Structural studies of eukaryotic cytochrome *c* modified at methionine-65

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(Received 1 July 1980/Accepted 8 September 1980)

¹H n.m.r. spectra were recorded in both oxidation states for the following species: tuna cytochrome *c*, tuna [carboxymethylmethionine-65]cytochrome *c*, horse cytochrome *c* and horse [homoserine-65]cytochrome *c*. The experiments give the assignments of the singlet methyl resonances of methionine-65 and the *N*-terminal acetyl group. The modification at methionine-65 is shown to cause an extremely small structural perturbation to one part of the molecule close to the site of modification.

In order to define its structure eukaryotic cytochrome *c* has been studied by many different techniques (see Harbury & Marks, 1973; Lemberg & Barrett, 1973; Dickerson & Timkovich, 1975), including high-resolution n.m.r. spectroscopy (Redfield & Gupta, 1971; McDonald & Phillips, 1973; Dobson *et al.*, 1975; Keller & Wüthrich, 1978; Moore & Williams, 1980*c,d*). One of the problems with the application of n.m.r. to the study of complex molecules is the assignment of resonances to specific nuclei. Various n.m.r. techniques provide assignments to types of nuclei, and specific assignments can sometimes be made by using these and certain structural data, such as the amino acid sequence and X-ray crystallographic structure. Often, however, an assignment cannot be made with complete confidence by using only these methods. One way of assisting in the task of resonance assignment is to compare spectra of specifically modified proteins with the spectra of their native forms. In the present paper we report the results of an n.m.r. study of eukaryotic cytochrome *c* specifically modified at Met-65.

As well as yielding precise assignments for a number of resonances, these studies allow structural perturbations caused by modification of Met-65 to be defined. This is of particular importance, since many semi-synthetic derivatives of cytochrome *c* used to define regions of the protein that are essential for biological activity are also modified at position 65 (Ando *et al.*, 1966*a,b*; Stellwagen, 1968; Corradin & Harbury, 1974; Boon *et al.*, 1979*a,b*; Wallace & Offord, 1979; Nix & Warne, 1979),

and previous work does not define which regions of the tertiary structure of the protein are affected by the alterations in the side chain of residue 65. In addition, it is of interest to ascertain to what degree changes of single amino acid residues can affect the structure and properties of proteins. Modification of Met-65 is particularly appropriate for such a study, since we are able to vary the size and charge of the modified group. The proteins studied are: native cytochrome *c* (horse and tuna); [Hse⁶⁵]cytochrome *c* ([homoserine-65]cytochrome *c*) (horse); [Met(Cm)⁶⁵]cytochrome *c* ([carboxymethylmethionine-65]cytochrome *c*) (tuna). The modifications are shown below to cause only small structural perturbations.

Materials and methods

Cytochromes *c* of the following species were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) (the type numbers given are those used by the supplier): horse (type VI), cow (type V), dog (type XVIII), rabbit (type XV), pigeon (type XIV), chicken (type X) and tuna (type XI). Donkey cytochrome *c* was obtained from Miles Laboratories (Slough, Berks., U.K.). DL-*N*-Acetylmethionine, iodoacetic acid, ascorbic acid and K₃[Fe(CN)₆] were purchased from BDH Chemicals (Poole, Dorset, U.K.). Oxygen-free argon was obtained from Air Products (Bracknell, Berks., U.K.), ²H₂O (99.8%) was from Merck, Sharp and Dohme (Montreal, Que., Canada, NaO²H (40% in ²H₂O, isotopic purity 99.0%) and ²HCl (35% in ²H₂O, isotopic purity 99.6%) were from CIBA (Duxford, Cambridge, U.K.), and CNBr was from Kodak

Abbreviations used: Cm, carboxymethyl; Hse, homoserine.

(Liverpool, U.K.). $K_3[Cr(CN)_6]$ was obtained from Alfa Inorganics (Karlsruhe, W. Germany) and recrystallized from water before use. Tris(biguanide) chromium(III) chloride, $[Cr(C_2N_5H_7)_3]Cl_3$, was prepared by the method of Ray (1960).

