemes, which already carry a unipositive charge prior to bombardment with the primary ion beam in the spectrometer, we commonly obtain the [M]$^+$ ion as the most intense signal. The features detected in N-acetylated samples at $m/z = 1944$, 1945, and 1946 correspond to the [M − 1]$^+$, [M]$^+$, and [M + 1]$^+$ ions of bis(N-acetylated) HUP. The presence of residual unacetylated HUP was never detected in samples. Occasionally, signals at $m/z = 1904$ and 1926, corresponding to the [M + 1]$^+$ and [M + 23]$^+$ ions of mono-(N-acetylated) HUP, were just resolved from the accompanying noise in the case of some preparations. However, it was possible to demonstrate that such samples were incompletely acetylated by the trinitrobenzenesulfonic acid test described in the Experimental Section. The data reported throughout the rest of this paper were obtained using preparations which contained no detectable levels of heme peptide species other than the bis-(N-acetylated) HUP derivative (*N*-Ac-HUP) according to the trinitrobenzenesulfonic acid assay. The mass spectral results are summarized in Table 1.

Table 1. Mass Spectral (LSIMS) Data for N-Acetylated Heme Undecapeptide Preparations

species[a]	*m/z* calcd	*m/z* measd
[protoheme + 1]$^+$	617	617[b]
[HUP + 1]$^+$	1862	not detected
[HUP + 23]$^+$	1884	not detected
[mono(*N*-acetyl)HUP + 1]$^+$	1904	not detected[c]
[mono(*N*-acetyl)HUP + 23]$^+$	1926	not detected[c]
[bis(*N*-acetyl)HUP − 1]$^+$	1944	1944
[bis(*N*-acetyl)HUP]$^+$	1945	1945[d]
[bis(*N*-acetyl)HUP + 1]$^+$	1946	1946
[tris(*N*-acetyl)HUP + 1]$^+$	1968	not detected[e]

[a] In LSIMS one usually obtains the molecular ion + H^+ and, if sodium ions are present, + Na^+ (see ref 12). [b] This rearranged fragment is obtained as the base peak in all heme peptide LSIMS (see ref 1). [c] This is qualified in the text under Results and Discussion. [d] We commonly observe the unprotonated parent ion in LSIMS of ferric hemes. [e] In some samples known to contain residual sodium ions a small peak with *m/z* 1968 was observed, but this almost certainly corresponds to the species [bis(*N*-acetyl)HUP + 23]$^+$ and not a tris(N-acetylated) derivative.

The starting HUP molecule contains two primary amine groups prior to acetylation. These are the α-amino group of the N-terminal valine and the ϵ-amino group of lysine.[2] Consequently, the indication in the mass spectra that the product is bis(N-acetylated) is extremely encouraging. The similarity of the electronic absorption spectra of certain monomeric HUP and *N*-Ac-HUP derivatives, such as their pH 2 forms and their pyridine hemochromagens, is compelling evidence that the structure of the heme is not changed when HUP is converted to *N*-Ac-HUP, as would reasonably be predicted. Accordingly, our proposed structure for *N*-Ac-HUP is given schematically in Figure 1.

(ii) Aggregation State at Neutral pH. The electronic absorption spectrum of *N*-Ac-HUP at pH 7.5 in phosphate buffer is shown in Figure 2 (solid line). The Soret band at 397 nm and visible region bands at 494 and 622 nm are clearly indicative of a high-spin ferric heme, being very like the same features observed in the spectra of spermwhale metmyoglobin and horseradish peroxidase.[13] This is quite distinct from the absorption spectrum of HUP recorded under the same conditions and also shown in Figure 2 (broken line). Above pH 6, ferric HUP is known to exist as a mixture of low-spin forms, aggregated due to intermolecular coordinate bond formation between hemes and primary amines.[2,14] The obvious difference between the spectra of Figure 2 is, therefore, good evidence that this interaction has been broken in *N*-Ac-HUP, exactly as expected if both available primary amines per molecule have been N-acetylated.

