The binding of platinum complexes to tuna cytochrome *c*

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The binding of $[\text{PtCl}_4]^{2-}$ and *cis*- $[\text{PtCl}_2(\text{NH}_3)_2]$ to methionine-65 of tuna cytochrome *c* was investigated by ^1H n.m.r. The modification at methionine-65 is shown to cause an extremely small structural perturbation to the protein at the site of modification.

Cytochromes *c* from many different species have been obtained and their natural variations in amino acid sequences used as an indication of the essential structural requirements of the cytochrome *c* molecule (Dayhoff, 1968; Dickerson & Timkovich, 1975; Margoliash, 1980). In addition, many chemically modified derivatives of cytochrome *c* have been described in order to define its functional characteristics further (Ferguson-Miller *et al.*, 1979). As a consequence of this large body of data, cytochrome *c* is an excellent choice to study the range and type of structural perturbations accompanying minor chemical modifications. In the present paper we report a ^1H n.m.r. study of the binding of two small platinum complexes, $[\text{PtCl}_4]^{2-}$ and *cis*- $[\text{PtCl}_2(\text{NH}_3)_2]$, to Met-65 of tuna cytochrome *c* and to *N*-acetylmethionine. These modifications were chosen because a previous study (Boswell *et al.*, 1981) found that, whereas one modification (Met-65 → Hse-65) caused only local structural perturbations, another modification [Met-65 → Met(Cm)-65] caused far-reaching perturbations that affected residues up to 1.5 nm away from the site of modification.

Platinum complexes have long been used in the preparation of heavy-atom derivatives for X-ray structural analyses, including that of tuna cytochrome *c*, where $[\text{PtCl}_4]^{2-}$ and $[\text{Pt}(\text{CN})_4]^{2-}$ have been shown to bind to Met-65 (Takano *et al.*, 1973). $[\text{PtCl}_4]^{2-}$ has also been used as an n.m.r. probe for resonances of exposed residues; Sadler *et al.* (1974) have shown it binds to Met-29 of ribonuclease. The work reported in the present paper shows that the binding of $[\text{PtCl}_4]^{2-}$ and *cis*- $[\text{PtCl}_2(\text{NH}_3)_2]$ to Met-65 of tuna cytochrome *c* causes only a local, small, structural perturbation to the protein.

Materials and methods

Tuna cytochrome *c* was obtained from Sigma

Abbreviations used: Hse, homoserine; Cm, carboxymethyl.

Chemical Co. (Poole, Dorset, U.K.). DL-*N*-Acetylmethionine, ascorbic acid and $\text{K}_3[\text{Fe}(\text{CN})_6]$ were purchased from BDH Chemicals (Poole, Dorset, U.K.), and oxygen-free argon from Air Products (Bracknell, Berks., U.K.). $^2\text{H}_2\text{O}$ (99.8%) was obtained from Merck, Sharp and Dohme (Montreal, Que., Canada), and NaO^2H (40% in $^2\text{H}_2\text{O}$, 99% isotopic purity) and ^3HCl (35% in $^2\text{H}_2\text{O}$, 99.6% isotopic purity) from CIBA (Duxford, Cambridge, U.K.). The platinum complexes were kindly given by Dr. M. J. Cleary (Johnson Matthey, Reading, Berks., U.K.).

Cytochrome *c* was purified after reduction with ascorbic acid at pH 6 by passage through a Sephadex G-25 column (30 cm × 1.5 cm) and dialysis against three changes (approx. 1000 ml) of double-distilled water. The ferrocycytochrome *c* was then freeze-dried. N.m.r. samples were prepared by dissolution of the required amount of protein in 0.4 ml of $^2\text{H}_2\text{O}$ and the pH was adjusted to 5.6. Quoted pH values are direct pH-meter readings uncorrected for isotope effects (Kalinichenko, 1976).

The ferrocycytochrome *c* derivatives were prepared by the addition of a 0.1 M excess of $\text{K}_2[\text{PtCl}_4]$ or *cis*- $[\text{PtCl}_2(\text{NH}_3)_2]$ dissolved in $^2\text{H}_2\text{O}$ to ferrocycytochrome *c* in an n.m.r. tube under argon. The mixture was left at room temperature for 24 h before the n.m.r. studies were performed. The analogous ferricytochrome *c* derivatives were prepared by oxidation of the modified ferrocycytochrome *c* with $\text{K}_3[\text{Fe}(\text{CN})_6]$ to avoid any binding to Met-80 in the oxidized protein by direct reaction of ferricytochrome *c* with the platinum complexes. The iron hexacyanides were removed by passage of the sample through a Sephadex G-25 column, the eluate was freeze-dried and n.m.r. samples were prepared as above.