The native cytochromes *c* were purified, after reduction by ascorbic acid at pH 6 or oxidation with $K_3[Fe(CN)_6]$ at pH 6, by passage through a Sephadex G-25 column (1.5 cm $\times$ 30 cm) and dialysis against three changes (approx. 1000 ml) of double-distilled water. The proteins were then freeze-dried. N.m.r. samples were obtained by dissolution of the required amount of freeze-dried protein in 0.4 ml of 2H_2O and the pH was adjusted with 2HCl and NaO^2H . The pH values quoted in the paper are uncorrected for the isotope effect (Glascoe & Long, 1960). The final protein concentration was usually 5 mM. Samples of reduced cytochrome *c* were transferred to n.m.r. tubes that had been previously flushed with argon, and the tubes were flushed again and then sealed.

Tuna $[Met(Cm)^{65}]$ ferrocytochrome *c* (50 mg) was prepared from tuna ferrocytochrome *c* by an adaptation of the method of Ando *et al.* (1966a,b) for bovine cytochrome *c*. To ensure that Met-80 remained unmodified, the reaction with iodoacetic acid was performed at pH 5.6 (Ando *et al.*, 1966a,b). The modified protein was purified at 4°C by passage down a column (1.5 cm $\times$ 30 cm) of Sephadex G-25 equilibrated with 0.1 M-NaCl, followed by dialysis against three changes (approx. 1000 ml) of double-distilled water (final estimated purity > 95%). The oxidized form of the modified protein was obtained by treating the reduced protein with a slight excess of $K_3[Fe(CN)_6]$, followed by dialysis against three changes (approx. 1000 ml) of double-distilled water. The solution was freeze-dried and n.m.r. samples were prepared as described for native cytochrome *c*. The carboxymethyl derivative of *N*-acetylmethionine was prepared by the same procedure.

Horse $[Hse^{65}]$ cytochrome *c* was prepared in accordance with published procedures (Boon *et al.*, 1979a; Wallace & Offord, 1979). Ferricytochrome *c* (approx. 200 mg) was treated with a 3-fold excess of CNBr in accordance with the method of Corradin & Harbury (1974), and the mixture of fragments was filtered on a column (2 cm $\times$ 135 cm) of Sephadex G-50 (fine grade) in 7% (v/v) formic acid. The pure preparations of fragments 1–65 and 66–104 were freeze-dried, and the fragments were redissolved in 0.1 M-potassium phosphate buffer, pH 7.0, such that the resulting solution was 0.1 mM in each. The mixture was reduced and maintained in the reduced state for 24 h by the procedure described by Wallace & Offord (1979), and the solution was then filtered on a Sephadex G-50 (fine grade) column. The first peak to emerge was

resynthesized $[Hse^{65}]$ ferrocytochrome *c*. After being freeze-dried the protein was redissolved in 0.1 M-potassium phosphate buffer, pH 7.0, made 8 M in urea, in accordance with the procedure of Boon *et al.* (1979a), and filtered through a Sephadex G-25 column (1.5 cm $\times$ 30 cm) in 0.1 M-potassium phosphate buffer, pH 7.0. The haem-containing peak was loaded on to a CM-cellulose 23 column (1.5 cm $\times$ 30 cm) and was eluted as a single peak with a 0.1–0.2 M-potassium phosphate gradient at pH 7.0. The resulting solution contained pure $[Hse^{65}]$ cytochrome *c* (final estimated purity > 95%). $K_3[Fe(CN)_6]$ was added to ensure complete oxidation, or ascorbic acid was added to ensure complete reduction. Samples for n.m.r. were prepared by using an Amicon ultrafiltration cell fitted with a UM10 membrane. Four cycles of concentration and dilution with 2H_2O at pH 7.0 were conducted. The final volume of the sample was 0.4 ml and the protein concentration was 5 mM.

The proton n.m.r. spectra were obtained with a 270 MHz spectrometer operating in the Fourier-transform mode. Convolution difference spectra (Campbell *et al.*, 1973) and Carr–Purcell spectra (Campbell *et al.*, 1975) were obtained as previously described. 1,4-Dioxan was used as an internal standard, but all chemical shifts are quoted in parts per million (p.p.m.) downfield from the methyl resonance of 4,4-dimethyl-4-silapentane-1-sulphonate.

