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Mössbauer spectra of the heme peptide (HP) 1–50 and the heme peptide:non-heme peptide (NHP) non-covalent complex 1–50:51–104 derived from cytochrome *c*: evidence for cytochrome *c* iron site solvation in aqueous solution

Paul A. Adams*, Raymond C. de L. Milton† & Jack Silver‡

**MRC Biomembrane Research Unit and †MRC Regulatory Peptides Research Unit, Department of Chemical Pathology, University of Cape Town Medical School, Observatory, Republic of South Africa and ‡Department of Chemistry and Biological Chemistry, University of Essex, Colchester, Essex, UK*

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Mössbauer spectroscopic studies on a heme peptide (HP) derived from cytochrome *c* and on the HP recombined non-covalently with the remaining cleaved section are reported. The results suggest that the environment of the heme site in the known crystal structure of cytochrome *c* may differ in detail from the environment of the heme in the working protein.

Keywords: cytochrome *c*, heme peptides, Mössbauer spectroscopy

Introduction

The heme peptide (HP) fragments obtained by 'chemical' or enzymatic cleavage of the heme protein cytochrome *c* (cyt-*c*) comprise an extensive and useful suite of compounds with which to model redox kinetic and O_2 transport properties of heme proteins/enzymes (Adams *et al.* 1994). In addition to the heme peptide fragments described above, an extensive series of non-covalent highly stable complexes are formed between HP and non-HP (NHP) fragments resulting from the cyt-*c* cleavage. These HP:NHP complexes have been utilized extensively in structure–function studies relating to cyt-*c* and, in particular, to investigate the relationship between biological activity and integrity of the 'bottom loop' (residues 36–59) of ctc-*c* (Stellwagen 1978, Proudfoot *et al.* 1986, Proudfoot & Wallace 1987, Wallace & Proudfoot 1987). In solution at pH 6 HP:NHP complexes exhibit the 695 nm absorption characteristic of an intact cyt-*c* heme cleft (i.e. the sixth ligand of the iron is Met-80); however, the pK_a for the 'alkaline transition' (characterized by loss of the 695 nm band), which is indicative of ligand exchange at the sixth position, is lower in the non-covalent complexes as is the redox potential, this being in part ascribed to varying degrees of solvent accessibility to the heme redox center (Wallace & Proudfoot 1987).

HP1–50 and NHP51–104 are the peptides which result from acid catalyzed cleavage of the peptide bond between residues 50 and 51 of the cyt-*c* primary structure. As part of a continuing program utilizing HPs as chemical models for heme protein functionality (Peterson *et al.* 1980, 1983, Adams 1990) we report here a preliminary Mössbauer study comparing the HP1–50 and the HP1–50:NHP51–104 non-covalent complex.

Materials and methods

The HP:NHP complex was prepared by acid hydrolysis of horse heart cyt-*c* (Sigma, St Louis, MO) type III and purified using the chromatographic procedure of Wallace & Proudfoot (1987). The HP1–50 fragment was prepared from the complex by preparative reverse phase HPLC as described previously for the short HPs MP-8 and MP-11 (Adams *et al.* 1989). Mössbauer spectra were obtained at 78 K using a Harwell spectrometer (Waveform, generator MWG 200, servoamplifier MSA 200, proportional counter

Address for correspondence: J. Silver, Department of Chemistry and Biological Chemistry, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, UK.

MPC 200, vibrator MV 200) and Canberra (multichannel analyzer series 30, HV power supply 3105 Amplifier 2012, pre amplifier 200 BE). The source was ^{57}Co (25 mCi) in rhodium (Radiochemical Centre, Amersham, UK). The spectrometer was operated in a 'saw tooth' mode and calibrated with a 25 μM thick natural iron reference absorber. All isomer shifts are referred to this as zero shift. Samples were placed in 2 mm lead holders (radius of hole 0.25 cm), quench frozen to liquid N_2 temperature and transferred to an E.S. Technology cryostat. All spectra were computer analyzed using a simple non-linear least-squares fitting program.

