The Alkaline Transition of Bis(N-acetylated) Heme Undecapeptide[⊥]

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Alkaline forms of the ferric bis(N-acetylated) heme undecapeptide of cytochrome *c* (*N*-ac-HUP) and some of its derivatives have been studied by electronic absorption and electron paramagnetic resonance spectroscopies. Surprisingly, even at pH >12, no evidence could be found for the formation of a hydroxyl ion adduct, in direct contrast to a previous report concerning ferric heme peptides encapsulated in detergent micelles (Mazumdar et al. *Inorg. Chem.* **1991**, *30*, 700−705). A spectroscopically determined pK_a of ∼9 is assigned to the deprotonation of the constituent histidine ligand of heme iron in *N*-ac-HUP. The present findings are not entirely in keeping with those of an earlier study concerning the properties of N-acetylated heme octapeptide (Wang et al. *J. Biol. Chem.* **1992**, *35*, 15310−15318), the differences observed being attributed to the buffering media employed in the two investigations. The implications of the current results in relation to a better understanding of the alkaline transitions observed in hemoglobins and myoglobins is considered.

Introduction

There are several different kinds of well-documented "alkaline transition" known in hemoproteins, all of which involve either exchange, or deprotonation, of an axial ligand to the heme. For example, upon raising the pH: (1) the distal aquo ligand present in methemoglobin and metmyoglobin becomes deprotonated to a hydroxyl species;[1] (2) the imidazole adducts of metleghemoglobin and metmyoglobin undergo deprotonation of the exogenous ligand to imidazolate;[2,3] (3) the endogenous (native) methionine ligand is displaced by one or more lysine ligands in cytochrome *c*;[4,5] (4) the endogenous (native) histidine ligand becomes deprotonated to histidinate in certain other cytochromes.[6,7] In all the preceding cases, the alkaline forms of the hemoproteins are low-spin ferric derivatives. Alkaline transitions leading to the formation of high-spin ferric forms at elevated pH are more unusual, but include the following: (5) the displacement of the distal aquo ligand by tyrosinate in some hemoglobins (see ref 8 and references therein); (6) the deprotonation of the proximal histidine in some five-coordinate mutant myoglobins (see ref 9 and references therein).

The factors which determine the precise nature of any given alkaline transition are not well delineated. In particular, it is not entirely clear which of the examples cited above, if any, reflect mainly the properties of the heme group and axial ligands, rather than essential contributions from the protein structure beyond the first coordination sphere. In some cases, it has been possible to partly address the problem using mutant proteins (see, e.g., refs 5 and 9). However, this methodology does not facilitate direct examination of heme-localized properties and occasionally suffers from some other drawbacks. For instance, mutation of a single surface residue in yeast cytochrome *c* was unexpectedly found to lead to substantial refolding of the molecule and exchange of one axial heme ligand.[10] The general difficulty is that cetain individual amino acid residues in a protein structure are likely responsible, in conjunction with other residues, for multiple functional and structural properties of the molecule. Consequently, some experiments in which site-directed mutants are employed to test a specific characteristic of a protein, while attempting to maintain all other significant properties identical with those of the native (wild-type) protein, will necessarily yield ambiguous results.

Heme peptides, obtained by proteolytic digestion of cytochrome *c*, have proven useful in various experimental circumstances as biomimetic hemoprotein models.[11,12] In addition to

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[⊥] Abbreviations: CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; EPR, electron paramagnetic resonance; H8P, heme octapeptide; 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; TRIS, tris-(hydroxymethyl)aminomethane.

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the covalently bound heme, these relatively small molecules (M_r < 2000) also retain the fifth ligand of the heme iron in the native cytochrome (i.e., histidine 18) while the sixth ligand is variable.[12,13] As all hemoproteins known to exhibit an alkaline transition have at least one histidine ligand, the heme peptides quite obviously represent extraordinarily useful models for studying this process in the absence of encapsulating protein. The bis(N-acetylated) heme undecapeptide (*N*-ac-HUP) does not oligomerize in aqueous media to the same degree as its underivatized precursor (HUP).[14] Consequently, *N*-ac-HUP is a better model system than HUP (and other unacetylated heme peptides) for probing the physicochemical properties of hemoproteins.

In this paper, we report the characterization, by electronic absorption and electron paramagnetic resonance (EPR) spectroscopies, of some *N*-ac-HUP forms obtained in alkaline aqueous solutions. The molecule exhibits a distinct alkaline transition under mildly basic conditions, which, not surprisingly, is clearly different from that previously reported for HUP.[13] More interestingly, however, upon an increase in the pH, no evidence could be found for deprotonation of the sixth exogenous aquo ligand of the heme iron in *N*-ac-HUP. In fact, the precise nature of the alkaline transition observed in ferric N-acetylated heme peptides is unlike those reported for both native and mutant hemoglobins, myoglobins, and cytochromes. The results appear to have some interesting implications with regard to our understanding of the alkaline transition associated with the heme group in hemoglobins and myoglobins particularly.