At pH <3, the absorption spectrum of *N*-Ac-HUP exhibits a Soret maximum at 394 nm (not shown). This is analogous to the spectrum of the acid form of HUP described by Wilson *et al.*[2] and suggested to be associated with a bis(aquo)-coordinated heme. Moreover, the species responsible for the 394 and 397 nm Soret bands of Figure 2 are interconvertible in an acid−base process with a pK_a of 3.4 at 20 °C. Again, this is entirely consistent with the data of Wilson *et al.*[2] concerning the unacetylated HUP. Therefore, we are inclined to agree with these previous authors that the process with pK_a 3.4 represents deprotonation of the side chain of the histidine residue present

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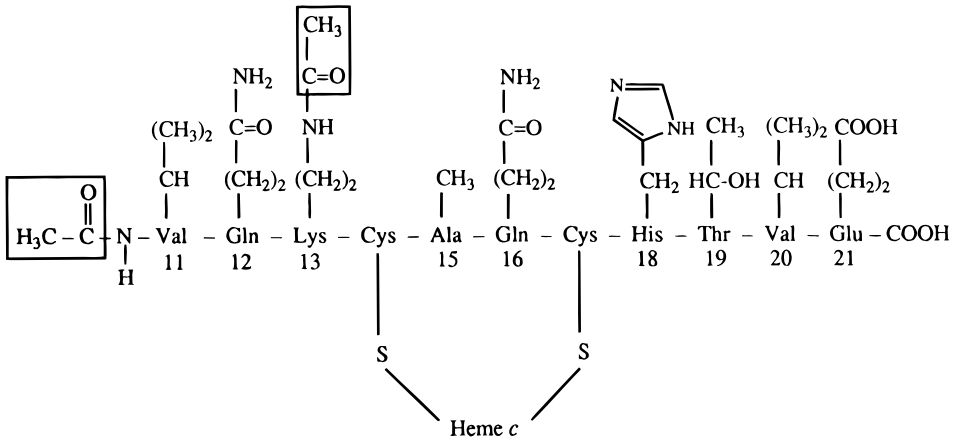

Figure 1. Schematic representation of the structure proposed for the N-acetylated heme undecapeptide (*N*-Ac-HUP). The functional groups of the amino acid side chains are shown and the sites of N-acetylation indicated with boxes. The numbering refers to the amino acid sequence of native beef (and/or horse) cytochrome *c*, from which the molecule is derived. The *c*-type heme group is covalently attached to the peptide via two thioether linkages as in other heme peptides and the native cytochrome (see ref 1).

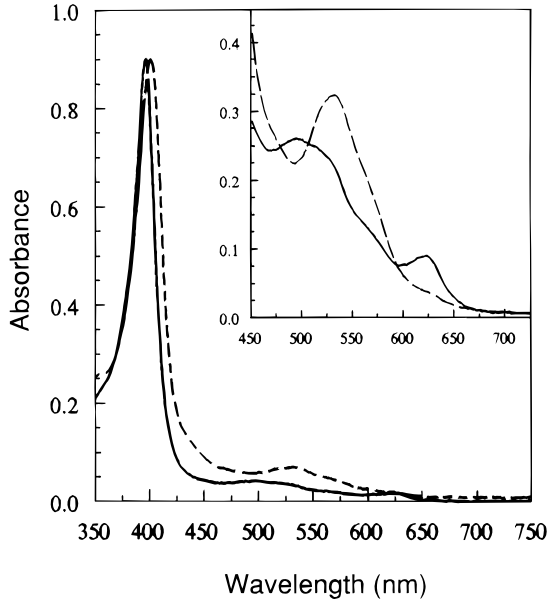

Figure 2. Comparison of the electronic absorption spectra of *N*-acetyl heme undecapeptide (*N*-Ac-HUP, solid line) and heme undecapeptide (HUP, broken line) at pH 7.0 in 10 mM sodium phosphate buffer, 20 °C, 1.00 cm path length. Main figure: 5.5 μM *N*-Ac-HUP, 6.0 μM HUP. Inset: 25 μM *N*-Ac-HUP and HUP.

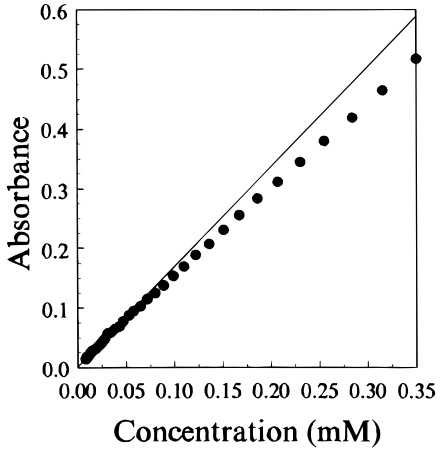

Figure 3. Concentration dependence of the electronic absorption spectrum (λ = 522 nm) of *N*-acetyl heme undecapeptide (*N*-Ac-HUP) at pH 7.0 in 50 mM sodium phosphate buffer, 20 °C, 1.0 mm path length. The solid line represents an extrapolated fit to the data points in the low (<60 μM) concentration range.

from an imidazolonium structure to an imidazole ring, which then binds to the heme iron as its fifth ligand.

