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N.m.r. and e.p.r. characterization of [4-carboxy-2,6-dinitrophenyl-lysine]cytochromes *c*

Karl-Erik FALK, Per Åke JOVALL and Jonas ÅNGSTRÖM
Department of Biochemistry and Biophysics, Chalmers Institute of Technology and University of Göteborg, S-412 96 Gothenburg, Sweden

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Monosubstituted [4-carboxy-2,6-dinitrophenyl-lysine]cytochromes *c* were investigated by n.m.r. and e.p.r. Modification of Lys-13 or Lys-72 in ferricytochrome *c* by 4-chloro-3,5-dinitrobenzoate yields either of two different conformers that are rapidly exchanging in the native form. The equilibrium involves small local changes in the conformation of Met-80 (the sixth ligand) and Phe-82, as a result of whether Lys-13 is in the 'on' or 'off' position in the Lys-13–Glu-90 salt bridge.

Cytochrome *c* singly modified at different lysine residues by incorporation of a 4-carboxy-2,6-dinitrophenyl moiety has yielded important conclusions about the binding domains for the physiological redox partners of this protein (Ferguson-Miller *et al.*, 1978; Speck *et al.*, 1979; König *et al.*, 1980). Through kinetic studies it was shown that the domains to a large extent overlap, involving the upper part of the solvent-exposed haem edge.

It was further concluded that the changes introduced in the cytochrome *c* molecule were rather minor, consisting largely of the change in charge and bulkiness of the lysine residue in question (Brautigan *et al.*, 1978*b*). However, these derivatives are potentially able to give important information about the conformational stability of cytochrome *c*, in that a well-defined local change in the structure can be monitored at many different sites on the molecule. We present below an n.m.r. and e.p.r. characterization of different 4-carboxy-2,6-dinitrophenyl derivatives of cytochrome *c*.

Experimental

Cytochrome *c* was prepared from horse hearts and freed from deamidated forms as described by Brautigan *et al.* (1978*c*). Monosubstituted 4-carboxy-2,6-dinitrophenyl derivatives of cytochrome *c* were prepared by the method outlined by Brautigan *et al.* (1978*a*). The different 4-carboxy-2,6-dinitrophenyl derivatives were identified from the elution patterns from Whatman CM-32 CM-cellulose columns and peptide 'mapping'.

^{1}H n.m.r. spectra were recorded on a Bruker WH 270MHz spectrometer, and chemical-shift values are quoted as positive downfield relative to 2,2-dimethyl-2-silapentane-5-sulphonate. pH values are given as the uncorrected pH-meter readings and are designated pH*. E.p.r. spectra were obtained at 15K on a Varian E-9 spectrometer operating at 9.12GHz.

Results and discussion

^{1}H n.m.r. spectra of different 4-carboxy-2,6-dinitrophenyl derivatives and the native form of ferricytochrome *c* in unbuffered solutions (pH* 7.0 and 300K) were recorded at 270MHz. Shift differences relative to the native protein for some of the hyperfine shifted resonances are given in Table 1. It is evident that only the Lys-13 and Lys-72 derivatives show significant shift differences, and the discussion below is restricted to these.

It has been shown that the direction of the electronic *g*-tensor (in the haem plane) is dependent on the chiral binding mode of the methionine ligand (Senn *et al.*, 1980). Thus in horse ferricytochrome *c* the maximal unpaired spin density in the haem ring is found on methyl groups 3 and 8 (Keller & Wüthrich, 1978*a*), whereas in *Pseudomonas aeruginosa* cytochrome *c*-551 methyl groups 1 and 5 have the largest spin density (Keller & Wüthrich, 1978*b*). The underlying reason for this behaviour can be found in the fact that the orientation of the lone-pair orbital of the methionine sulphur with respect to the d_{xz} and d_{yz} orbitals of the iron to a large extent influences the spin-density distribution in the haem ring (Senn *et al.*, 1980). The description given above suggests that the results obtained in the

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present study may be rationalized as a perturbation of the methionine ligand, since the chemical-shift changes given in Table 1 most probably occur as a consequence of altered hyperfine contributions to the observed shifts.