Experimental Section

Bis(N-acetylated) heme undecapeptide (*N*-ac-HUP) was prepared by N-acetylation of proteolytic digests of cytochrome *c* (Sigma, beef or horse heart) and purified as previously described.[14] After incubation in 0.1 M NaOH overnight at 20 °C, the stability of the *N*-ac-HUP molecule toward alkaline media was verified in the following manner: (i) a pyridine hemochrome assay[15] showed that neither significant decomposition nor modification of the heme chromophore had taken place; (ii) a colorimetric test using the reagent trinitrobenzenesulfonic acid[16] showed that hydrolysis of the N-acetylated amino acids did not occur. Amines employed as ligands in these studies were purchased from Aldrich (highest available purity in each case) and used without further purification. In experiments where detergent was added, the tetramethylammonium bromide/sodium dodecyl sulfate recipe of Mazumdar et al.[17] was followed, using reagents obtained from Sigma without further purification. In deuterated buffer, the negative logarithm (base 10) of the hydronium ion concentration (i.e., pD) was taken as the pH meter reading + 0.4 unit.[18]

Electronic absorption spectra were recorded on 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.[15] 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.

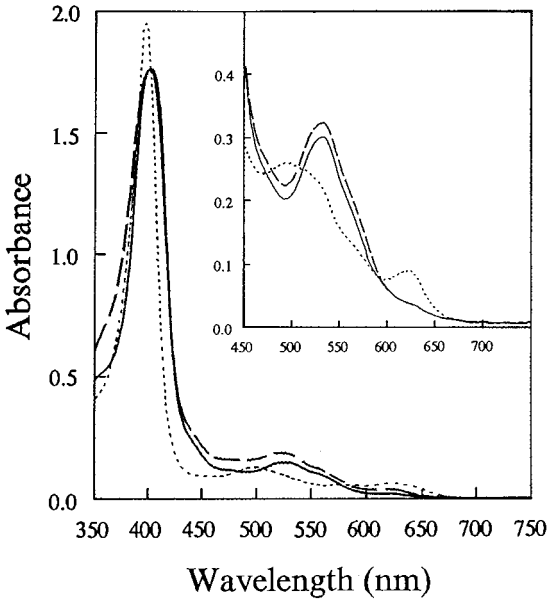

Figure 1. Electronic absorption spectra of heme peptides at 20 °C: *N*-ac-HUP, pH 10.0 in 10 mM sodium tetraborate (solid traces), pH 9.5 in 10 mM sodium tetraborate, 5% (w) sodium dodecyl sulfate, 0.1 M tetramethylammonium bromide (dotted traces); HUP, pH 7.0 in 10 mM sodium phosphate (broken traces). Main figure: 110 μM heme peptides, 1.0 mm path lengths. Inset: 22 μM heme peptides, 1.0 cm path lengths.

Results

In Figure 1 is shown the absorption spectrum of *N*-ac-HUP at pH 10.0 in sodium tetraborate buffer (solid line). For comparison, the spectrum of HUP at pH 7.0 in sodium phosphate buffer is also shown (broken line). At neutral pH, the HUP is known to be in a low-spin form.[12,13,19] Thus, the spectrum of alkaline *N*-ac-HUP appears similar to that expected for a low-spin ferric heme, quite distinct from its spectrum at pH 7.0, which is that of a high-spin system when recorded under suitably dilute conditions.[14] Following the addition of detergent and 15−20 min incubation at room temperature, the spectrum of *N*-ac-HUP at pH 9−10 changes to that shown by the dotted line in Figure 1. With a Soret band at 398 nm and visible region maxima at 494 and 623 nm, this is also clearly the spectrum of a high-spin ferric system.[20] These results argue strongly that, under alkaline conditions and in the absence of detergent, *N*-ac-HUP exists in some aggregated form, which, like HUP,[17] readily becomes monodispersed if encapsulated in detergent micelles. The absorption spectrum of *N*-ac-HUP at pH 10.0 in tetraborate buffer without detergent (Figure 1, solid line) obeys Beer's law in the range ~300−3 μM, indicating the oligomeric form persists upon dilution. This is unlike its behavior at pH 7.0 in phosphate buffer reported previously;[14] where, in similar dilution experiments, there was clearly a change in linearity (from one slope to another) as the concentration was reduced to less than about 60 μM.

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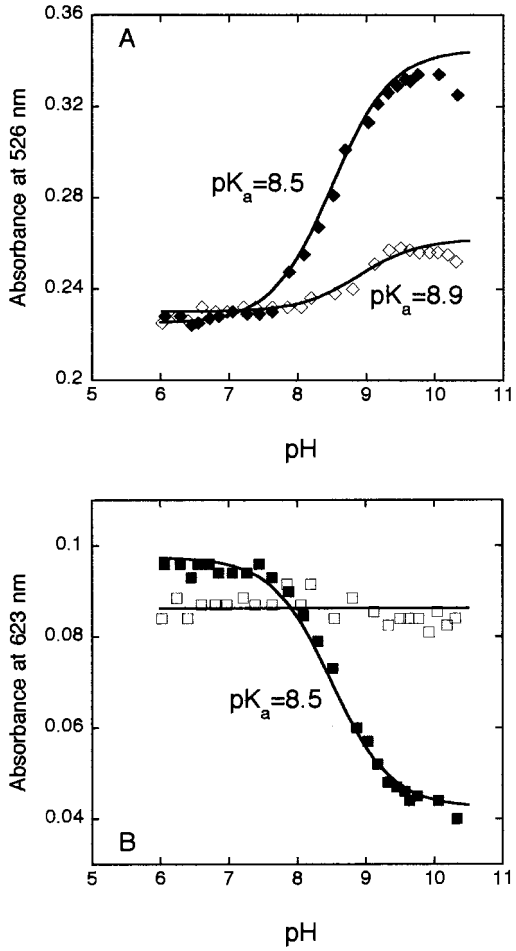

Figure 2. *N*-ac-HUP pH titration in unbuffered aqueous solution at 20 °C. Solid symbols: [*N*-ac-HUP] = 25 μM, 0.3 M NaCl, 1.0 cm path length. Open symbols: same as above, except also 5% (w/w) sodium dodecyl sulfate, 0.1 M tetramethylammonium bromide. A: Monitored at 526 nm. B: monitored at 623 nm. The solid lines are theoretical fits to the data representing single processes with associated pK_a's as indicated on the diagrams.