The *N*-Ac-HUP absorption spectrum of Figure 2 is, in fact, concentration dependent. The best way to appreciate this is by inspection of the data shown in Figure 3, where we plot absorbance at 522 nm (ordinate) versus *N*-Ac-HUP concentration (abscissa). Beer's law requires a linear relationship between these two parameters to be maintained and extrapolate through the origin. However, a distinct deviation from Beer's law is apparent at above 60 μM in these data. This is exactly the kind of behavior to be expected if the system is monomeric with histidine−aquo coordinated hemes at low concentration but exhibits the well-known tendency of hemes to form μ-oxo-bridged structures in aqueous solution as the concentration is raised.[15] The EPR spectrum of *N*-Ac-HUP at pH 7.0 and 545 μM concentration in phosphate buffer is shown in Figure 4A. Being featureless, it is fully consistent with the presence of μ-oxo-bridged structures, where the ferric hemes are antiferromagnetically coupled and so, diamagnetic. The spectrum is quite unlike that of aggregated HUP at pH >6, which exhibits low-spin ferric heme EPR signals consistent with the presence of amine adducts.[4]

In Figure 4B is shown the EPR spectrum of *N*-Ac-HUP at 665 μM concentration and again at pH 7.0, but this time in HEPES buffer instead of phosphate. Astonishingly, the spectrum is now clearly that of a monomeric high-spin ferric heme, with g_x, g_y = 5.9 and g_z = 2.0, very like the spectra of metmyoglobin and other well-characterized high-spin ferric hemoproteins.[16] The signals of Figure 4B are not due to a minority species, since their doubly integrated intensity decreases linearly with dilution in solvents known to promote monomerization in heme peptides.[4]

The result that the aggregation state of *N*-Ac-HUP depends on the buffer composition is both dramatic and perplexing. HEPES cannot be preventing the formation of μ-oxo-bridged structures by coordinating via its tertiary amine groups to the heme iron, because this would lead to the formation of low-spin ferric derivatives (see below). That this is not the case is immediately obvious by inspection of Figure 4B. The HEPES must be undergoing an interaction with the oligopeptide portion

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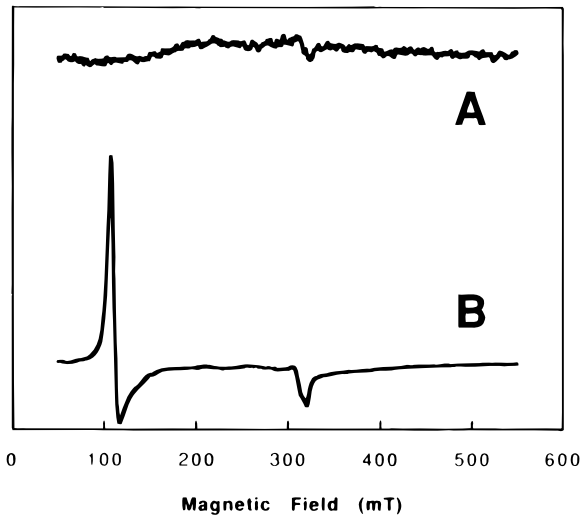

Figure 4. X-band EPR spectra of *N*-acetyl heme undecapeptide (*N*-Ac-HUP) at 11 K, 10 G modulation amplitude, 0.2 mW microwave power: (A) 545 μM *N*-Ac-HUP, pH 6.5 in 200 mM sodium phosphate buffer, 1×10^5 amplifier gain. The apparent small signals around 200 and 320 mT are due to cavity contaminants. (B) 665 μM *N*-Ac-HUP, pH 7.0 in 10 mM HEPES, 3.2×10^4 amplifier gain.

of the molecule and/or heme peripheral groups, involving the formation of a zwitterionic complex, or simple ion-pairing. The resulting structure must then be such that subsequent μ-oxo-bridge formation is sterically prohibited. This represents an entirely plausible explanation for the observed spectra, but it cannot easily be verified. It should be stressed, however, that while the precise nature of this interaction may presently be unclear, its existence is unequivocally supported by the data of Figure 4B which quite obviously represents a magnetically dilute (and therefore monodispersed) high-spin heme, while that of Figure 4A indicates just the opposite.

