

Studies on the Undeca-peptide of Ferricytochrome c Using ESR, Mössbauer and Visible Spectroscopies *

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Undeca-peptide of Ferricytochrome c, Electron Spin Resonance, Mössbauer Spectroscopy, Visible Spectroscopy

The ESR, Mössbauer, and visible spectra of the undeca-peptide of ferricytochrome c (HPp) were measured for both the solid peptide and solutions of the peptide at pH 1.5, 7, and 10. At the low pH, the iron exists in the HPp in the high-spin species. At neutral and alkaline pHs, the low-spin ferric species predominate. The results of these measurements support the previous suggestions concerning the ligands in the fifth and sixth position of HPp at the neutral and alkaline pHs. The spectra of lyophilized preparations of HPp show the presence of both high and low-spin ferric species. The study of the Mössbauer spectra of the lyophilized preparations as function of temperature shows that there exists a spin-spin equilibrium between the two spin states. The relative amounts of the two components vary with the preparation. In these preparations there exist two components, one of which exists in the equilibrium mixture and the second in the high-spin form.

Introduction

Considerable amount of experimental data has been accumulated for hemoenzymes relating structures and physiological activities. Cytochrome c has been studied in great detail since it plays an important role in the cellular electron transport system. It differs from other hemoproteins in that the protein is covalently bonded through thiol ether bridges to the porphyrin ring. This permits the formation of small peptides in which the integrity of the peptide chain between the cysteine residues remains even after treatment with enzymes such as pepsin and trypsin. Such peptides have been studied as models for cytochrome c since they retain a similar chemical environment around the iron atom in the heme structure, but the molecular weight is much less than that of the parent protein, cytochrome c.

One peptide that has been extensively studied is the undeca-peptide (HPp), which is prepared by the peptic digestion of the parent protein¹. The amino acid sequence in HPp has been determined with

amino acid residues 11 to 21 of the cytochrome c retained².

HPp has been studied by different physiochemical methods to determine the nature of the bonding between the peptide moiety and the heme ring. These properties have been found to be strongly pH dependent indicating that the bonding may be altered in different pH environments. There are several groups in the amino acids of this peptide that could bind to the heme iron: these include the imidazole nitrogen of the histidine, the ϵ -amino group of the lysine, the amino group of the glutamine, the α -amino group of the terminal valine, and the carboxyl groups of the terminal glutamic acid. This present work was undertaken to gain additional information about the structure of lyophilized and dissolved HPp and the changes with pH using ESR, Mössbauer and visible spectroscopies. These spectra are sensitive to changes in the oxidation state, spin states, and configuration of the ligands around the iron atom in the heme ring.

Experimental

Preparation of HPp

The HPp was prepared by the peptic digestion of horse heart ferricytochrome c (Calbiochem) and purification by column chromatography following the procedure of Harbury and Loach³. The purity of the product was checked by an amino acid analysis using a Beckman Model 120C automatic amino acid analyzer and iron analyses of several samples

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* A preliminary report of the Mössbauer spectral studies was presented at the 3rd Symposium on Low Energy X- and Gamma Ray Sources and Applications, 1970 (L. May, R. Nassif, and M. Sellers, Applications of Low Energy X- and Gamma Rays [C. A. Ziegler, ed.], p. 257—263, Gordon and Breach, London 1971). The values of the parameters have been revised in this present paper since we have obtained improved spectra.



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using a spectrophotometric method⁴. The amino acids and amounts found were equal to those predicted by the formula. The iron contents of our samples were 2.2%, which agrees with published values ranging from 2.2 to 2.9%^{3, 5-7}. The pH of the solutions were adjusted with 1 N perchloric acid (pH 1.5) and 0.05 N sodium bicarbonate-sodium hydroxide buffer for pH 10. Lyophilized HPp was prepared from water solutions of the peptide.

Mössbauer studies

The Mössbauer spectrometer was of the constant acceleration type with moving absorber geometry⁸. A gas-flow proportional counter using 10% methane and 90% argon was used as the detector⁹. A cryostat described by Travis and Spijkerman¹⁰ was used for low temperature measurements and was modified for variable temperature operation¹¹. The source was 40 mCi ⁵⁷Co in a palladium matrix and was maintained at the same temperature as the absorber. The absorbers were mounted in aluminium foil cups. The absorption due to the iron in the aluminium was about 0.02%. The spectra were measured with lyophilized samples (approximately 50 mg) and frozen solutions (1 ml) in which the concentration of HPp was about 10 mM. The Mössbauer parameters were obtained by fitting the spectra to the best least-square Lorentzians subtracting out the parabolic background of the instrument using a computer curve fitting program¹². All values are reported relative to sodium nitroprusside. The total counts at each velocity for all spectra were at least 10⁶.