It should be noted that, occasionally, the absorption spectra of some preparations of *N*-ac-HUP encapsulated in detergent micelles show evidence of contamination with low-spin ferric species. This happens even around pH 7 at concentrations at which *N*-ac-HUP is known to be high-spin and monodispersed in the absence of detergent.[14] The effect is almost certainly associated with the tetramethylammonium bromide used as a counterion in the sodium dodecyl sulfate micelle. Since this is normally employed at around 10^3- to 10^4-fold excesses over the heme peptide, the presence of relatively small amounts of trimethylammonium bromide (the most likely contaminant and a strong-field ligand) is potentially a significant problem. The use of an alternate quaternary ammonium salt might not be that helpful, since all are going to be subject to contamination with similar N-donor species. We did not attempt further purification of the tetramethylammonium bromide as at least one batch appeared to be essentially free of the strong-field contaminant.

If *N*-ac-HUP is dissolved in 1.0 mM NaOH (0.3 M in NaCl for constant ionic strength) and titrated with 100 mM HCl, a fully reversible process occurs, with an associated pK_a of 8.5, as the molecule is converted to its neutral form (Figure 2, filled symbols). The interconversion of acidic and alkaline forms of the unacetylated HUP occurs with an associated pK_a of 5.9,[13,19] quite different from the present data set, as might reasonably be expected. The *N*-ac-HUP titration was monitored at both 526 nm (Figure 2A) and 623 nm (Figure 2B), corresponding to the positions of absorption bands indicative of respectively low-spin ferric-like and high-spin ferric species. The titration was repeated in the presence of detergent, the other conditions being the same as before. These results are represented by the open symbols in Figure 2. The data obtained at 623 nm (Figure 2B) show that, with added detergent, *N*-ac-HUP remained essentially high-spin (and, therefore, monodispersed) in keeping with the results of Figure 1. Interestingly, the titration data obtained at 526 nm in the presence of detergent (Figure 2A) showed some evidence of a small spectral change, but the associated pK_a was about 8.9, slightly shifted with respect to that observed in the absence of detergent.

There are several further issues that need to be raised concerning Figure 2. First, there is clear evidence in both sets of data obtained at 526 nm (Figure 2A) for at least one additional process with an apparent pK of >10. We have not yet investigated this particular behavior further, because our current interest is in alkaline transitions that occur under mildly basic conditions, since these seem more relevant to the hemoprotein-related problems we wish to address. Second, when the titration experiments were repeated in deuterated media, the data obtained were found to be fully superimposable on those of Figure 2. This is to be expected if the experimentally determined process is a proton transfer, but not so if, for example, the mechanism actually involves displacement of bound hydroxyl ion by a water molecule. Third, while it is quite clear that the spectral changes observed upon raising the pH of an initially neutral *N*-ac-HUP solution involve deprotonation of a group associated with the chromophore, interpretation of the titration data is not entirely straightforward. In the absence of detergent, the oligomerization which also takes place masks the true pK_a; that is, the value 8.5 is merely an apparent pK_a. With detergent present, the oligomerization is certainly suppressed, but the effect of encapsulation in a detergent micelle on the value of the measured pK_a is of unknown magnitude. Furthermore, the spectral changes measured are now rather small and so, while the value 8.9 is a true pK_a, it has not been determined with great precision. In summary, it can at least be stated that upon an increase in the pH of a neutral *N*-ac-HUP solution, the deprotonation of a heme-associated group occurs which, in the absence of other added ligands (see below), has a true pK_a value in the vicinity of 9.

The nature of the alkaline species formed above pH 9 was further investigated by EPR spectroscopy. The X-band EPR spectrum of *N*-ac-HUP at pH 9.5−10.5 in 10 mM tetraborate buffer is essentially featureless (Figure 3A). This is in keeping with the formation of oligomers in which the ferric hemes are antiferromagnetically coupled, leading to diamagnetic ground states. For instance, μ-oxo-bridged dimers would certainly be consistent with this result, but there is another possibility. Wang et al.[21] have previously suggested imidazolate-bridged dimers to be present in highly alkaline solutions of *N*-acetyl heme octapeptide (*N*-ac-H8P). The spectrum of Figure 3A could also be accounted for by the presence of imidazolate-bridged oligomers. This is the preferred interpretation, since, in addition to the EPR evidence, it appears to provide a convenient explanation of both the low-spin-like appearance of the absorption spectrum in Figure 1 (solid line) and the origin of the apparent pK_a in the data of Figure 2 (solid symbols). If, however, the sample is prepared at pH 9.5−10 in 10 mM CAPS buffer, the EPR spectrum of Figure 3B is obtained. The "g =

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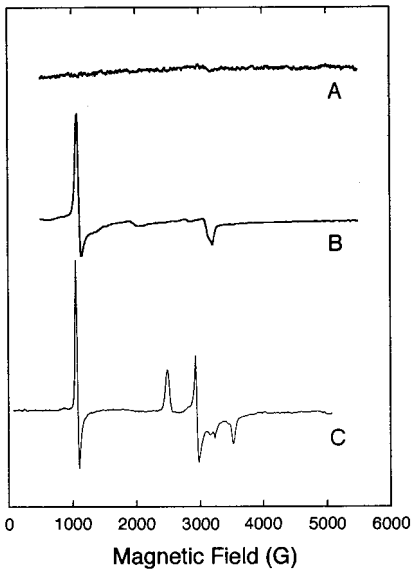