(iii) Mixed-Axial-Ligand Derivatives. All commonly encountered heme peptides contain histidine 18, the fifth ligand on the heme iron in the native cytochrome.[1,8] It is widely accepted that this is coordinated to iron in heme peptide derivatives at neutral pH.[2−6] In the case of dilute aqueous solutions of *N*-Ac-HUP around neutral pH, the sixth ligand on the heme iron is almost certainly a water molecule. The spectral data of Figures 2 and 4B are fully consistent with these notions and rule out, for example, the possibility of hydroxide ion as the sixth ligand, since this would yield low-spin signals,[4] in addition to excluding an amine or a μ-oxo bridge.

In Figure 5 are shown the results of an experiment in which sodium azide is titrated into an aqueous solution of *N*-Ac-HUP at pH 7.0. The electronic absorption spectra exhibit a well-maintained isosbestic point in the Soret region (Figure 5A) and the Scatchard plot is linear with an intercept at 1.0 (Figure 5B). This clearly demonstrates that one azide ion is bound per heme. The EPR spectrum of the *N*-Ac-HUP−azide adduct exhibits low-spin signals at g_z, g_y, $g_x = 2.84$, 2.21, 1.75, very similar to the spectrum of the metmyoglobin−azide adduct. The data comparing the properties of model compound and hemoprotein derivatives for this and some other exogenous ligand species are collected in Table 2. With the evidence of Figure 5 and Table 2, there can be no reasonable doubt, whatsoever, that around pH 7 *N*-Ac-HUP forms exclusively mixed ligand adducts histidine−L, where L is an exogenous ligand.

It is noteworthy that data for azide binding like those of Figure 5, *i.e.* straightforward to interpret, have been impossible to obtain previously using unacetylated heme peptides, which always give complicated results. Except in the case of very strong field

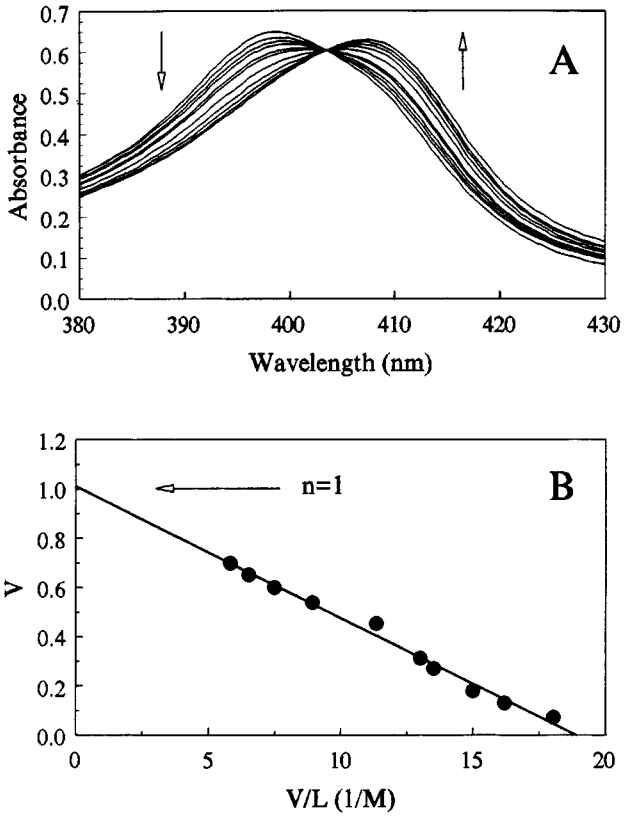

Figure 5. Titration of 4 μM *N*-acetyl heme undecapeptide (*N*-Ac-HUP) with sodium azide at 20 °C in 10 mM sodium phosphate buffer, pH 7.0: (A) change in the Soret band at 1 cm path length following the addition of sodium azide to zero, 4, 8, 12, 20, 24, 40, 60, 80, 100, and 120 mM concentrations; (B) Scatchard plot prepared from the data of panel A, where *V* is the ratio of azide bound to unbound heme (sixth ligand) sites and *L* is the molar concentration of free azide.

ligands such as cyanide and imidazole, it is often not possible to add an exogenous ligand (L) to unacetylated heme peptides and obtain a homogeneous sample with mixed axial ligands histidine−L, due to competition for the sixth ferric site between the exogenous ligand and the peptide's primary amines.[4] Some authors[5,6] have attempted to overcome this difficulty by using micellar preparations of unacetylated heme peptides. However, there is a serious drawback to this approach stemming from the fact that detergents are not routinely available in highly purified form. The cocktails used typically include at least one component which is a quaternary amine. Unfortunately, this means that likely contaminants will in all probability include some level of the tertiary amine derivatives of such components, which are potential strong field ligands.