Results

Native cytochrome *c*

The aliphatic regions of the convolution difference spectra of horse ferrocytochrome *c*, tuna ferrocytochrome *c*, horse ferricytochrome *c* and tuna ferricytochrome *c*, all in 2H_2O at pH 5.25 and 57°C, are given in Fig. 1.

The resonances of major concern in the present work are the three-proton-intensity singlet resonances in the region 1.8 p.p.m. to 2.2 p.p.m., which arise from methyl groups, and which are designated M10, M11, M*19, M*20, TM8, TM9, TM*16 and TM*17 (Fig. 1). [The resonance nomenclature scheme is used to simplify the discussion and to preserve continuity with previous reports. It is fully described by Moore & Williams (1980a,b,e).]

The addition of small portions of the paramagnetic complex tris(biguanide) chromium(III) chloride to solutions of horse cytochrome *c* produced broadening of resonances M10 and M*20 at high cytochrome *c*/Cr(III) ratios. The result of an experiment at a ferricytochrome *c*/Cr(III) ratio of 40:1 is given in Fig. 2. Apart from the singlet methyl resonance, M*20, only a doublet methyl resonance at 1.2 p.p.m. and complex multiplet resonance at approx. 2.4 p.p.m. and approx. 3.0 p.p.m. were perturbed. No

Fig. 1. Aliphatic region of the convolution difference spectra of horse ferrocyanochrome *c* (a), tuna ferrocyanochrome *c* (b), horse ferricytochrome *c* (c) and tuna ferricytochrome *c* (d) at pH 5.25 at 57°C

For details see the text. Methyl resonances of Met-65 are marked in accordance with the resonance nomenclature scheme (see the text).

other resonances in the spectral region 12 p.p.m. to -2 p.p.m. were affected by the addition of tris(biguanide) chromium(III) chloride up to ferricytochrome *c*/Cr(III) ratios of 20:1. The analogous experiment with horse ferrocyanochrome *c* produced similar results; only M10, a doublet methyl resonance at 1.15 p.p.m., and complex multiplet resonances at approx. 2.3 p.p.m. and approx. 3.0 p.p.m. were perturbed. A similar set of experiments with $K_3[Cr(CN)_6]$ resulted in the broadening of resonances M*19 and M11, along with a number of other resonances. In these experiments resonances M*20 and M10 were not perturbed by the addition of $K_3[Cr(CN)_6]$ (A. P. Boswell, G. R. Moore & R. J. P. Williams, unpublished work).

Before turning to a description of the structural perturbations accompanying modification of Met-65 we shall assign the singlet methyl resonances of cytochrome *c*. A total of seven such resonances are expected for horse and tuna cytochromes *c*: four from the haem group, and one each from Met-80, Met-65 and the *N*-acetyl group (Dayhoff, 1968; McDonald & Phillips, 1969). The methyl resonances

of the haem group (Redfield & Gupta, 1971; Keller & Wüthrich, 1978) and Met-80 (Redfield & Gupta, 1971) have been firmly assigned, and therefore the two singlet methyl resonances between 1.8 p.p.m. and 2.2 p.p.m. must come from Met-65 and the *N*-acetyl group. Assignment of these resonances is indicated from the effects of binding the paramagnetic probes tris(biguanide) chromium(III) chloride and $K_3[Cr(CN)_6]$. The data permit the cross-assignment of these resonances as follows: resonance M11 corresponding to resonance M*19, and resonance M10 corresponding to resonance M*20. This conclusion was confirmed by obtaining spectra of mixtures of ferricytochrome *c* and ferrocyanochrome *c*, where relatively rapid electron exchange occurred (A. P. Boswell, G. R. Moore & R. J. P. Williams, unpublished work). Specific assignment of the methyl resonances is indicated by the paramagnetic-probe studies to be: resonances M11 and M*19 to Met-65, and resonances M10 and M*20 to the *N*-acetyl group. This tentative assignment scheme is based on the observations that Met-65 serves as a binding site

Fig. 2. Aliphatic region of the convolution difference spectra of 5 mM horse ferricytochrome *c* (a) and 5 mM horse ferricytochrome *c* and 0.125 mM-tris(biguanide) chromium(III) chloride at pH 5.25 at 37°C (b), and also the difference spectrum [(a)-(b)] (c)

For details see the text.