Figure 3. X-band EPR spectra of alkaline *N*-ac-HUP and hemoglobin at 11 K, 10 G modulation amplitude, 0.2 mW microwave power. A: 0.45 mM *N*-ac-HUP, pH 10.5 in 20 mM sodium tetraborate buffer, 1×10^5 receiver gain. B: 0.66 mM *N*-ac-HUP, pH 10.0 in 10 mM CAPS, 2×10^4 receiver gain. C: 0.14 mM methemoglobin (Deer Lodge), pH 8.3 in 50 mM TRIS buffer, 2.5×10^4 receiver gain.

6" signal (at ca. 1100 G) is characteristic of high-spin ferric heme[22] and is, therefore, indicative of a magnetically dilute (i.e., monodispersed) system. Importantly, the absorption spectrum of *N*-ac-HUP in CAPS buffer is essentially identical to that of the detergent encapsulated system (Figure 1, dotted traces). Furthermore, the high-spin ferric signal of Figure 3B is indistinguishable from that obtained following the addition of detergent to tetraborate-buffered samples, confirming that this spectrum represents monodispersed *N*-ac-HUP. It was previously shown that *N*-ac-HUP yielded detectable EPR signals at pH 7 in HEPES buffer but not in phosphate buffer.[14] The result that the aggregation state of *N*-ac-HUP depends on the buffer composition, both at pH 7 and 10, is dramatic and perplexing. It is important to note, with regard to the present sample, that CAPS cannot be preventing the formation of bridged structures by coordinating via its tertiary amine group to the heme iron, because this would lead to the formation of low-spin ferric derivatives which are easily identified by EPR. In fact, in HEPES, CHES, or CAPS buffer, if the concentration of the buffering species is raised to 50−100 mM and the pH adjusted to the alkaline side of the apparent pI of the particular buffer molecule, then one does observe minority low-spin species with EPR spectra suggestive of histidine/amine coordination. However, this is clearly not the case in Figure 3B. CAPS is presumably undergoing an interaction with the oligopeptide portion of the molecule and/or heme peripheral groups, involving the formation of a complex or simple ion-pairing. The resulting structure must then be such that subsequent bridge formation is sterically prohibited. While the precise nature of the proposed interaction between CAPS and *N*-ac-HUP is unclear, its existence is very strongly supported by the spectrum of Figure 3B, which unambiguously represents a monodispersed high-spin heme, while that of Figure 3A indicates just the opposite.

The obvious difference between the spectra of Figure 3A and Figure 3B is an important observation, but there is another key issue here. Neither these spectra nor any other heme peptide

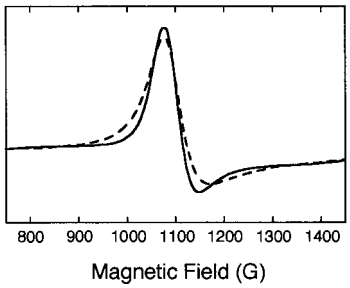

Figure 4. Comparison of X-band EPR spectra of alkaline and neutral forms of monodispersed *N*-ac-HUP. Solid line: 0.66 mM *N*-ac-HUP, pH 10.0 in 10 mM CAPS, 2×10^4 receiver gain. Broken line: 0.66 mM *N*-ac-HUP, pH 7.0 in 10 mM HEPES, 3.2×10^4 receiver gain. The spectrum of *N*-ac-HUP in 10 mM sodium tetraborate buffer, pH 10.0, 5% (w/w) sodium dodecyl sulfate, 0.1 M in tetramethylammonium bromide (not shown) is essentially indistinguishable from the solid trace.

EPR data we have collected have ever given any indication of the presence of a derivative where histidine and hydroxyl ion can be assigned as the axial heme ligands. This includes the results of experiments in which 0.1 M NaOH was the solvent, both with and without added detergent. To make the point absolutely clear, consider the EPR spectrum of human methemoglobin (Deer Lodge) at pH 8.3, shown in Figure 3C. There is no particular significance in the fact that the spectrum was obtained using a mutant protein, except that it is the best example we have with which to illustrate the point in question. In addition to the high-spin ($g = 6$) signal, there is a distinct rhombic low-spin signal with *g*-values of 2.59, 2.18, and 1.83. Double integration of these signals suggests that the low-spin component represents about 80% of the total heme present in this particular sample. Low-spin EPR signals with these *g*-values have been shown to arise from ferric hemes with histidine/hydroxyl axial coordination,[23,24] and when present, they are not easily missed. The absence of such signals from Figure 3B establishes that the monodispersed form of *N*-ac-HUP at pH ∼10 is definitely not a histidine/hydroxyl axially coordinated species.

It has been reported by others that unacetylated heme peptides encapsulated in detergent micelles undergo an aquoferric → hydroxylferric transition with an associated pK_a of 7.2.[17] In our hands, no such process occurs, and we have previously suggested[12] the results of the earlier authors to be due to an amine contaminant in their experiments. The absence of any detectable process with a pK_a of ∼7.2 in the present data set concerning *N*-ac-HUP represents further convincing evidence that the earlier study[17] is in error.