Significantly, despite using added excesses of azide and cyanide greater than 10^4-fold over the heme, by EPR we have never observed any tendency of these ligands to displace the covalently bound histidine in *N*-Ac-HUP in solutions around neutral pH. This is also the case for various unacetylated heme peptides in ethanolic solutions and is not unexpected given that there is a chelate effect operative. Therefore, we were surprised by the assertion of Othman *et al.*[6] to have observed heme octapeptide bisligated by imidazole added at about a 10^4-fold excess in aqueous media. It is not clear that the electronic absorption spectral changes reported by these previous authors can unambiguously be attributed to displacement of endogenous histidine by added imidazole. For example, a π-stacking interaction between the exogenous ligand and the heme macrocycle, rather than the iron, cannot be excluded as a plausible explanation.

Table 2. EPR Parameters of Low-Spin Ferric *N*-Ac-HUP and Hemoprotein Derivatives

	N-Ac-HUP			metmyoglobin[b]		
sixth ligand[a]	g_z	g_y	g_x	g_z	g_y	g_x
amine	3.22	2.08	1.14[c]	3.33	2.05	1.13[d]
	3.25	2.08	1.05[e]	3.38	2.05	1.14[f]
	3.27	2.09	0.97[g]			
	3.27	2.10	0.95[h]			
	3.20	2.12	1.12[i]			
azide	2.84	2.21	1.75	2.79	2.21	1.72
cyanide	3.33	2.07	0.79[j]	3.45	1.89	0.93
imidazolate				2.80	2.25	1.67
				2.82	2.29	1.69[k]
				2.78	2.26	1.74[l]
imidazole	2.95	2.24	1.51	2.93	2.22	1.52
	2.96	2.25	1.49	2.98	2.24	1.54[k]
				3.02	2.24	1.51[l]
hydroxide				2.59	2.17	1.88
				2.54	2.24	1.84[k]
				2.59	2.18	1.83[m]
1-methylimidazole	2.98	2.26	1.49	2.98	2.26	1.50[k]
propanethiol	2.48	2.24	1.91	2.37	2.24	1.93
tetrahydrothiophene	2.95	2.29	1.43[n]	3.07	2.23	1.26[o]
tert-butylphenol	2.65	2.29	1.75[n]	2.65	2.24	1.86[k]

[a] The fifth ligand in all the hemoprotein derivatives is either imidazole or the imidazole ring of a histidine residue. [b] Beef or horse metmyoglobin (from ref 19). [c] Ammonia adduct. [d] Horse cytochrome *c*, pH 11. [e] Ethanolamine adduct. [f] Butylamine adduct of leghemoglobin. [g] Piperazine adduct. [h] Triethylamine adduct. [i] HUP derivative (from ref 4). [j] In keeping with common practice, where g_x was difficult to observe, it was calculated from the relationship $16 = g_x^2 + g_y^2 + g_z^2$ (see ref 17). [k] Soybean leghemoglobin derivative. [l] Iron protoporphyrin IX derivative (from ref 18). [m] Human hemoglobin derivative (from ref 3). [n] In 95% ethanol, all other *N*-Ac-HUP derivatives were prepared in aqueous HEPES buffer, pH 6.5 - 7.5. [o] Native horse cytochrome *c*.

(iv) Lysine−Histidinate Axial Coordination? Using the equations derived by Taylor,[17] to obtain the ligand field parameters tetragonality (Δ/λ) and rhombicity (V/λ) from *g*-values determined by EPR, Peisach *et al.*[18] have developed a protocol for the identification of axial ligands in low-spin ferric hemes. The presentation of Figure 6, which in the manner recommended by Peisach *et al.*,[18] shows a plot of the EPR-derived parameters tetragonality (abscissa) against rhombicity (ordinate) for some low-spin ferric hemes exhibiting variable axial coordination. It can readily be seen that, for any given pair of axial ligands, the data points fall into a distinct cluster. The circles on Figure 6 were constructed by calculating the mean of the points in a cluster and then scribing a circle centered on the position of the mean and having the minimum radius required to encompass all the points in that cluster. Clearly, the present data indicate that quite different EPR parameters are obtained when the axial ligands are histidine−amine, compared to histidine−imidazole or histidine−imidazolate. While there are some important limitations to the protocol of assigning completely unknown pairs of axial ligands to ferric heme on the basis of this kind of information, it has been suggested[19] that EPR alone might provide the best available means of distinguishing histidine−amine ligated ferric heme from coordination by other kinds of endogenous nitrogen donors. Until now, this position was supported by very little data.[20] Consequently, the fact that the histidine−amine data of Table 2 and Figure 6 were obtained using primary, secondary, and tertiary amines, containing quite different sterically hindered nitrogen donors, is of some significance.