Results and discussion

Mössbauer spectra obtained at 80 K on lyophilized samples of HP1–50 and HP:NHP are presented in Figure 1(a and b) and Mössbauer parameters are shown in Table 1 along with relevant literature values. The spectrum (Figure 1a and footnote to Table 1) has been fitted according to a two-site model with both low- and high-spin Fe(III) species. Mössbauer parameters for the high-spin Fe(III) site are not complete nor is it possible to evaluate complete parameters from the data. This is because such sites have Mössbauer spectra at 80 K that are typified by a low velocity line (in our case the line we 'fitted') that is resolved and a broad high velocity line that in our case is somewhere under the spectrum envelope (accordingly, no attempt has been made to fit it). Such a spectrum has been reported for octaethylporphyrin iron chloride and indeed at a 91.2 K temperature the high velocity line becomes so broad and diffuse that it almost 'disappears' into the background of the spectrum and the resulting spectrum takes on the appearance of a single line (Fitzsimmons *et al.* 1976, Sams & Tsin 1978). However, such a site is clearly present (Figure 1a) (a similar site being previously found for HP11–21) (Peterson *et al.*, 1980) and must represent either a five-coordinate or six-coordinate Fe(III) site.

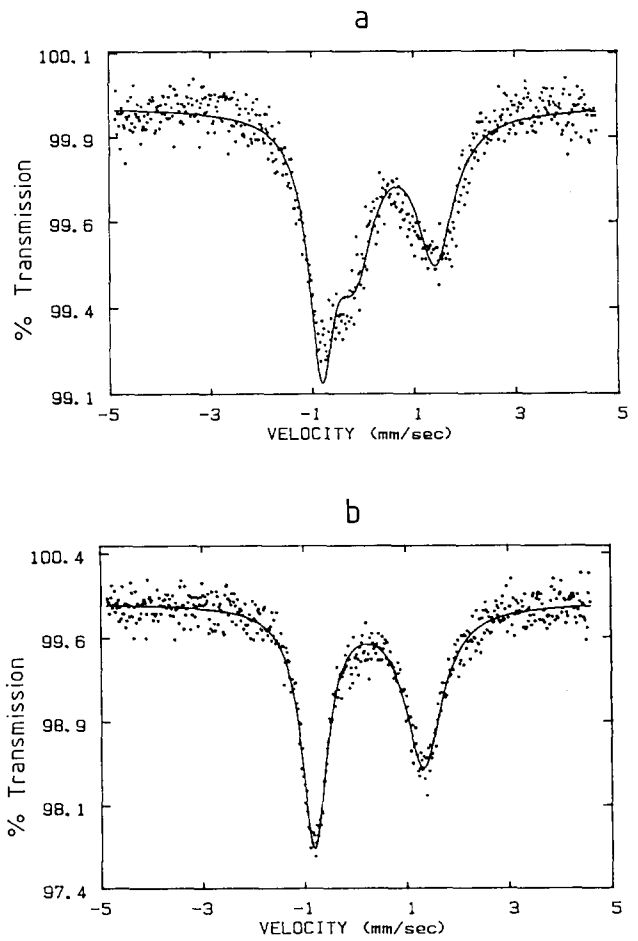

Figure 1. Mössbauer spectra at 80 K of lyophilized samples of (**a**) HP1–50 and (**b**) HP1–50:NHP51–104 non-covalent complex.

In the case of HP1–50 such an iron site would come about if in the lyophilized sample the exposed heme iron either did not acquire a sixth ligand or it was a six-coordinate complex having gained its sixth ligand from the small molecules in the solution or from intramolecular bonding to other HP1–50 molecules during lyophilization, i.e. aggregation. Six-coordinate heme Fe(III) high-spin sites result only if the sixth ligand is early in the spectrochemical series (Miller *et al.* 1987), in this case water or carboxylate (if originating from other HP1–50 molecules). If the ligand was picked up from solution, it would be a OH^-, H_2O or CF_3COO^- (however, preparative considerations where reverse-phase HPLC is carried out at $pH_{opt} \sim 2.5$ in 0.1% CF_3COO^- would argue against OH^-).