Another crucial matter with regard to the arguments we wish to develop concerns the high-spin ferric ($g = 6$) signal of Figure 3B. As stated already, this signal is indistinguishable from that obtained following the addition of detergent to tetraborate-buffered samples. However, while it is also similar to that reported previously[14] for *N*-ac-HUP at pH 7 in HEPES buffer, it is not exactly the same. In Figure 4 is shown the $g = 6$ signal associated with the monodispersed form of *N*-ac-HUP at pH ∼10 (solid line) compared with the analogous signal of the monodispersed form at pH ∼7 (broken line). The latter signal is clearly broader, indicating that the two spectra arise from different species.

It was desirable to establish whether the imidazole ring of the constituent histidine ligand could become deprotonated in

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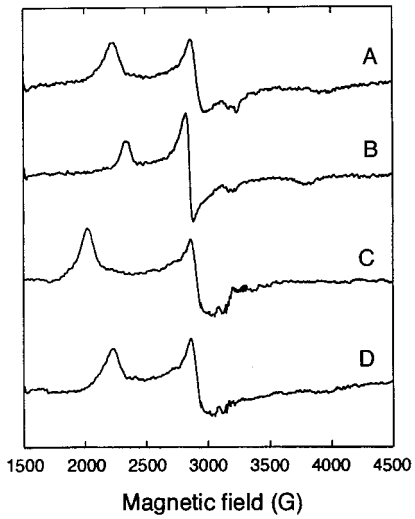

Figure 5. X-band EPR spectra of 0.8 mM *N*-ac-HUP 1-methylimidazole (A, B) and ethanolamine (C, D) adducts at 11 K, 10 G modulation amplitude, 0.2 mW microwave power. A, C: pH 8.5 in 10 mM CHES, 5×10^4 receiver gain. B, D: pH ~13 in 0.1 M NaOH, 5% (w/w) sodium dodecyl sulfate, 0.1 M tetramethylammonium bromide, 3.2×10^4 receiver gain.

the experimental pH range of current interest. In Figure 5 are shown the X-band EPR spectra of the 1-methylimidazole adduct of *N*-ac-HUP at pH 8.5 (Figure 5A) and in 0.1 M NaOH (Figure 5B). This system converts from a low-spin derivative with g-values of 2.96, 2.26, and 1.50 to another with g-values 2.76, 2.27, 1.71 upon raising the pH. These signals are consistent with the presence of histidine/1-methylimidazole axial coordination at low pH and histidinate/1-methylimidazole coordination at high pH.[2,23] The pK_a for the interconversion of the imidazole ring of the histidine ligand in the present derivative is close to 11, since samples prepared in the pH range 10.5−11 exhibited both sets of signals (not shown). An entirely analogous pH-dependent process appears to take place in the case of the ethanolamine adduct of *N*-ac-HUP, where the system converts from one low-spin form with observable *g*-values of 3.25 and 2.08 (Figure 5C) to another with *g*-values of 2.86 and 2.23 (Figure 5D). A summary of these low-spin ferric heme derivatives and their associated EPR signals, together with some additional relevant data, is given in Table 1. To better demonstrate the ability of these EPR spectra to distinguish between possible axial ligand pairs, a correlation chart prepared in the manner suggested by Peisach et al.[25] is also presented in Figure 6. Using the equations of Taylor,[26] the parameters tetragonality (abscissa) and rhombicity (ordinate) were calculated from the data in Table 1. It can readily be seen that, for any given pair of axial ligands, the data points fall into a distinct cluster. The circles in 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 of minimum radius required to encompass all the points in that cluster. There are some important limitations to assigning completely unknown pairs of axial ligands to low-spin ferric hemes on the basis of this kind of information.[23,24] However, in the present examples, where at least two out of three EPR *g*-values were readily measured in all cases and only a few axial combinations were possible, the protocol is trustworthy. The important conclusion to be drawn from the data presented in Figures 5 and 6 is that the constituent histidine of *N*-ac-HUP clearly can become deprotonated in aqueous media. The systems in which this can be demonstrated with reasonable certainty exhibit associated pK_a's of around 11, but these are adducts of strong-field ligands

Table 1. EPR Spectral Parameters of Ferric N-ac-HUP and Various Hemoprotein Derivatives

	EPR parameters (g_{zyx})					
	N-ac-HUP[a]			metmyoglobin[b]		
exogenous ligand						
(i) Fifth Ligand: Histidine						
ammonia	3.22	2.08	(1.13)[c]			
ethanolamine	3.25	2.08	(1.05)			
piperazine	3.27	2.09	(0.97)			
triethylamine	3.27	2.10	(0.95)			
butylamine				3.38	2.05	(1.14)[d]
lysine	3.20	2.12	1.12[e]	3.33	2.05	(1.13)[f]
(ii) Fifth Ligand: Histidine						
imidazole	2.95	2.24	1.51	2.93	2.22	1.52
	2.96	2.25	1.49[g]			
	3.02	2.24	1.51[h]			
1-methylimidazole	2.98	2.26	1.49	2.98	2.26	1.50[d]
(iii) Fifth Ligand: Histidinate						
buytlamine	2.87	2.22	1.67			
ethanolamine	2.86	2.23	1.67			
trimethylamine	2.89	2.22	1.66			
lysine				2.87	2.18	1.60[i]
(iv) Fifth Ligand: Histidinate[j]						
histidine				2.82	2.29	1.69[d]
imidazole	2.76	2.27	1.72	2.80	2.25	1.67[k]
	2.78	2.26	1.74[h]			
1-methylimidazole	2.76	2.27	1.71			
	2.78	2.27	1.70[l]			
(v) Fifth Ligand: Histidine						
hydroxyl				2.59	2.17	1.88
				2.54	2.24	1.84[d]
				2.59	2.18	1.83[m]