For the model complex studies three n.m.r. samples were made: (a) 10 mM-*N*-acetylmethionine, (b) 10 mM-*N*-acetylmethionine and 10 mM- $\text{K}_2[\text{PtCl}_4]$, and (c) 10 mM-*N*-acetylmethionine and 10 mM-*cis*- $[\text{PtCl}_2(\text{NH}_3)_2]$, all at pH 6 and in 0.4 ml of $^2\text{H}_2\text{O}$.

Table 1. Chemical shift of the S-CH₃ group in *N*-acetylmethionine and tuna cytochrome *c*, free and bound to platinum complexes

For experimental details see the text. — indicates an upfield shift.

Species	Chemical-shift values (p.p.m.)		
	Free	Bound	Difference
(i) Bound to PtCl ₄ ²⁻			
<i>N</i> -Acetylmethionine (pH 7)	2.04	2.34	0.30
Tuna ferrocytochrome <i>c</i> (pH 5.6)	2.11	1.96	—0.15
Tuna ferricytochrome <i>c</i> (pH 5.6)	1.96	1.91	—0.05
(ii) Bound to <i>cis</i> -[PtCl ₂ (NH ₃) ₂]			
<i>N</i> -Acetylmethionine (pH 7)	2.04	2.33	0.29
Tuna ferrocytochrome <i>c</i> (pH 5.6)	2.11	2.05	—0.06
Tuna ferricytochrome <i>c</i> (pH 5.6)	1.96	1.96	0.00*

* This solution was reduced to confirm that the platinum complex was still bound.

The ¹H n.m.r. spectra were obtained with a 270 MHz spectrometer. Convolution difference spectra were obtained as previously described (Campbell *et al.*, 1973). 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

Reaction of *N*-acetylmethionine with the Pt²⁺ complexes produces perturbations to the ¹H n.m.r. spectrum of *N*-acetylmethionine. Most significantly, the resonance of the S-CH₃ group is shifted 0.30 p.p.m. downfield. The relevant chemical-shift values are given in Table 1. The data tabulated are for the central component of the triplet resonances resulting from coupling between the S-CH₃ group and ¹⁹⁵Pt. (¹⁹⁵Pt has only approx. 30% natural abundance; the more abundant isotope has no nuclear spin.) The interaction of *N*-acetylmethionine with K₂[PtCl₄] has been thoroughly studied by Dr. P. Sadler and Mr. I. Ismail (Birkbeck College, London, U.K.) by using ¹H, ¹³C and ¹⁹⁵Pt n.m.r. We are indebted to these authors for discussion and free access to their work before publication. Our results are completely compatible with theirs.

Complex-formation between the Pt²⁺ complexes and cytochrome *c* results in perturbations to the S-CH₃ resonance of Met-65 in both ferrocytochrome *c* and ferricytochrome *c* (Table 1). Fig. 1 shows a small region of the spectra of tuna ferrocytochrome *c* and its two derivatives in ²H₂O at pH 5.6 and 57°C. In this region of the spectrum are found two singlet methyl resonances, which are due to the *N*-acetyl group and Met-65 (Moore & Williams, 1980; Boswell *et al.*, 1981); that of the latter is marked in each spectrum. The Met-65 resonance is shifted upfield on the binding of the Pt²⁺ complexes.

A number of other assigned resonances experience small shifts, although none is larger than 0.05 p.p.m. The resonances that shift are: TM4 (Ile-57), TA4 and TA23 (Trp-59) and the resonance of haem methyl 8. Resonances TM5, TM6 and TM7 do not shift. This is a significant difference between the modifications reported in the present paper and those reported earlier (Boswell *et al.*, 1981); these resonances are significantly affected by the conversion of Met-65 into Met(Cm)-65: for example, resonance TM6 is shifted 0.11 p.p.m. downfield. Resonance TM6 has not been firmly assigned, but resonances TM5 and TM7 arise from Val-20 (Moore & Williams, 1980, and unpublished work). [The resonance nomenclature scheme is used to simplify the discussion and to preserve continuity with previous reports. It is described for the resonances given above by Boswell *et al.* (1981).]