If the sixth ligand were a nitrogenous one, then it would give rise to a low-spin Fe(III). Such low-spin sites have been found by one of us previously (Peterson *et al.* 1980, 1983) for HP11–21 and HP14–22, and were attributed to intra- and inter-molecular nitrogen ligands bonding, respectively. Clearly, the presence of a low-spin site in the spectrum in Figure 1(a) represents such an iron coordination. This low-spin Fe(III) site of HP1–50 has an isomer shift of 0.29(2) mm s^{-1} and ΔE_Q of 2.25(2) mm s^{-1}, which is larger than previously reported for other HPs (Peterson *et al.* 1980, 1983) and accordingly must reflect inter- or intramolecular coordination of an aliphatic nitrogen of the peptide to the sixth position. However, it is noted that

Table 1. Mössbauer parameters of cyt-*c* and HPs of cyt-*c*

Material	T (K)	δ ($mm\,s^{-1}$)	ΔE_Q ($mm\,s^{-1}$)	r[a] ($mm\,s^{-1}$)	Reference
Ferricytochrome-*c*	300	0.15(3)	1.87(5)	NG[b]	Fitzsimmons *et al.* (1976)
(lyophilized)	195	0.20(3)	1.94(5)	NG[b]	Fitzsimmons *et al.* (1976)
Ferricytochrome-*c* (solution)	195	0.21(3)	2.14(5)	NG[b]	Fitzsimmons *et al.* (1976)
Ferricytochrome-*c*	200	NG[b]	1.88(2)[c]	NG[b]	Sams & Tsin (1978)
(lyophilized)	100	0.18(2)	1.90(2)[c]	NG[b]	Sams & Tsin (1978)
Ferricytochrome-*c*	200	NG[b]	2.25(2)	NG[b]	Sams & Tsin (1978)
(solution)	100	0.18(2)	2.26(2)	NG[b]	Sams & Tsin (1978)
HP 11–21 (lyophilized)	200	0.20(1)	1.97	0.23(2) 0.24(2)	Peterson *et al.* (1980)
		0.15(7)[d]	0.0	0.35(15)	
	80	0.27(1)	2.07(2)	0.34(2) 0.54(3)	Peterson *et al.* (1980)
		0.11(6)[d]	0.0	0.35(8)	
HP14–22 (lyophilized)	230	0.19(1)[e]	2.09(2)	0.22(2) 0.22(2)	Peterson *et al.* (1980)
	80	0.26(2)[e]	2.08(2)	0.39(3) 0.47(1)	Peterson *et al.* (1983)
HP14–22 (solution pH 5.9)	80	0.23(3)[e]	2.21(6)	0.25(5) 0.25(5)	Peterson *et al.* (1983)
HP14–22 (solution pH 8.5)	80	0.22(2)[e]	2.06(4)	0.26(4) 0.26(4)	Peterson *et al.* (1983)
HP1–50 (lyophilized)	80	0.24(2)[f]	2.25(2)	0.30(2) 0.45(3)	This work
		−0.17(2)[g]	0.0	0.48	This work
HP:NHP (lyophilized)	80	0.24(2)	2.14(2)	0.30(1) 0.44(2)	This work

[a]Half width at half height. [b]Not given in reference. [c]Recalculated from curves shown in Peterson *et al.* (1980). [d]Minor iron(III) component. [e]Relevant site only reported from Peterson *et al.* (1983). [f]This fit (Figure 1a) to the data was constrained so the peak areas of the two parts of this douplet were equal. Each was 33% of the total spectum absorption. [g]This parameter is one part of a high spin Fe(III) spectrum. The weaker positive line was not fitted. This parameter should only be taken as evidence for the presence of a high spin Fe(III) site. This site accounts for approximately 33% of the total spectrum absorption.

although HP1–50 appears monomeric at concentrations up 5×10^{-6} mol dm^{-3} in aqueous solution at pH 7 (Adams, unpublished studies), and no evidence for low-spin Fe(III) is found in solution, the sixth ligand must bind (to an extent of no greater than 66% in our sample) as the concentration increases on lyophilization. This result indicates some aggregation on lyophilization. The 66% is based on the absorption area of this site in the Mössbauer spectrum. This in fact will be an overestimate as the high-spin Fe(III) site is not fully fitted and indeed may have a different Mössbauer absorption 'f' factor.