for $[\text{PtCl}_4]^{2-}$ in crystals of horse cytochrome *c* (Dickerson *et al.*, 1971) and that the *N*-acetyl group is in a particularly acidic region on the surface of the protein close to the carboxy groups of Asp-2, Glu-4 and Asp-93 (Dickerson *et al.*, 1971; Swanson *et al.*, 1977; Takano *et al.*, 1977); there are both carboxy groups and lysine residues close to Met-65. The broadening effect caused by the paramagnetic complexes is due to dipolar relaxation of neighbour-

ing nuclei after formation of a complex of the paramagnetic ions with the protein (Dwek, 1973). The formation of the complex is based on electrostatic interactions. The data reported in the next section for $[\text{Hse}^{65}]$ cytochrome *c* confirm these assignments.

The chemical-shift values of the corresponding resonances of the cytochromes *c* from the animals donkey, cow, dog, rabbit, chicken and pigeon are virtually the same as those of horse and tuna cytochromes *c*. These data are summarized in Table 1.

Horse $[\text{Hse}^{65}]$ cytochrome *c*

The convolution difference spectra of horse ferrocytochrome *c* and horse $[\text{Hse}^{65}]$ ferrocytochrome *c*, both in $^2\text{H}_2\text{O}$ at pH 7.0 and 57°C, are presented in Fig. 3. The spectrum of the modified protein is very similar to that of the native protein: the few differences that have been observed are given below. The similarity between the two spectra indicates that the modification has not seriously perturbed the structure of the protein.

The resonances of major interest are the singlet methyl resonances M10 and M11, which were discussed in the preceding section. There is not an analogous resonance to M11 in the spectrum of $[\text{Hse}^{65}]$ ferrocytochrome *c*, whereas resonance M10 is unaffected by the modification; the disappearance of M11 confirms the assignment of this resonance to Met-65. Similarly, in the spectrum of $[\text{Hse}^{65}]$ -ferricytochrome *c*, resonance M*20 is unaffected by the modification, but resonance M*19 is abolished by it. Thus M*19 is the corresponding resonance to M11.

A number of resonances of ferrocytochrome *c* shifted as a consequence of the modification (Fig. 3), but the shifts were relatively small (Table 2). $[\text{Hse}^{65}]$ ferricytochrome *c* was not studied in the same detail as was the reduced protein. However, it was notable that the co-ordination centre was not disrupted by the modification, since the haem methyl resonances and Met-80 resonances were not significantly perturbed.

Tuna $[\text{Met}(\text{Cm})^{65}]$ cytochrome *c*

N-Acetylmethionine and its carboxymethyl derivative were used to obtain the primary chemical-shift position (the chemical-shift value of the resonances unperturbed by effects associated with the folding of a protein) of the various methyl resonances. For the $\text{N}-\text{CO}-\text{CH}_3$ resonance and the $\text{S}-\text{CH}_3$ resonance of *N*-acetylmethionine these were 2.05 p.p.m. and 2.13 p.p.m. respectively, and for the carboxymethyl derivative of *N*-acetylmethionine they were 2.05 p.p.m. and 2.92 p.p.m. respectively. Thus the $\text{S}-\text{CH}_3$ resonance is shifted

Table 1. Assignment of the methyl resonances of the *N*-acetyl terminal residues and Met-65 residues of various eukaryotic cytochromes *c*
For details see the text. Chemical-shift values are given in p.p.m. at pH 5.3 at 57°C.

Protein source	Chemical shift (p.p.m.)			
	Ferrocycytochrome <i>c</i>		Ferricycycytochrome <i>c</i>	
	Met-65	<i>N</i> -Acetyl	Met-65	<i>N</i> -Acetyl
Horse	2.11 (M11)	2.07 (M10)	1.94 (M*19)	1.97 (M*20)
Tuna	2.14 (TM9)	2.05 (TM8)	1.96 (TM*16)	1.96 (TM*17)
Donkey	2.11	2.07	1.94	1.97
Cow	2.12	2.06	1.94	1.97
Dog	2.14	2.07	1.95	1.98
Rabbit	2.12	2.08	1.94	1.98
Chicken	2.09	2.05	1.91	1.97
Pigeon	2.12	2.08	1.92	1.99

Fig. 3. Convolution difference spectra of horse [Hse⁶⁵]ferrocycytochrome *c* (a) and horse ferrocycytochrome *c* (b) at pH 7 at 57°C

For details see the text. The resonances of Met-65, Ile-57 and Tyr-74 are marked in accordance with the resonance nomenclature scheme (see the text).