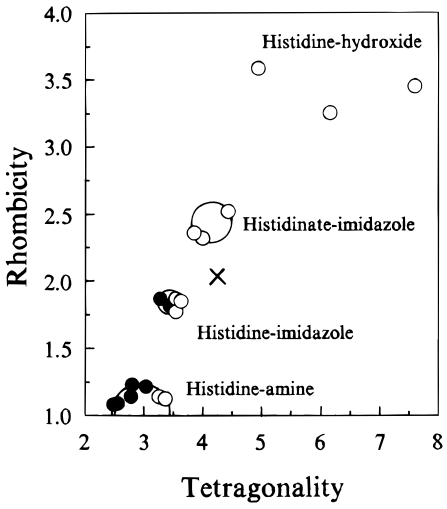

Figure 6. EPR correlation diagram prepared in the manner suggested by Peisach *et al.*[18] for low-spin ferric derivatives of hemoproteins (open symbols) and heme peptides (filled symbols). Tetragonality and rhombicity are respectively the crystal field parameters Δ and V expressed in units of spin−orbit coupling. These were calculated from the data in Table 2 using the following expressions given by Taylor:[17] $V/\lambda = g_x/(g_z + g_y) + g_y/(g_z - g_x)$; $\Delta/\lambda = g_x/(g_z + g_y) + g_z/(g_y - g_x) - {}^1/_2(V/\lambda)$.

Ubbink *et al.*[21] recently reported the existence of an alkaline form of the cytochrome *c* from *Thiobacillus versatus*, with EPR *g* values 2.87, 2.18, 1.60, which they tentatively associate with lysine−histidinate axial coordination of the heme, formed by deprotonation of the native histidine. The position occupied by this species in the correlation diagram of Figure 6 is marked by a cross. These signals are quite different from those of other low-spin hemes exhibiting histidine−amine coordination and consequently, the present work lends support to the idea that the spectrum obtained from the alkaline *Thiobacillus* cytochrome *c* represents reasonable evidence for lysine−histidinate coordination.

(v) Unusual Heme Spin States? Wang and Van Wart have reported[7] the preparation of ferric N-acetylated heme octapeptide (*N*-Ac-H8P) and identified two forms in solution at room temperature and neutral pH by Raman spectroscopy. In addition to the anticipated high-spin component, these same authors assert that "since there are no strong field ligands available...," the second species must be an intermediate-spin ($S = {}^3/_2$) moiety. It appears unlikely in the extreme that *N*-Ac-H8P and *N*-Ac-HUP will not be comparable systems so far as this issue is concerned. If an intermediate-spin species were present in the case of *N*-Ac-HUP, as either a pure spin state or an admixture of intermediate- and high-spin states, one would expect to see a readily detectable feature in the EPR spectra of Figure 4 in the range $5.9 > g > 4.2$,[22] which is clearly not present. Furthermore, it is, in fact, quite well established that histidine−hydroxide ion axial coordination renders ferric hemoproteins low spin.[19] However, there is no evidence in the spectra of Figures 2 and 4 for a hydroxide adduct around neutral pH, and so, we are also inclined to discount this as a plausible explanation for the appearance of the two species in solutions of *N*-Ac-H8P identified by Raman spectroscopy.

Curiously, Wang and Van Wart[7] do not seem to have seriously considered the likely formation of diamagnetic μ-oxo-

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Table 3. Solvent Dependence of Spectral Parameters of *N*-Acetyl Heme Undecapeptide Derivatives

	aqueous buffer				95% ethanol			
axial ligands[a]	Soret λ_{max}, nm	g_z	g_y	g_x	Soret λ_{max}, nm	g_z	g_y	g_x
histidine−azide	410	2.84	2.21	1.75	414	2.82	2.24	1.73
histidine−cyanide	412	3.33	2.07	0.79[b]	416	3.38	2.06	0.78[b]
histidine−imidazole	406	2.95	2.24	1.51	410	2.97	2.24	1.51

[a] Prepared by the addition of the exogenous ligand as excess solid−sodium salts in the case of anionic species. [b] In keeping with common practice, where g_x was difficult to observe, it was calculated from the relationship $16 = g_x^2 + g_y^2 + g_z^2$ (see ref 17).