Electron spin resonance

The ESR was measured at liquid nitrogen temperature using the Varian Model V-4500 spectrometer equipped with 100 kc field modulation.

Visible spectrum

All visible spectra were measured using a Beckman model DB-G grating spectrophotometer. The spectrum of the solid was obtained as a Nujol mull¹³. The solution spectra were measured at a HPp concentration of 1 mM.

Results

Effects of pH

The ESR spectrum of the acid solution (Fig. 1) in which two signals near *g*-values of 5.8 and 2.0 are observed, is characteristic of high-spin ferrihemoproteins¹⁴. The visible spectrum (Fig. 2) reveals absorption maxima at 495 nm and 620 nm.

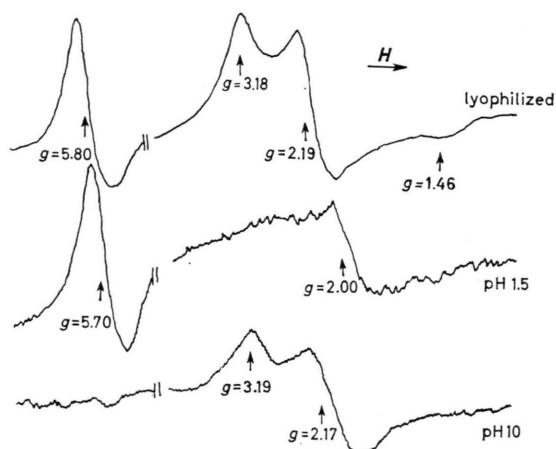


Fig. 1. ESR spectra of HPp at 77 °K.

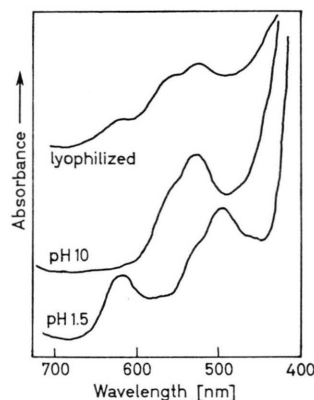


Fig. 2. Visible spectra of HPp.

The positions of these bands are characteristic of the spectra of high-spin ferrihemoproteins¹⁵. The Mössbauer spectrum of a frozen solution of HPp at pH of 1.5 is given in Fig. 3 and the parameters in Table I. The spectrum is typical of high-spin ferric complexes¹⁶. For these complexes, the ground elec-

Table I. Mössbauer parameters of HPp solutions at different pHs*.

pH	Outer lines		Inner lines	
	Quadru- pole splitting	Isomer shift	Quadru- pole splitting	Isomer shift
1.5	—	—	0.32	0.46
7 (Slurry)	2.04	0.40	0.35	0.44
10	2.15	0.43	0.40	0.40

* The error in quadrupole splitting is ± 0.03 and in isomer shift ± 0.01 . All values at 140 K are in mm/s relative to sodium nitroprusside.

tronic state is symmetric (6A_1), and no quadrupole splitting is expected. The small observed splitting (0.33 mm/s) is due to lattice effects. Paleus, Ehrenberg, and Tuppy⁷ studied dilute solutions of HPp by means of spectrophotometry, magnetometry, and titrimetry and found that the HPp exists in the high-spin ferric form at low pHs.

The Mössbauer spectrum of HPp at pH 7 was that of a slurry (Fig. 3, Table I) due to the low

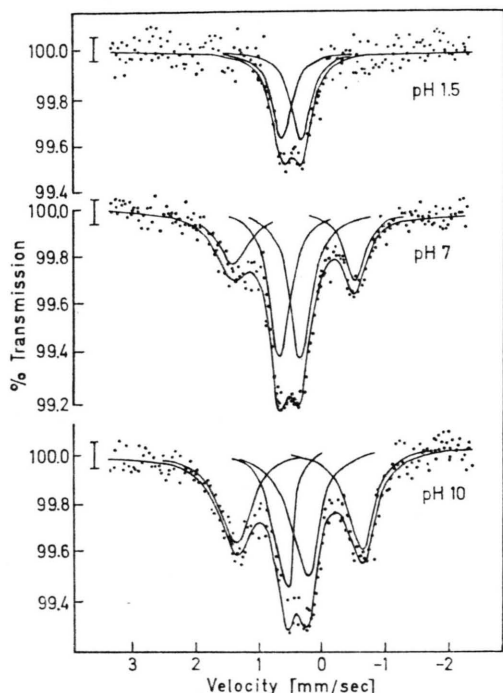


Fig. 3. Mössbauer spectra of HPp solutions at 140 °K. The outer lines at positive velocities are broadened as is often found with low-spin ferrihemoproteins.