The *g*-values of the Lys-13 and Lys-72 derivatives were measured from e.p.r. spectra obtained at 15K and compared with those of the native form (Brautigan *et al.*, 1977): Lys-13, 3.07, 2.24, 1.25; Lys-72, 3.06, 2.27, 1.25; native, 3.06, 2.25, 1.25. These values are quite similar, and it can be calculated that from the differences in the *g*-values between the derivatives and the native form maximally 0.1 p.p.m. of the observed shift differences can be ascribed to changes in the pseudo-contact shift for haem methyl groups 3 and 8. For methyl groups 1 and 5 at most 0.05 p.p.m. would be expected. This means that further changes in the pseudo-contact shifts must stem from the angular part of the expression used to calculate these shifts (Dwek, 1973), i.e. a minor displacement of the *g*-tensor in the haem plane.

We therefore suggest that the shift differences given in Table 1 occur as a consequence of small movements of the $-S-CH_3$ position of Met-80, as indicated in the schematic view of the haem group shown in Fig. 1. From considerations of the expected changes in contact and pseudo-contact contributions to the shifts of the haem methyl resonances it is readily seen that the signs of the shift differences are qualitatively reproduced for methyl groups 3 and 8. In this case changes in the contact shift will dominate. Alterations in the contact and pseudo-contact shifts for haem methyl groups 1 and 5 will be appropriately equal but of opposite sign and thus cancel each other. An upward movement of the $-S-CH_3$ position of Met-80 corresponds to the Lys-72 derivative, whereas a downward movement is expected for the Lys-13 derivative. This shift mechanism must also perturb the resonances of the axial ligands, mainly through changes in the contact shift, which indeed is the case. The signs of these shifts also fit in with the general description given above. Small shifts on haem substituents other than those given in Table 1 support the mechanism presented above.

In the aromatic region of the n.m.r. spectra only Phe-82 showed any significant changes. The overlapping resonances from this residue are found at 5.9 p.p.m. (300K) (Boswell *et al.*, 1980). The linewidth of the overlapping resonances in native ferricytochrome *c* is approximately 25 Hz, whereas 22 Hz and 34 Hz are found for the Lys-13 and Lys-72 derivatives respectively. These changes in linewidth probably originate from a slight perturbation of Phe-82, causing this residue to experience different ring-current shifts from the haem.

It has been found that the resonance stemming from haem methyl group 3 in ferricytochrome *c* could be resolved into two peaks by lowering the temperature (<278K) in the neutral pH range (Burns & La Mar, 1979). This behaviour was ascribed to two conformers of the protein coming into slow exchange at low temperature, and it was estimated that the two states were equally populated at 298K. The shift difference found for the split haem-methyl-group-3 resonance (0.4 p.p.m.) is close to the difference between the same resonance in the Lys-13 and Lys-72 derivatives (see Table 1). This fact suggests that modification of Lys-13 or Lys-72 results in either of the two conformers involved in the equilibrium mentioned above. Furthermore, it is not unexpected that only the haem-methyl-group-3 resonance can be brought into slow exchange at low

Table 1. *Comparison of chemical shifts of the hyperfine shifted resonances for different [4-carboxy-2,6-dinitrophenyl-lysine ferricytochromes c and native ferricytochrome c*

Chemical group*	Chemical shifts in native cytochrome *c*† (p.p.m.) relative to 2,2-dimethyl-2-silapentane-5-sulphonate	Chemical shifts of 4-carboxy-2,6-dinitrophenyl derivatives of cytochrome *c* relative to native cytochrome *c*‡ (p.p.m.)			
		Lys-13	Lys-27	Lys-60	Lys-72
Haem substituents§					
Methyl 8	34.75	−0.24	−0.08	+0.06	−0.53
Methyl 3	31.89	+0.17	−0.04	0	−0.36
Methyl 5	10.22	+0.02	0	+0.04	0
Methyl 1	7.2	~0	~0	~0	~0
Axial ligands					
His-18 C-4	23.67	−0.34	−0.04	0	+0.26
Met-80 CH_3	−23.79	+0.30	+0.06	+0.05	−0.29
Met-80 γ-CH	−27.62	−0.14	0	0	−0.96

* Assignments are taken from Redfield & Gupta (1971) and Keller & Wüthrich (1978*a*).
† Chemical-shift values were measured at pH* 7.0 and 300K.
‡ A positive sign indicates a shift to lower field.
§ The haem methyl groups are numbered in accordance with Fig. 1.