bridged structures as a possible explanation for their data. Instead, in support of their claim to have detected an intermediate-spin ($S = {}^3/_2$) component, they cite the paper by Jehanli *et al.*,[14] who reported a low value for the room-temperature magnetic moment of a nominally monomeric form of ferric HUP. Now, an extensive set of methods for obtaining meaningful magnetization data from bioinorganic samples was recently developed and described.[23,24] A critical aspect of the overall protocol is that conclusions not be drawn from data obtained at a single applied magnetic field and narrow temperature range. Furthermore, the method employed by Jehanli *et al.*[14] was clearly unreliable in two ways. The diamagnetic correction to be applied was estimated by calculation instead of being measured, and also, the HUP concentration was determined by weight, which requires an assumption about the amount of water present. A much better procedure, one might think, is to determine the heme peptide concentration spectrophotometrically and, then, to measure the diamagnetic correction to be applied directly using a diamagnetic derivative (*e.g.*, the ferrous imidazole adduct). If these variations are adopted, the room-temperature magnetic moment found for the acid form of HUP is that calculated for a high-spin ($S = {}^5/_2$) ferric system from the spin-only formula (unpublished observations of J. Peterson and K. Kimura). Unfortunately, however, as the experimental uncertainty is quite large, this result really does not differ significantly from the previous low result[14] and therefore, the method is clearly of limited value in probing these systems.

The EPR spectrum of Figure 4B and data summarized in Tables 1 and 2 exhibits evidence of low-spin ($S = {}^1/_2$) and high-spin ($S = {}^5/_2$) feric hemes only. That is, the model compound derivatives exhibit the same spectral features as are observed for mononuclear hemoproteins[16] and not other kinds of signal. The EPR data are quite definitive in this matter, being a direct method of probing the oxidation number and spin state of the heme iron. We previously measured the EPR spectra of numerous heme octapeptide, nonapeptide, and undecapeptide derivatives[4] and did not find a single piece of evidence for an intermediate-spin ferric heme in any heme peptide sample. In summary, it is our opinion that the two species observed by Wang and Van Wart[7] were most likely a high-spin ferric species and either a μ-oxo, or some other, bridged structure.

(vi) Solvent Dependence of Spectra. In the case of complicated, multicomponent hemoproteins, such as cytochrome *c* oxidase, the deconvolution of spectral characteristics arising from the various chromophores and their detailed interpretation to yield usable structural information are a crucially important issue.[25] The extent to which the solvent environment can perturb the spectral properties of a heme group is a case in point. This might provide an indication of whether a membrane protein, for example, can have substantially different solvent environments associated with a constituent heme in two protein conformations.

A comparison of the electronic absorption and EPR spectral characteristics of some low-spin *N*-Ac-HUP derivatives in aqueous media and 95% ethanol is given in Table 3. It is apparent that the solvent environment has a very small, but measurable, effect on the observed EPR parameters. This is entirely reasonable, since an electron located primarily in a d orbital of the ferric ion would be expected to be largely shielded from solvent effects by its first coordination sphere, *i.e.* the porphyrin ring and axial ligands. In contrast, the derivatives in Table 3 exhibit a consistent 4 nm (*ca.* 240 cm^{-1}) red shift in the positions of their Soret maxima when the solvent is changed from an aqueous to ethanolic medium. The Soret band is principally due to *x,y*-polarized porphyrin $\pi-\pi^*$ transitions,[13] the electron having moved further away from the centrally coordinated ferric ion in the excited state. Consequently, the observed red shift could conceivably represent changes in the levels of the electronic excited states, ground states, or both. However, because the heme iron d orbitals will almost certainly be mixed with the electronic ground state porphyrin π orbitals to a greater extent than the porphyrin π^*, the EPR spectrum can reasonably be expected to remain relatively unaffected by a change in the solvent environment if the primary effect of this is to shift the potential energy of the excited state involved in the Soret transition. This appears to be the most plausible explanation for the results documented in Table 3.

In the case of *N*-Ac-HUP, one side of the heme in its entirety, or about 50% of the total heme surface area, is exposed and, therefore, subject to interaction with solvent. Since, in a protein, the total heme area exposed to solvent will inevitably be much less, the expected magnitude of observed spectral shifts due to a changing solvent environment must arguably be less than the observed 240 cm^{-1}, *i.e.* less than 4 nm in the vicinity of the Soret transition (Table 3). Consequently, the 12 nm variation in the position of the Soret maximum in certain cytochrome *c* oxidase derivatives reported by Holm *et al.*,[26] as these authors suggest, cannot be ascribed to "solvent effects".