solubility of the peptide at this pH. Four lines are present. The two center lines give parameters that are typical of high-spin ferric species. The parameters of the outer lines are similar to those of the ferricytochrome *c*^{17, 18}, which contains low-spin ferric ions. The presence of the two spin states was also reported by Paleus, Ehrenberg, and Tuppy⁷ for dilute solutions of HPp at this pH.

The ESR spectrum of HPp dissolved in a buffer solution at pH of 10 (Fig. 1) has signals near *g*-values expected for low-spin ferrihemoproteins¹⁴ (3.25, 2.10, 1.46). A weak signal appears at a *g*-value of 5.9 indicating the presence of high-spin ferric ion¹⁴. Four lines appear in the Mössbauer

spectrum of HPp dissolved in a buffer solution at pH 10 (Fig. 3). The parameters (Table I) of the outer lines are different from the parameters of the outer lines found in the spectrum of HPp at pH 7 and are consistent with the assignment to low-spin ferric iron¹⁶. The inner lines are characteristic of high-spin ferric iron indicating that both spin states are present at this high pH. The visible spectrum (Fig. 2) shows only bands that are assigned to the low-spin species at 530 nm with a shoulder near 560 nm¹⁵. The visible spectrum is not completely comparable with the ESR and Mössbauer spectra since at the concentrations needed to obtain the latter spectra, some precipitation occurred when the solutions were frozen at 77 °K. The ESR spectrum was measured with the same solution used for the Mössbauer measurements after it had been diluted 1:1 with the buffer. The iron in the precipitate is in a mixed spin state as was found with the lyophilized solid (see below). Paleus, Ehrenberg, and Tuppy⁷ report low-spin ferric iron present in dilute solutions of HPp in alkaline pH.

Effect of lyophilization

Signals in the ESR spectrum of lyophilized HPp (Fig. 1) are found at *g*-values of 5.8, 3.18, 2.19, and 1.46. The *g*-values at 5.8 is characteristic of the high-spin species and the remaining signals are characteristic of the low-spin species¹⁴. The visible spectrum (Fig. 3) of the lyophilized HPp confirm the presence of both spin states since absorption bands present in the spectrum are characteristic of both high-spin and low-spin ferrihemoproteins. The Mössbauer spectrum (Fig. 4) has four lines whose parameters are listed in Table II. The magnitudes of these parameters indicate that two spin states are present, high-spin (inner lines) and low-spin (outer lines) confirming the ESR measurements at liquid nitrogen temperature.

The Mössbauer spectra of the lyophilized HPp were measured at several different temperatures (Fig. 4). The quadrupole splittings of the outer lines were higher at lower temperatures (Table II) as would be expected for low-spin ferric iron. Measurements of the areas of both the outer and inner lines also indicated that the ratio of the area of the outer lines to the areas of all the four lines decreased with increasing temperature (Fig. 5). This suggests that there exists an equilibrium between the two spin states.

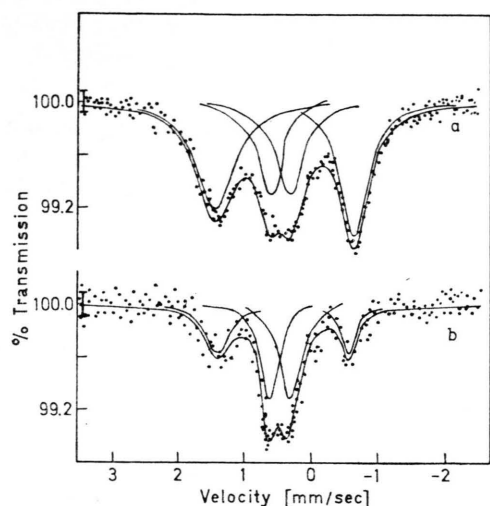


Fig. 4. Mössbauer spectra of lyophilized HPP: a. 160 °K; and b. room temperature. The outer lines at positive velocities are broadened as is often found with low-spin ferrihemoproteins.

Table II. Mössbauer parameters of lyophilized HPP at different temperatures*.