0.79 p.p.m. downfield on modification, a consequence of the sulphur bearing a positive charge.
The convolution difference spectra of tuna ferrocycytochrome *c* and tuna [Met(Cm)⁶⁵]ferrocycytochrome *c*, both in ²H₂O at pH 5.6 and 57°C, are presented in Fig. 4. It can readily be seen that the spectrum of the carboxymethylated derivative is

very similar to that of the native protein, showing that the modification has not seriously perturbed the structure of the protein. In particular, the resonances of Met-80 are not affected by the modification. A number of specific differences between the spectra are apparent, and these are described below.

Table 2. Comparison of horse ferrocyclochrome *c* and horse [Hse⁶⁵]ferrocyclochrome *c*

Resonance designates are taken from Moore & Williams (1980a,b) and from Boswell *et al.* (1980). These references summarize the assignments. The primary chemical-shift values were taken from standard compilations (McDonald & Phillips, 1969; Bundi & Wüthrich, 1979). For other details see the text. Chemical-shift values are given in p.p.m. at pH 7.0 at 57°C.

Resonance	Assignment	Chemical shift (p.p.m.)		
		Primary position	Ferrocyclochrome <i>c</i>	[Hse ⁶⁵]Ferrocyclochrome <i>c</i>
M1	Met-80 CH ₃	2.13	-3.28	-3.28
M11	Met-65 CH ₃	2.13	2.11	Not present
M10	<i>N</i> -Acetyl CH ₃	2.05	2.07	2.07
M4	Ile-57 δ-CH ₃	0.89	-0.43	-0.48
M5	Val-20 or Leu-98 CH ₃	0.97	0.07	0.07
M7	Val-20 or Leu-98 CH ₃	0.94	0.43	0.43
M6	Ile-75 or Ile-81 γ-CH ₃	0.94	0.38	0.42
A5	Trp-59 C-4 or C-7	7.65 or 7.50	7.60	7.57
A30	Trp-59 C-6 or C-5	7.24 or 7.17	5.74	5.78
A23	Phe-82 <i>ortho</i>	7.34	6.71	6.72
A10	Phe-82 <i>meta</i>	7.34	7.40	7.39
A11	Tyr-74 <i>ortho</i>	7.15	7.22	7.21
A26	Tyr-74 <i>meta</i>	6.86	6.64	6.61

Fig. 4. Convolution different spectra of tuna [Met(Cm)⁶⁵]ferrocyclochrome *c* (a) and tuna ferrocyclochrome *c* (b) at pH 5.6 at 57°C

For details see the text. The resonances of Met-65, Ile-57, Tyr-74 and Tyr-48 are marked in accordance with the resonance nomenclature scheme (see the text).

The methyl resonances TM8 and TM9 are of particular interest: whereas resonance TM8 is unaffected by the modification, resonance TM9 shifts downfield to 2.93 p.p.m. Spectra obtained with the Carr-Purcell A pulse sequence (Campbell

et al., 1975) were required to locate resonance TM9 for the modified protein; these are given in Fig. 5. The data show that the site of modification is Met-65 and gives the assignment of the singlet methyl resonances (Table 1).

Fig. 5. Aliphatic region of the Carr-Purcell A spectra of tuna [Met(Cm)⁶⁵]ferrocyclochrome *c* (a) and tuna ferrocyclochrome *c* (b) at pH 5.6 at 57°C
For details see the text.

A number of other resonances shifted as a consequence of modification, but these shifts all appear to be small; the shifts for some assigned resonances are given in Table 3. The largest shift other than that for resonance TM9 was for resonance TM6, a methyl resonance that has not yet been firmly assigned. Resonances of Trp-59 and Ile-57 were also shifted. Some resonances between 0.5 p.p.m. and 1.8 p.p.m. shifted on modification and the general profiles of the spectra in this region are slightly different (Fig. 4).