(vii) Catalytic Activity. Various unacetylated heme peptides are available from commercial sources as "microperoxidases", the name describing their well-known peroxidatic behavior.[27] It was, therefore, natural to determine the peroxidase activity of *N*-Ac-HUP. This information is presented in Table 4, where the results for the *N*-Ac-HUP-catalyzed peroxidation of pyrogallol are compared with those obtained using other heme peptides and horseradish peroxidase in a standard assay procedure.[28] It is clear that the heme peptides all exhibit reasonable peroxidase activity, with activities only a few orders

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Table 4. Pyrogallol Assay of Peroxidatic Activity for Various Heme Peptides

catalyst	H_2O_2 consumption, mmol of $H_2O_2 \cdot min^{-1} \cdot (\mu mol \text{ of heme})^{-1}$ (20 °C, pH 7.0)
N-Ac-HUP	0.9
heme octapeptide	1.0
heme nonapeptide	1.3
heme undecapeptide	1.1
horseradish peroxidase	170[a]

[a] Method and data taken from ref 28.

of magnitude less than the enzyme at room temperature. The fact that *N*-Ac-HUP has essentially the same activity as the other heme peptides is not surprising because the heme concentration was around 400 nM in all these assays. At this low concentration, the unacetylated heme peptides might be expected to show less tendency to aggregate. Alternately, the off rate of amine ligands present could well be considerably faster than that of a subsequent rate-determining step.

The results of attempted catalase (*i.e.*, hydroperoxidase) activity assays on *N*-Ac-HUP were considerably more interesting. Following the addition of hydrogen peroxide to a 10^3-fold excess (phosphate buffer, pH 7.0, 20 °C) and in the absence of a second substrate, like pyrogallol, complete bleaching of the heme was observed within seconds. No loss of absorption intensity at 240 nm, where hydrogen peroxide absorbs, could be detected. This presumably indicates that the heme chromophore was simply destroyed by hydrogen peroxide, and therefore, *N*-Ac-HUP does not exhibit any catalase activity. This is contrary to a previous report[29] concerning HUP where some degree of hydrogen peroxide disproportionation was thought to have been detected by stopped-flow spectrophotometry. However, the earlier work involved an oligomeric heme peptide, and consequently, it is entirely possible that the process observed was, in fact, a peroxidatic oxidation of the amino acid side chain of one HUP molecule by the heme group of another. The present data obtained with the monodispersed *N*-Ac-HUP constitute a much less ambiguous result.

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Conclusions

The conversion of HUP to *N*-Ac-HUP with acetic anhydride is perfectly straightforward. The reaction can readily be monitored and shown to be essentially 100% complete by a simple colorimetric test as described. Purification is then easily achieved in a single step by size-exclusion chromatography.

N-Ac-HUP is very soluble in aqueous media where it exists as a monodispersed system at pH 6.5−7.5 in HEPES buffer (20 mM, or less) at concentrations of at least 0.6 mM. The electronic absorption and EPR spectral characteristics of its paramagnetic ferric derivatives are hemoprotein-like, exhibiting recognizably high-spin and low-spin features only, with no indication of intermediate-spin states. As might reasonably have been expected, *N*-Ac-HUP displays significant peroxidatic behavior, but, perhaps more unexpectedly, no measurable catalase activity.

The unionized structure of *N*-Ac-HUP, confirmed by mass spectrometry, is as indicated in Figure 1. The native structure of the *c*-type heme of the cytochrome remains unchanged, with two covalent thioether linkages to the peptide. In this and all other heme peptides, the proximal heme ligand of the cytochrome (histidine 18) is retained but the distal ligand (methionine 80) is missing. Consequently, using exogenous ligand excesses of 10^4-10^5-fold over heme, it is routinely possible to prepare various mixed-ligand addducts of *N*-Ac-HUP in aqueous solution. In general, it is not possible to do this with other unacetylated heme peptides unless very strong field ligands are used, either in the presence of detergents or in aqueous−alcoholic solvents.

N-Ac-HUP is undoubtedly a much better model system for hemoproteins than its unacetylated precursor, HUP, or other similar heme peptides with underivatized primary amine groups. The molecule provides a range of useful adducts which can be employed to investigate the spectral, magnetic, oxidation−reduction, and ligand exchange properties of protein-free hemes, without having to deal with the problems of aggregation or of insolubility in aqueous solution, so often encountered with heme models. The presence of the constituent histidine is especially useful, given that this is by far the most commonly encountered proximal heme ligand in biomolecules.

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