Temp. [°K]	Outer lines		Inner lines		% Low spin**
	Quadru- pole splitting (±0.02)	Isomer shift (±0.04)	Quadru- pole splitting (±0.02)	Isomer shift (±0.02)	
140	2.07	0.33	0.38	0.34	65
160	2.07	0.33	0.39	0.48	63
220	2.02	0.35	0.41	0.44	58
280	1.95	0.31	0.41	0.48	34

* All values are in mm/s relative to sodium nitroprusside.

** % Low spin =, (Area of outer lines ; areas of outer and inner lines) × 100.

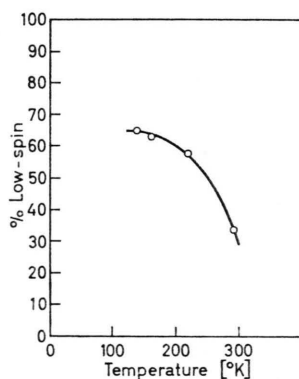


Fig. 5. Variation of the low-spin fraction in lyophilized HPP with temperature.

Discussion

Previous results have been obtained with solutions of HPP in which the concentration was much lower (about 0.1 mM) than the concentrations used in this present study (10 mM). The sedimentation studies of Ehrenberg and Theorell⁵ showed that the HPP is a monomer in acid pH and a monodisperse polymer at alkaline pHs. Using molecular models and assuming that the peptide chain is an α -helix, they concluded that the imidazole N is bound to the iron atom at position 5 and polymerization can take place when a primary amino group of another molecule coordinates in the sixth position (Fig. 6).

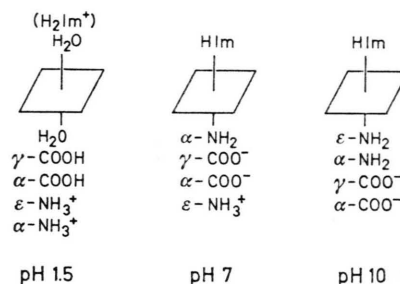


Fig. 6. Possible ligands in HPP at pHs of 1.5, 7 and 10. The plane represents the heme ring. The groups coordinated to the iron are shown directly over (Position 5) and under the plane (Position 6). The remaining functional groups of the peptide are given below.

The formation of intramolecular bonding at alkaline pHs was suggested by Margoliash, Frohwirt, and Wiener². That this is possible was shown with an atomic model requiring an unfolded peptide chain of four amino acid residues between the two presumed coordination groups. There was a position of the chain in which the imidazole group and the ϵ -amino group of the lysine residue next to the cysteine could coordinate with the central iron atom from opposite sides of the heme plane. The monomeric and polymeric forms of the HPP were examined using optical rotatory dispersion and circular dichroism¹⁹ and a head-to tail alignment was suggested for the polymer. Ligands such as lysine, imidazole, histidine and ammonia disperse the HPP aggregate³. In view of the ligand effects and the non-aggregation properties of the heme octapeptide¹⁹ in which the lysine group has been removed, the ϵ -amino group of the lysine was assumed to effect the polymerization. The titrimetric studies are not very conclusive and identification of the groups involved in the ionization processes cannot be made

on the basis of the observed pK values alone⁶. These authors suggested that in the monomeric form at low pH only one of the coordination positions is occupied by a nitrogenous base and the other ligand is water or the carboxyl group of the glutamic acid residue, whereas in the polymeric form, both ligands are nitrogenous bases (Fig. 6). This would be consistent with the observations that at low pHs (monomeric form), the iron is high-spin ferric in a weak crystal field with two oxygen ligands. The polymeric form with the two nitrogenous ligands would have a stronger crystal field than at the lower pHs and the iron would be in the low-spin ferric form as is observed with ESR, magnetic, Mössbauer, and optical measurements.

These measurements indicate that at low pHs, the monomeric form is present and as the pH is raised, mixed spin states occur with the presence of the polymeric HPP. The quadrupole splittings of the low-spin forms are different at pH 7 and 10 (Table I). This suggests that there are different ligands at the different pHs. The different possible ligands are shown in Fig. 6, where the scheme is similar to the working hypothesis presented by Harbury and Loach⁶. At pH 1.5, water could be the ligand in both the fifth and sixth positions since the carboxylate groups are protonated at this pH. The weaker crystal field due to the water molecules compared to that with nitrogen ligands would account for the high-spin configuration at low pH and low quadrupole splitting (0.33 mm/s) while at higher pHs, the nitrogen ligands would produce stronger crystal fields and low-spin configuration. The polymeric forms at pH 7 and 10 involve different ligands at the sixth position with the imidazole nitrogen (pK = 6) complexed at the fifth position. The greater quadrupole splitting at pH 10 suggests that the strength of the ligand in the sixth position at pH 10 is greater than that of the ligand at pH 7. This is consistent with the conclusion of Harbury and Loach⁶ that the ligand at pH 10 is the ϵ -amino group and the ligand at pH 7 is the α -amino group.