The resonances TA15 and TA22 arise from Tyr-48, an aromatic residue whose rotation is hindered (Campbell *et al.*, 1976; Moore & Williams, 1980e). The corresponding resonances of the carboxymethylated protein are not shifted relative to the

native protein, but they are of greater apparent intensity (see Fig. 4).

It was also observed that the NH protons of the carboxymethylated ferrocyclochrome *c* exchanged for deuterons much faster than did the NH protons of native ferrocyclochrome *c* at the same temperature, pH and protein concentration. Neither of these effects, which are associated with the conformational flexibility of the protein structure (Moore & Williams, 1980c,d,e), have yet been investigated further.

The spectrum of tuna [Met(Cm)⁶⁵]ferrocyclochrome *c* was not studied in the same detail as was the spectrum of the reduced derivative. This was partly due to the fact that spectra of reduced cytochrome *c* are more easily interpreted than are the spectra of their oxidized forms, since there are no complicating paramagnetic effects, and partly because tuna [Met(Cm)⁶⁵]ferrocyclochrome *c* is less stable than native tuna ferrocyclochrome *c* and begins to denature at approx. 50°C. Nevertheless, a number of observations made at pH 5.6 and at 27°C are significant. Tuna [Met(Cm)⁶⁵]ferrocyclochrome *c* retains its Met-80 ligand to the iron, since the haem methyl resonances at 35.2 p.p.m. and 32.7 p.p.m. and the Met-80 methyl resonance at -23.4 p.p.m. were not significantly perturbed by the modification. The singlet resonance TM*17 was not affected by the modification, but resonance TM*16 was shifted downfield to 2.80 p.p.m. As with ferrocyclochrome *c*, these data assign the singlet methyl resonances (Table 1).

Discussion

In the present paper we are primarily concerned with the structures of two derivatives of eukaryotic cytochrome *c*, namely [Hse⁶⁵]cytochrome *c* (horse) and [Met(Cm)⁶⁵]cytochrome *c* (tuna). These derivatives were prepared by established procedures: [Hse⁶⁵]cytochrome *c* by reconstitution of CNBr-cleaved fragments (Corradin & Harbury, 1974; Boon *et al.*, 1979a; Wallace & Offord, 1979), and [Met(Cm)⁶⁵]cytochrome *c* by reaction of ferrocyclochrome *c* with iodoacetic acid (Ando *et al.*, 1966a,b). Detailed carboxymethylation studies of horse and bovine cytochrome *c* have shown that under the reaction conditions that we have used only His-33 and Met-65 are carboxymethylated (Ando *et al.*, 1966a,b; Stellwagen, 1968). We used tuna cytochrome *c* for the carboxymethylation reaction in order to eliminate the side reaction with His-33; tuna cytochrome *c* contains a tryptophan residue at position 33 (Dayhoff, 1968).

The resonance assignments that follow from the modifications are summarized in Table 1. These assignments are a useful addition to the list of assignments for cytochrome *c*, although the

Table 3. Comparison of tuna ferrocyclochrome *c* and tuna [Met(Cm)⁶⁵]ferrocyclochrome *c*

Resonance designates and resonance assignments are taken from Moore & Williams (1980e) and from Boswell *et al.* (1980). The primary chemical-shift values were taken from standard compilations (McDonald & Phillips, 1969; Bundi & Wüthrich, 1979). For other details see the text. Chemical-shift values are given in p.p.m. at pH 5.6 at 57°C.

Resonance	Assignment	Chemical shift (p.p.m.)		
		Primary position	Ferrocyclochrome <i>c</i>	[Met(Cm) ⁶⁵]Ferrocyclochrome <i>c</i>
TM1	Met-80 CH ₃	2.13	-3.25	-3.26
TM9	Met-65 CH ₃	2.13	2.14	2.93
TM8	<i>N</i> -Acetyl CH ₃	2.05	2.05	2.05
TM4	Ile-57 δ -CH ₃	0.89	-0.34	-0.39
TM5	Val-20 or Leu-98 CH ₃	0.97	0.11	0.11
TM7	Val-20 or Leu-98 CH ₃	0.94	0.54	0.50
TM6	Ile-75 or Ile-81 (?) γ -CH ₃	0.94	0.37	0.48
TA4	Trp-59 C-4 or C-7	7.65 or 7.50	7.58	7.56
TA23	Trp-59 C-6 or C-5	7.24 or 7.17	5.76	5.83
TA16	Phe-82 <i>ortho</i>	7.34	6.73	6.74
TA6	Phe-82 <i>meta</i>	7.34	7.41	7.43
TA9	Tyr-74 <i>ortho</i>	7.15	7.22	7.21
TA19	Tyr-74 <i>meta</i>	6.86	6.67	6.65
TA22	Tyr-48	7.15 or 6.86	6.14	6.14