Apart from the resonance of Met-65, other resonances between 0.8 p.p.m. and 2.0 p.p.m. were perturbed on modification, resulting in different profiles for the three spectra (Fig. 1). These shifts are not readily explicable, and without assignments cannot be interpreted. However, the regions of the spectra of Fig. 1 are no more different than are the corresponding regions of the spectra of horse and tuna cytochromes *c* (Boswell *et al.*, 1981). This comparison serves to emphasize the minor nature of the perturbation.

Discussion

The binding constants of Cl⁻ and NH₃ to Pt²⁺ in H₂O are very high (Petsko *et al.*, 1978), and, in the solvent used in our experiments, unbuffered ²H₂O, there is unlikely to be any solvent substitution before or after reaction with Met-65. Assuming that the complexes are bound to only the sulphur atom of

Fig. 1. Part of the aliphatic regions of the 270 MHz ^1H n.m.r. spectra of (a) PtCl_3 -tuna ferrocytochrome c, (b) $\text{cis-}[\text{PtCl}(\text{NH}_3)_2]$ -tuna ferrocytochrome c and (c) tuna ferrocytochrome c

All spectra were measured at pH 5.6 and 57°C . The marked resonance in each spectrum is from Met-65.

Met-65 and to no other protein group the modifications are:

There is a difference in charge of -2 between the two species.

P. Sadler and I. Ismail (personal communication) have shown that in an equimolar solution of *N*-acetylmethionine and $[\text{PtCl}_4]^{2-}$ the predominant species is the 1 : 1 complex

The co-ordination of the methionine sulphur to Pt^{2+} causes a 0.30 p.p.m. downfield shift to the S-CH_3 resonance (Table 1) due to deshielding of the methyl-group protons by the sulphur positive charge. A similar shift is produced by the co-ordination of *N*-acetylmethionine to $\text{cis-}[\text{PtCl}_2(\text{NH}_3)_2]$ (Table 1). The shifts of the Met-65 S-CH_3 resonances of cytochrome *c* on reaction with the Pt^{2+} reagents are in the opposite direction (Table 1). This change from the model system suggests that either (a) the mode of binding by *N*-acetylmethionine is different from that of cytochrome *c*, or (b) the configuration of the Met-65 side chain is altered by the complex-formation so that the S-CH_3 group is placed in a different environment to that in the native protein.

It is unlikely that there is a second ligand from the protein, since the remaining three sulphur-containing residues of cytochrome *c*, which are located far from Met-65, are unaffected by the modification and there are no nitrogen-containing groups available for co-ordination at pH 5.6. Thus the shift of the Met-65 S-CH_3 resonance compared with that of *N*-acetylmethionine (Table 1) that accompanies the complex-formation must result from a perturbation of the structure of cytochrome *c*.

The structural perturbations are small, as there are no large changes to the cytochrome *c* spectrum. Yet the perturbation provides an effective shift to the S-CH_3 resonance of 0.45 p.p.m. on complex-formation with $[\text{PtCl}_4]^{2-}$ (Table 1). The other assigned resonances that shifted on complex-formation were also affected by carboxymethylation of Met-65. These resonances arise from groups close to each other in space on the same side of the protein as Met-65 (Takano *et al.*, 1977). The shifts result from a small displacement of the aromatic ring of Trp-59, and possibly that of Tyr-74, which leads to different ring-current shifts for resonances of neighbouring

groups (Boswell *et al.*, 1981). The S-CH_3 resonance of $[\text{Met}(\text{Cm})\text{-65}]$ ferrocytochrome *c* was not perturbed compared with that of *N*-acetylcarboxymethylmethionine, which indicates that there is an additional perturbation to the structure of cytochrome *c* induced by the Pt^{2+} complexes. This

additional perturbation does not affect other assigned resonances. There are no far-reaching perturbations affecting Val-20 and Tyr-97 similar to those of [Met(Cm)-65]cytochrome *c*. [In a previous paper the tyrosine resonances were incorrectly assigned. They come from Tyr-97, not Tyr-48 (Keller & Wüthrich, 1981; A. P. Boswell, C. G. S. Eley, G. R. Moore & R. J. P. Williams, unpublished work).] Thus, in summary, the structural perturbations induced by the Pt²⁺ complexes are small and confined to the side of the protein where the site of modification is located. This contrasts with the perturbations induced by carboxymethylation, which are more far-reaching.

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