[a] Buffer: HEPES/CHES, pH 7.0−8.5; or 0.1 M NaOH, 5% (w/w) sodium dodecyl sulfate, 0.1 M tetramethylammonium bromide, pH ~13. [b] Taken from ref 23. Unless indicated to the contrary, proteins were buffered at pH 7.0−7.4. [c] Where g_x was difficult to observe, in keeping with common practice (e.g., see ref 23) it was calculated from the relationship $16 = g_x^2 + g_y^2 + g_z^2$. [d] Leghemoglobin derivatives. [e] Oligomeric heme undecapeptide. [f] Cytochrome *c*, pH 11. [g] Heme undecapeptide in 95% ethanol.[12] [h] Iron-protoporphyrin IX.[25] [i] *Thiobacillus versutus* cytochrome *c*, pH 11.6.[6] [j] Or imidazolate. [k] Myoglobin, pH 11.7. [l] 0.1 M NaOH with no detergent. [m] Two equivalent spectra obtained for human A_0 and Deer Lodge methemoglobins.

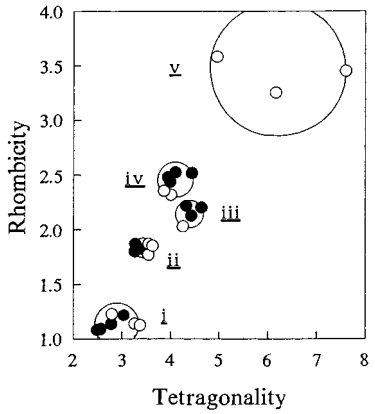

Figure 6. EPR correlation chart (after Peisach et al.[25]) for the assignment of low-spin ferric heme adducts with exogenous ligands. The data for both model compounds (●) and hemoprotein derivatives (○) are taken from Table 1. The clusters of points correspond to the following pairs of axial heme ligands: (i) imidazole/amine; (ii) imidazole/imidazole; (iii) imidazolate/amine; (iv) imidazolate/imidazole; (v) imidazole/hydroxyl.

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which might have some considerable trans influence on the basicity of the endogenous histidine ligand.

Discussion

There are two important issues that need to be addressed before proceeding further. First, in the case of certain ferrihemoprotein derivatives, the existence at room temperature of equilibrium mixtures of species exhibiting different ground-state spins has been known for some considerable time.[27] In the specific case of hydroxyl adducts, one finds predominantly high-spin forms at ambient temperatures and predominantly low-spin forms at cryogenic temperatures.[28] Although we have not shown all the data, whenever a derivative was observed by cryogenic EPR, its presence in the same spin state at room temperature was confirmed by measurement of the electronic absorption spectrum of an analogous sample. No examples of temperature-dependent spin equilibria were observed in these investigations, fully corroborating our conclusion that a hydroxyl adduct of *N*-ac-HUP was not formed. Second, using the colorimetric test described in the Experimental Section, it was repeatedly shown that, following overnight incubation in 0.1 M NaOH at 20 °C, measurable hydrolysis of the N-acetylated amino acid residues of *N*-ac-HUP did not occur and, in fact, there was no detectable level of HUP present in *N*-ac-HUP samples used in these experiments. Consequently, it should be quite clear that none of the observations reported here can, in any way, be ascribed to complications arising from reconversion of *N*-ac-HUP to HUP or from the presence of contaminating HUP.

(i) Comparison of the Properties of *N*-ac-H8P and *N*-ac-HUP. There is no readily apparent reason to suppose that the heme-dependent properties of the various common heme peptides should differ very much once their primary amines have been derivatized. Therefore, the behavior of the hemes in *N*-ac-HUP and other N-acetylated heme peptides ought to be directly comparable. In an earlier study concerning the properties of N-acetylated heme octapeptide (N-ac-H8P),[21] the authors were unaware of the profound effect that the buffer composition can have on the aggregation state of a ferric heme peptide, and unfortunately, many of their data were obtained using samples in phosphate buffer, which we now know leads to oligomerization of these systems.[14] This complicates a direct comparison of their results with the current data. Nevertheless, the previous authors did report some useful information derived from a combination of EPR, magnetic circular dichroism, and resonance Raman measurements performed on N-ac-H8P in the pH range 1−12. Some especially noteworthy observations were as follows: (1) no evidence could be found for μ-oxo-bridged structures; (2) no evidence could be found for hydroxyl adducts; (3) it was proposed that, at around neutral pH, there is an equilibrium mixture of six-coordinate high-spin ($S = {}^5/_2$) and intermediate-spin ($S = {}^3/_2$) forms; (4) the predominant form at pH 10 was stated to be a five-coordinate high-spin species.

Regarding these observations, the following remarks are made taking into account both our previous[14] and current findings. In the case of observations 1 and 2, there is no conflict. The absence of any evidence for hydroxyl adducts is a particularly important assertion, which is in full agreement with the present results. If substantiated, the claim made in observation 3, concerning the existence of a six-coordinate intermediate-spin ferric heme, announces what would be a highly unusual example of a biological heme derivative. This suggestion really needs to be supported by some unambiguous evidence. Neither in our own data nor in those of Wang et al.[21,29] can we find any such compelling evidence in support of this claim. The absence of any EPR signal that could reasonably be attributed to ferric heme in an intermediate-spin state is especially troublesome. We are also in disagreement over observation 4, since, in our data, there is no evidence for a five-coordinate high-spin species. The EPR spectrum of Figure 3B is that typical of a six-coordinate high-spin ferric heme, quite unlike the more rhombic signals exhibited by five-coordinate metmyoglobins (e.g. see ref 9). It is our opinion that this apparent discrepancy in the behavior of the hemes in N-ac-H8P and *N*-ac-HUP most likely stems from the different buffering media used in the two laboratories.