chemical-shift values of the Met-65 and *N*-acetyl methyl resonances provide only limited structural information. This is because the difference between the chemical-shift values of the Met-65 and *N*-acetyl methyl resonances and the chemical-shift values of analogous resonances in simple peptides are so small. Such differences in chemical-shift values, usually referred to as secondary shifts, arise from through-space perturbations resulting from the folding of a protein into an ordered structure. For the resonances of Table 1 the maximum secondary shift is 0.16 p.p.m. The observations that the secondary shifts of the methyl resonances are small, that the resonances are affected by extrinsic paramagnetic ions, and that Met-65 can be readily modified without a large conformation change accompanying the modification, all indicate that neither Met-65 nor the *N*-acetyl group is near to a ring-current centre and that both groups are on the surface of the protein. The X-ray crystallographic structure (Dickerson *et al.*, 1971; Tanaka *et al.*, 1975; Swanson *et al.*, 1977; Takano *et al.*, 1977) shows that these groups are indeed on the surface of the protein, far from aromatic residues.

We now turn our attention to the structural perturbations accompanying modification of Met-65. The resonances of ferrocyclochrome *c* most affected by the modification are those of residue 65 itself, Trp-59 and Ile-57, and the methyl resonances M6/TM6 and M7/TM7 (resonances that are tentatively assigned to Ile-75 or Ile-81 and to Val-20 or Leu-98 respectively) (Tables 2 and 3). In addition the *meta* proton resonance of Tyr-74 is slightly perturbed. Some of these residues are

relatively far from Met-65; for example Trp-59 is approx. 1.3 nm away, Tyr-74 is approx. 0.95 nm away and Ile-57 is approx. 1.1 nm away (Swanson *et al.*, 1977; Takano *et al.*, 1977). However, although the resonance shifts are relatively small, they are significant (Figs. 3 and 4). How, then, does the modification of Met-65 cause such perturbations? The following proposal, based on our previous work showing that the region of the protein about Ile-57 is a conformationally sensitive region (Moore & Williams, 1980c,d,f), provides a plausible explanation for the perturbations involving resonances of Ile-57, Trp-59 and Tyr-74. The δ -methyl resonances of Ile-57 (M4 and TM4) are shifted upfield by the ring-current fields of Tyr-74 and Trp-59 (Moore & Williams, 1980b; Perkins, 1980). The hydroxy group of Tyr-74 and the carboxy group of Glu-66 are probably hydrogen-bonded to each other, and conformational perturbations that affect Glu-66 may also affect Tyr-74, resulting in variation of the chemical-shift values of resonances M4 and TM4. We assume that modification of Met-65 does perturb Glu-66 and Tyr-74. The shift of the Trp-59 resonances (Tables 2 and 3) may be associated with the same structural perturbation. The Trp-59 resonances will only experience a small ring-current shift from Tyr-74, and thus the shifts of resonances A30 and TA23 probably arise from a change in the Trp-59-haem orientation (Moore & Williams, 1980a).

In summary, modification of Met-65 causes a small rearrangement of one side of the protein.

The small perturbations of resonances M5/TM5, M6/TM6 and M7/TM7 and shifts of several methyl

resonances between 0.6 p.p.m. and 1.8 p.p.m. (Figs. 3 and 4) are not readily explicable. Resonances M6 and TM6 have been tentatively assigned to either Ile-75 or Ile-81 (Moore & Williams, 1980*b,e*), and the mechanism proposed above to account for the shift of the Ile-57 resonances provides support for the Ile-75 assignment, since the methyl groups of Ile-75 are relatively near to Tyr-74 (Swanson *et al.*, 1977; Takano *et al.*, 1977). However, further work is required to clarify this assignment and to define the perturbations that affect the other unassigned methyl resonances, especially in view of the fact that resonances M5 and M7 have been tentatively assigned to Val-20 or Leu-98, both of which are 1.5 nm away from Met-65.