An alternative suggestion is that the Mössbauer spectrum of HP1–50 could be equally well fitted by two low-spin Fe(III) sites both with δ values around 0.25 $mm\,s^{-1}$, but one having a quadrupole splitting ~ 1 $mm\,s^{-1}$, the other around the value we record here. Such a low-spin Fe(III) site with a small quadrupole splitting of ~ 1 $mm\,s^{-1}$ is rare and to our knowledge has only been reported for [Fe TPP$(Py)_2$]Cl (Epstein *et al.* 1967). In the light of the fact that (i) no evidence for a strong sixth ligand is found for HP1–50 in dilute aqueous solution and (ii) there are few possible aromatic ligands available in the system to act as sixth ligand (such ligands would be required to bring about a quadrupole splitting of ~ 1 $mm\,s^{-1}$) we believe that the chemical evidence is not on the side of this suggestion.

Since the Mössbauer spectrum of HP:NHP (Figure 1b), which is a non-covalent complex of HP1–50 and NHP51–104, contains only one kind of iron site, and as there is clearly more than one site in HP1–50, these must rearrange into a single type of low-spin Fe(III) site on formation of the complex. The most likely explanation is that this asymmetric pattern of the Mössbauer spectrum of the HP:NHP complex represents an envelope of slightly different spectra. These individual spectra each originate from structurally similar six-coordinate low spin Fe(III) heme sites. The average quadrupole splitting of these sites is smaller than that of the similar site in Figure 1(a). The value of ΔE_Q (2.14 $mm\,s^{-1}$) found here for the complex is in the range of those found previously for lyophilized HPs (Peterson *et al.* 1980, 1983). However, it is significantly larger than that

found for lyophilized ferricytochrome-*c* from both 'food yeast' (Lang *et al.* 1968) and horse heart (Cooke & Debrunner 1968). It is accepted that the sixth ligand of the iron in the low pH form of the HP:NHP complex is Met-80 and that the complex essentially duplicates the conformation of the native protein (Wallace & Proudfoot 1987).

The value of ΔE_Q for the HP:NHP complex is in fact virtually identical to that found for ferricytochrome-*c* in (frozen) solution; the apparently larger δ found here (cf. Table 1) compared with the solution ferricytochrome-*c* values being readily explained in terms of the expected temperature dependence of the second-order doppler shift (Peterson *et al.* 1980). Clearly, ferricytochrome-*c* in solution possesses a slightly different iron environment to that found in the lyophilized state; furthermore, the iron environment in the former is (electronically) virtually identical to that found in the lyophilized HP:NHP complex. It must be pointed out that the major factor contributing to the redox 'inactivity' (relatively to cyt-*c*) of the HP:NHP complex is the solvation of the heme edge in the latter (Stellwagen 1978, Proudfoot *et al.* 1986). Solution kinetic studies using the HP:NHP complex indicate a considerable degree of solvent penetration (via a channel opened on scission of the 50–51 peptide bond) to the heme distal face, resulting in a relatively polar heme environment. This suggests that the differences observed in the Mössbauer spectra of lyophilized and 'solution' cyt-*c* result from a restricted degree of solvent penetration to the distal face of the porphyrin macrocycle in the region of the iron atom in aqueous solution, this is removed on lyophilization but retained in frozen solution. In the HP:NHP the solvent penetration is more extensive and some solvent molecules are likely to be retained in the heme cleft on lyophilization. These observations further suggest that the three-dimensional structure of the HP:NHP complex may more closely reflect the detailed environment of the iron in the solution state form of cyt-*c* than does the crystal structure of cyt-*c* itself.

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