(ii) Alkaline Transitions in *N*-ac-HUP Derivatives. The EPR spectrum of the 1-methylimidazole adduct of *N*-ac-HUP in highly alkaline solution (Figures 5B and 6) is very similar to that previously reported for analogous derivatives of leghemoglobin and myoglobin where one axial ligand is provided by an imidazolate nitrogen.[2,3] Under such conditions, the imidazole ring of the endogenous histidine ligand in *N*-ac-HUP is expected to be deprotonated, and therefore, this observation is entirely reasonable. Likewise, the EPR spectrum of the ethanolamine adduct of *N*-ac-HUP in highly basic solution (Figures 5D and 6) is very similar to that previously reported for the alkaline form of the cytochrome *c* from *Thiobacillus versutus* and tentatively associated with lysine/histidinate axial coordination of the heme.[6] In nearly all these systems, the observed pK_a's for the imidazole-to-imidazolate interconversion are around 11. Significantly, however, the pK_a for this transition in the leghemoglobin adduct is around 7, probably due to the influence of a histidine residue on the distal side of the heme pocket.[2] Similarly, another low pK_a associated with an endogenous histidine-to-histidinate interconversion in cytochrome b_{562} from *Escherichia coli* has been attributed to the existence of an identifiable hydrogen-bonding network in the protein.[7] Such networks are presumably absent in *N*-ac-HUP. Consequently, it appears that observation of a pK_a of approximately 11 for this kind of alkaline transition, as obtained in the present study and in previous studies of some hemoproteins, is probably indicative of there being no essential contribution from the protein tertiary structure in determining the precise value of the pK_a.

At concentrations less than about 60 μM, *N*-ac-HUP in neutral aqueous solution is monodispersed, with intramolecularly bound histidine as the fifth heme ligand and a water molecule as the sixth.[14] The data of Figure 2 show quite clearly that, upon an increase in the pH from around neutrality, something becomes deprotonated. Moreover, following this alkaline transition, the EPR spectrum of the system has changed (Figure 4). This argues very strongly that some alteration in the nature of the heme ligands has taken place. If a modification of the porphyrin ring had occurred, it would almost certainly be obvious from the electronic absorption spectra. As is the case for HUP,[13,30] with the constituent histidine bound in the fifth position, other potential ligands are sterically prevented from binding to the sixth position in an intramolecular manner. In the concentration range of current interest (<1 mM), intermolecular replacement of the water ligand with a carboxylate species from another *N*-ac-HUP molecule can be excluded for two reasons. First,

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the addition of detergent leads to an EPR spectrum which is indistinguishable from Figure 3B. Second, it is difficult to reconcile a pK_a >8 with such a process. Deprotonation of bound water has been conclusively refuted as a possibility, since this would lead to a characteristic low-spin ferric EPR signal (Figure 3C). Therefore, deprotonation of the imidazole ring of the histidine ligand to an imidazolate species seems to be left as the only possible explanation. Furthermore, the water in the sixth position appears to remain bound, since the spectra of five-coordinate ferric heme histidinate species are distinctly rhombic (see ref 9 and references therein), whereas that of Figure 3B is axial. The combination of axial ligands histidinate/water is not expected to exert a sufficiently strong ligand field to render ferric heme low spin.[24] Consequently, the spectrum of Figure 3B is fully consistent with this argument.

The authors of the previous report concerning the properties of N-ac-H8P also came to the conclusion that the fifth histidine ligand in that system becomes deprotonated under alkaline conditions with a pK_a of approximately 9.[21] Furthermore, two mutant metmyoglobins which do not undergo the normal distal aquoferric → hydroxylferric alkaline transition have been shown to exhibit proximal histidine → histidinate transitions with associated pK_a's of 9.3 and 9.5.[9] Consequently, there is now a growing body of evidence to suggest that, in the absence of a sixth strong-field ligand, the pK_a associated with interconversion of a ferric heme histidine ligand to histidinate will generally be found around pH 9−9.5. A substantial departure from this value in other systems should, therefore, be taken to indicate the presence of some significant perturbing influence exerted by the protein. The higher pK_a's associated with the histidine-to-histidinate interconversion in the butylamine and 1-methylimidazole adducts of *N*-ac-HUP can be readily understood in terms of the trans influence of these strong-field nitrogen donors. The lone pair on the nitrogen of the sixth exogenous ligand interacts with the same ferric ion d orbital as the fifth endogenous histidine ligand. The resulting inductive effect leads to an increase in electron density associated with the N−H bond of the histidine ligand compared to the situation where there is no nitrogen donor in the fifth position.

(iii) The Alkaline Transition in Hemoglobins and Myoglobins. At the beginning of the investigation and by analogy with the known chemistry of methemoglobins and metmyoglobins[1], raising the pH was naively anticipated to lead to deprotonation of the bound water molecule in the sixth coordination position, resulting in the formation of a histidine/hydroxyl axially coordinated heme. With the benefit of hindsight, however, it is unsurprising that this was not the case. The pK_a for the interconversion of uncomplexed imidazole to imidazolate is ∼14.[31] By definition (i.e., $[OH^-]/[H_2O]$ = unity), the pK_a for the interconversion of uncomplexed water to hydroxyl ion is unattainable in aqueous solution, being >14. Consequently, given a ferric heme at neutral pH with a water molecule and an imidazole group as its axial ligands, it is entirely reasonable to expect the imidazole to become deprotonated first upon raising the pH. Furthermore, once the charge on the coordinated ferric ion is counterbalanced by three negative charges (two on the porphyrin ring and one on the imidazolate), the aquo ligand might not exhibit much increased tendency to ionize relative to uncomplexed water. In view of this, a brief re-examination of what is known about some systems which do undergo the aquo-to-hydroxyl transition seems to be appropriate.