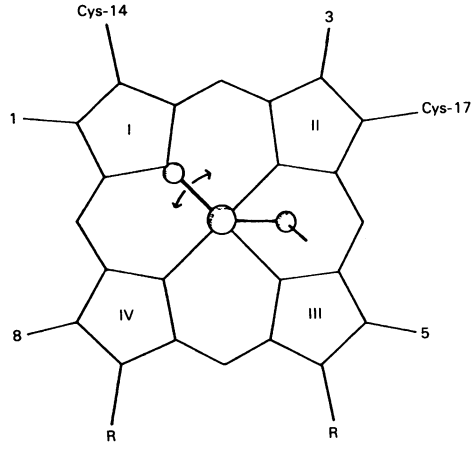

Fig. 1. *Schematic view of the haem group in cytochrome c showing a segment of the methionine ligand*
The segment of Met-80 is $-CH_2-S-CH_3$ with the methyl group pointing towards pyrrole ring I. The arrows indicate small movements of the $-S-CH_3$ group in the haem plane as a consequence of chemical modification at Lys-13 or Lys-72 by 4-carboxy-2,6-dinitrophenylation. The positions of the haem propionate groups (R), haem methyl groups (1, 3, 5 and 8) and the residues to which the haem is attached are indicated.

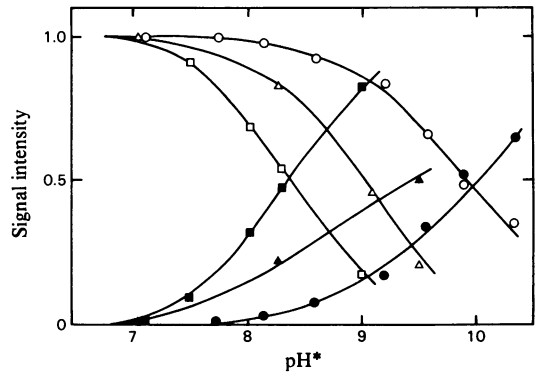

Fig. 2. *pH*-dependence of the downfield haem-methyl-group resonances in the ^1H n.m.r. spectra of the neutral and alkaline forms of three different [4-carboxy-2,6-dinitrophenyl-lysine]cytochromes c*
□, △ and ○, Disappearance of the haem-methyl-group resonances in the Lys-13, Lys-27 and Lys-72 derivatives respectively. ■, ▲ and ●, Appearance of the corresponding methyl-group resonances of the alkaline forms. The measurements were performed in unbuffered solutions at 300K. For experimental details see the text.

temperature, considering the chemical-shift differences in Table 1 in relation to the exchange rate between the conformers and the transverse relaxation rates of the hyperfine shifted resonances.

Fig. 2 shows the pH*-dependence of the downfield haem methyl groups for three derivatives. It has previously been shown (Gupta & Koenig, 1971; Morishima *et al.*, 1977; Andersson *et al.*, 1980) that the gradually appearing alkaline form of ferricytochrome *c* is in slow exchange with the neutral form, resulting in two superimposed spectra. Thus both the disappearance and formation of the two forms can be monitored directly. For native cytochrome *c* a p*K** of 9.0 was obtained, whereas the Lys-13 and Lys-72 derivatives gave values of 8.3 and 9.8 respectively. Other derivatives, represented by Lys-27 in Fig. 2, gave a p*K** close to that obtained for the native form. These results are in accord with results obtained from titrations of the 695nm band of ferricytochrome *c* (Osheroff *et al.*, 1980), except in the case of the Lys-72 derivative, where p*K*9.3 was obtained, which must be attributed to the fact that our titrations were performed in an unbuffered medium.

From single-crystal X-ray-crystallographic data (Takano *et al.*, 1977) it is found that Lys-13 forms a salt bridge with Glu-90, which contributes in maintaining the closed structure of the haem crevice. Modification of Lys-13 disrupts the salt bridge with a concomitant lowering of the p*K* of the alkaline isomerization, as found in the present study and by Osheroff *et al.* (1980). If Lys-72 is modified, on the other hand, a stabilization of the neutral form is obtained. This indicates that Lys-13 is involved in an 'on'–'off' equilibrium in the native form. Small local changes in the conformation of Met-80 and Phe-82 thus occur as a consequence of whether Lys-13 is in the 'on' or 'off' position in the Lys-13–Glu-90 salt bridge.

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