The resonance shifts that we have been discussing are very small, and they are no greater than the differences between horse and tuna ferrocyclochromes *c* (compare Table 2 with Table 3; Moore & Williams, 1980*e*). This serves to emphasize the minor nature of the structural perturbations, something that had previously been indicated by activity measurements: both [Hse⁶⁵]cytochrome *c* (horse) and [His(Cm)³³,Met(Cm)⁶⁵]cytochrome *c* (cow) are as active as the native proteins in biological assay systems (Ando *et al.*, 1966*a,b*; Boon *et al.*, 1979*a*; Wallace & Offord, 1979).

One of our interests in the cytochrome *c* molecule is to monitor the effects of amino acid changes on the structure of the surface of the protein in an attempt to understand the nature of the antigenicity of cytochrome *c*. Urbanski & Margoliash (1977) have described a number of antigenic determinants for a range of eukaryotic cytochromes *c*, and one of these determinants includes Ile-57. Structural perturbations near to Ile-57 resulting from amino acid substitutions affect the immunochemical behaviour of cytochrome *c* (Urbanski & Margoliash, 1977), and some of these structural perturbations have been observed by n.m.r. in an analogous manner to the perturbations involving Ile-57 described in the present paper for the modified cytochromes *c* (Moore & Williams, 1980*f*). In view of this correlation between Ile-57 δ -methyl chemical shift, amino acid substitutions and immunochemical properties, it is noteworthy that [Hse⁶⁵]cytochrome *c* (horse) and native horse cytochrome *c* have different affinities for the rabbit anti-(horse cytochrome *c*) sera directed against the Ile-57-containing determinant (H. A. Harbury, personal communication). It is not yet clear whether Met-65 is part of this determinant. To extend the description of the surface of cytochrome *c* we have compiled a list of the chemical-shift values of the Met-65 and *N*-acetyl methyl resonances of various eukaryotic cytochromes *c* (Table 1), although we have not performed the detailed experiments necessary to assign specifically the resonances for

all the proteins. The data show that there are differences in the environments of the various methyl groups resulting from the amino acid changes, although at present it is not possible to pinpoint the origin of the differences. This is in contrast with the analogous set of data for the Ile-57 δ -methyl resonances, and is an illustration of the fact that it is easier to draw structural conclusions when a set of resonances are subject to large secondary shifts, as those of Ile-57 are (Moore & Williams, 1980*c,d,f*).

The final point that we must consider is the difference in NH proton exchange rates and the difference in Tyr-48 mobility between tuna ferrocyclochrome *c* and tuna [Met(Cm)⁶⁵]ferrocyclochrome *c*. Both of these observations point to tuna [Met(Cm)⁶⁵]ferrocyclochrome *c* having a looser time-averaged structure than tuna ferrocyclochrome *c*. These observations are in accord with Stellwagen's (1968) conclusion, that [His(Cm)³³,Met(Cm)⁶⁵]cytochrome *c* (horse) had an increased susceptibility to basic and acidic denaturation compared with the native protein. Additionally, we observed that the thermal stability of the carboxymethylated protein was lower than that of the native protein. It is not clear whether [Hse⁶⁵]cytochrome *c* is similarly destabilized, and further experiments are required to compare the stabilities of [Hse⁶⁵]cytochrome *c*, [Met(Cm)⁶⁵]cytochrome *c* and native cytochrome *c*.

Conclusions

The structures of [Met(Cm)⁶⁵]cytochrome *c* (tuna) and [Hse⁶⁵]cytochrome *c* (horse) are very similar to those of tuna cytochrome *c* and horse cytochrome *c* respectively. In fact the differences between the native and modified proteins are no greater than the differences between tuna cytochrome *c* and horse cytochrome *c*.

We thank the Science Research Council (grant to A. P. B.), the Medical Research Council and The Royal Society for financial support. We thank Professor R. E. Offord for reading the manuscript and for helpful discussions. We also thank Dr. I. D. Campbell, Mr. R. Porteous and Mr. N. Soffe for technical assistance. R. J. P. W. is a member of the Oxford Enzyme Group.

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