Table 2. First pK_a Values for Deprotonation of Mononuclear Aquo−Iron(III) Species

species	pK_a	distal residue[a]	ref
$Fe^{II}(H_2O)_6$	6.7		32
$Fe^{III}(H_2O)_6$	2.5		32
deuterioferriheme	7.1		33
mesoferriheme	7.0		34
protoferriheme	∼7		35
micellar protoferriheme	6.5		36
ferric *N*-ac-HUP	>9		this work
ferric *N*-ac-H8P	>9		21
Chironomous methemoglobin	7.4	isoleucine	8
Aplysia methemoglobin	7.6	valine	8
Lucina methemoglobin I	9.6	unidentified[b]	8
human methemoglobin	8.0	histidine	1
horse methemoglobin	8.3	histidine	1
horse metmyoglobin	8.9	histidine	1
whale metmyoglobin	9.0	histidine	1

[a] Distal side heme pocket residue, commonly found to be histidine, which in physiologically active forms may interact directly with bound oxygen (e.g., corresponding to His64 in the horse myoglobin sequence). [b] Considered highly unlikely to be histidine.[8]

The pK_a values for the deprotonation of a single water molecule bound to a number of relevant ferric systems are given in Table 2. The first pK_a of the hexaaquoferric ion is 2.5, indicating the ferric ion to be a reasonably strong Lewis acid. Coordination of the iron to a porphyrin macrocycle results in a square-planar arrangement of nitrogen donor ligands around the metal ion. This complement of strong σ donors reduces the acidity of aquo ligands bound in the axial positions considerably, leading to values for the observed pK_a's of ∼7. In fact, typical pK_a values for protein-free ferrihemes are rather like that observed for the hexaaquoferrous ion, which is a considerably weaker acid than its ferric counterpart due to the reduced charge on the central metal ion. If a fifth nitrogen donor ligand is present in the coordination sphere, then the pK_a of the aquo ligand in the sixth position can clearly be expected to be significantly higher than 7. This follows because the fifth nitrogen ligand donates electron density into the same d orbital as the aquo oxygen, leading to a weakening of the interaction between the metal ion and aquo species, with consequent increase in the electron density of the aquo O−H bonds.

The histidine/aquo → histidine/hydroxyl transitions observed in various hemoglobins and myoglobins have associated pK_a's which are reported (see Table 2 references) to vary from 7.4 in *Chironomous* methemoglobin to 9.6 in *Lucina* methemoglobin I. In the present set of experiments, we have found no evidence for a similar process in *N*-ac-HUP up to approximately pH 9. This suggests that, at least in the case of those hemoglobins where this pK_a is less than about 9−9.5, the aquo-to-hydroxyl conversion is assisted by one or more basic groups on the distal side of the heme pocket. The mere presence or absence of such a basic group is not enough to explain the range of pK_a's observed, since mammalian hemoglobins and myoglobins both have distal histidine residues, yet their relevant pK_a's are respectively ∼8 and ∼9. That is, in the case of mammalian metmyoglobin, the distal histidine seems to have relatively little

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effect on the observed pK_a of the alkaline transition. Also, the methemoglobins from both *Chironomous* and *Aplysia* have pK_a values of respectively 7.4 and 7.6 associated with their alkaline transitions, but both lack a distal histidine residue. The factors governing any observed pK_a are clearly complicated beyond simple questions of the inherent basicity of the macrocycle plus fifth axial ligand and the identitiy of a single distal amino acid residue. The available data seem to suggest that there are probably some rather specific additional interactions involved in determining the observed pK_a in each individual hemoglobin where it is less than 9−9.5, the details of which will have to be determined on a case by case basis.

Undoubtedly, it can still correctly be asserted that the precise details of the interactions involved in the alkaline transitions of hemoglobins and myoglobins remain poorly understood. Nevertheless, the present work has established that there are two broad and perhaps interrelated questions which need to be answered concerning these processes, the relative importance of which depends on the individual pK_a's involved. First, in those instances where the histidine/aquo → histidine/hydroxyl transitions exhibit pK_a's of around 9 and greater, what are the structural features responsible for preventing the preferential deprotonation of the imidazole group of the proximal histidine? Second, what structural features on the distal side of the heme assist in deprotonation of the aquo ligand? The full significance of this second question has previously been improperly recognized. In the absence of evidence to the contrary, previous authors (e.g., see ref 37 and references therein) have drawn attention to the fact that the observed pK_a's of the alkaline transitions in the *Chironomous* and *Aplysia* methemoglobins are similar to those observed for bis(aquo)-coordinated protein-free ferrihemes (see Table 2). That is, the erroneous impression has been created that the alkaline transitions of these two hemoglobins might not be perturbed by the protein to any great extent. In direct contrast, the present findings strongly suggest that the *Chironomous* and *Aplysia* proteins, which do not contain a distal histidine residue, represent examples where the deprotonation of the aquo ligand is actually much more strongly influenced by the protein moiety than is the case in mammalian hemoglobins and myoglobins.

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