In the lyophilized HPP, two-spin states are found. Morton and Bohan²⁰ found evidence for two spin states in the ESR spectrum of lyophilized ferricytochrome c. The g -value of the high-spin component was at 6.05, which is higher than the g -value found with HPP. The g -values of the low-spin component are also different in the parent protein. No evidence

of two spin states has been found from Mössbauer measurements of lyophilized preparations of the protein^{17, 18}.

The variation of the Mössbauer spectrum with temperature shows that there is a change in the relative amounts of the low and high-spin ferric species with temperature (Table II, Figs 4 and 5). The Mössbauer measurements provide a means for making a direct determination of the relative relative amounts of the spin states as a function of temperature. For example, Lang, Asakura, and Yonetani²¹ observed the coexistence of the two spin states in cytochrome c peroxidase in the Mössbauer spectra at 4.6 and 195 °K. Similar behavior has been observed in inorganic compounds both ferric²² and ferrous²³. It was found that minor changes in the ligand substitution could produce complexes that are fully high-spin, mixed or fully low-spin.

Such a change in spin states is directly manifested by the observed values of effective magnetic moment associated with the ferric ion in a series of related hemoproteins. Depending upon the nature of the sixth ligand a spectrum of effective magnetic moments have been measured, ranging from the five electron spin-only value of 5.92 to the single electron value of 1.72²⁴. Similarly, if the ligands are kept fixed and the nature of the heme ring is varied only slightly, a change in the spin state observed¹⁵. In these hemoproteins, the crystal field strength must be close to the ${}^6A_1 \longleftrightarrow {}^2T_2$ crossover region because small changes are enough to make the ground state a sextet or a doublet²⁵.

Fig. 5 gives the relative population of the two spin states at different temperatures from the measurement of the line areas (Table II). Extrapolation of the curve to low temperatures indicates that a 100% low-spin configuration is not attainable at any temperature. This suggests that there are at least two different species present in the solid HPP, component I—existing entirely in the high-spin configuration and component II—a thermal mixture of low- and high-spin species. Similar results were obtained with MtMb·OCN complex²⁶. The magnetic data for this complex were interpreted assuming that two compounds were present, one in the low-spin state and the other one in a thermal mixture.

Different preparations of HPP made during this research gave varying ratios of low-spin to high-spin ferric iron but the same Mössbauer parameters.

These ratios ranged from 50 to 70%. This indicated that during various preparations different amounts of component I and component II are obtained. For that reason, samples obtained from one preparation were used for all the measurements.

The transition ${}^6A_1 \longleftrightarrow {}^2T_2$ involves the transfer of electrons from t_{2g} to e_g orbitals and *vice versa*, which should be followed by a change in the ionic radius with a modification of the molecular structure. Examination of crystals undergoing spin transitions²³ show that the molecules adopt different crystal structures. The existence of different structures for the molecule in the different electronic states would account for the observation of separate Mössbauer transitions for the two states. The slow relaxation time between the electronic states is probably due to the potential barrier between the two molecular forms, leading to slow interconversion. This mechanism is supported by the evidence of some hysteresis in the spin-spin equilibrium. After a series of runs had been completed from 120 to 300 °K with one sample, small changes in the relative population were observed upon returning to low temperatures with the same sample of HPP. Similar behavior has been observed in the Mössbauer spectra of methaemoglobin derivatives²⁷

Both similarities and dissimilarities have been found between the properties of HPP and its parent

protein, cytochrome c. HPP in solution exhibits the same change in the spin state of the ferric form as does the ferricytochrome c with pH as shown from magnetic susceptibility measurements for cytochrome c²⁸. The parameters of the Mössbauer spectrum of the protein¹⁸ at neutral pH is very similar to that of low-spin HPP. Upon drying, the quadrupole splitting of cytochrome c decreases to a greater extent than the splitting of the low-spin form of HPP. The presence of water appears to affect the electric field gradient to a greater extent in the protein than in the peptide by its hydration of the polypeptide chain complexed in the sixth position. The drying of HPP involved the formation of a high-spin component which was observed in dried preparations of the protein by ESR measurements²⁰. The differences in the binding to the iron between the protein and its peptide illustrates why lower molecular weight peptides are not necessarily simplified models for the protein.

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