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The interaction between cytochrome c and *trans*-$[PtCl_2(NH_3)_2]$ †

Lijuan Jiang,[a] Guozhi Tang[b] and Wenxia Tang *,[a]

[a] *State Key Laboratory of Coordination Chemistry, Coordination Chemistry Institute, Nanjing University, Nanjing 210093, People's Republic of China*
[b] *Department of Biochemistry, Nanjing University, Nanjing 210093, People's Republic of China*

The reaction between cytochrome c and *trans*-$[PtCl_2(NH_3)_2]$ has been studied under different conditions. The complex can not only bind to a Met-80 residue, which causes the Fe–S bond of ferricytochrome c to be broken, but can also induce a facile conversation of low-spin iron(III) cytochrome c to the high-spin form under mild conditions. Cytochrome c can also be cross-linked selectively by *trans*-$[PtCl_2(NH_3)_2]$. The binding sites of the platinum complex in the protein derivatives were identified by analysis of the molar ratio of the two metals (Pt and Fe), ^1H NMR and UV/VIS spectroscopy and differential-pulse voltammetry.

Cytochrome c (cyt c) is an important haemoprotein in mitochondrial electron transfer and has been a benchmark for studies of protein folding and stability, protein engineering and long-range electron transfer.[1] Ferricytochromes c have been known to exhibit five discrete pH-dependent conformational states.[2] Interest in these conformational forms of the protein has recently intensified as the result of evidence suggesting that cytochrome c may undergo related conformational changes upon binding to other electron-transfer proteins and in the protein folding.[3–5] To study the conformational changes of cytochrome c extreme conditions (such as high or low pH, high temperature or covalent modification) are required to rupture the Fe–S (Met-80) bond and to change the co-ordination of the haem iron atom.[6]

Here, the interaction of cyt c with *trans*-$[PtCl_2(NH_3)_2]$ is reported. This complex can bind to the Met-80 axial ligand of cyt c under very mild conditions, causing the Fe–S bond of ferricytochrome c to be broken, similar to the interaction of cytochrome c with $[Pt(dien)]^{2+}$ (dien = diethylenetriamine).[7] Moreover, it can induce low- to high-spin state conversion of ferricytochrome c at pH 5.5 and a stable Pt-modified high-spin cytochrome c derivative was obtained. Our studies confirm that iron(III) cytochrome c has the structural flexibility to accommodate spin-state changes readily. It is also interesting that cytochrome c can be cross-linked selectively by this complex and there are few reports of such cross-linking.[8]

Experimental

Materials

Horse heart cytochrome c (type VI) was obtained from Sigma Chemical Co. and purified prior to use.[9] The complex *trans*-$[PtCl_2(NH_3)_2]$ was synthesized as described.[10]

Preparation of platinum-modified cyt c

To cyt c (100 mg, 8 μmol) in 80 mmol dm^{-3} (pH 5.5 or 7) phosphate buffer solution (30 cm^3) was added *trans*-$[PtCl_2(NH_3)_2]$ (24 mg, 80 μmol). The mixture was kept in the dark at 37 °C for 2 d. The excess of complex was removed by dialysis into 80 mmol dm^{-3} phosphate buffer at pH 7. The protein solution was then placed on a pre-equilibrated Sephadex G-75 gel filtration column (1.6 × 100 cm) at 4 °C and eluted with 80 mmol dm^{-3} (pH 7) phosphate buffer solution at a flow rate of 5 cm^3 h^{-1}. Two components I and II were collected. Component I was then placed on a pre-equilibrated CM 52 cation-exchange column (2.6 × 70 cm) in 80 mmol dm^{-3} (pH 7) phosphate buffer and eluted with a gradient from 80 to 100 mmol dm^{-3} (pH 7) phosphate buffer solution at a flow rate of 30 cm^3 h^{-1}. The components were collected and dialysed against water to remove any inorganic salts.

Determination of molecular weights of protein derivatives

The molecular weight measurements of modified cyt c derivatives were performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 12% gel concentration.[11] Since the cyt c derivatives do not contain disulfide bonds and platinum labels might be displaced from the proteins by 2-sulfanylethanol, the latter was not used for electrophoresis. Typical concentrations of proteins were around 1 mg cm^{-3}.

NMR spectroscopy

All ^1H NMR spectra were recorded on a Bruker AM 500 spectrometer using dioxane as an internal reference. The protein-containing samples were dialysed into D_2O by ultrafiltration repeatedly to exchange all the labile protons.[12]

Magnetic measurements of some samples were also performed by the NMR method, essentially as developed by Evans.[13] Susceptibilities were calculated from the difference between the resonance of dioxane in the NMR tube (outside diameter 5 mm) containing paramagnetic cytochrome c derivative, *etc*, and that of dioxane in a capillary (2.5 mm) containing everything but the cyt c derivative (*e.g.* the internal reference).

Absorption spectroscopy and elemental analysis of metal

Electronic spectra were recorded with a Shamazhu UV-3100 spectrophotometer. The elemental analyses (Pt and Fe) were performed on an inductively coupled plasma quantomer (JARREL-ASH 1100–2000).

Voltammetry

Differential-pulse voltammograms were obtained with a PARC model 273 electrochemical analyser equipped with an Epson FX-850 recorder at 25 °C. All experiments employed a three-electrode cell with a saturated calomel electrode as reference, a platinum wire as auxiliary, and a gold disc (3.0 mm) as working electrode. The composition of the sample solutions was as follows: 0.2 mmol dm^{-3} cytochrome c and 10 mmol dm^{-3} 4,4′-bipyridyl as a mediator,[14] 0.1 mol dm^{-3} $NaClO_4$ dissolved in

† *Non-SI unit employed*: $\mu_B \approx 9.274 \times 10^{-24}$ J T^{-1}.

85 mmol dm^{-3} phosphate buffer at pH 7.0. A small, jacketed cell (10 cm^3) allowed experiments with 3 cm^3 samples. The solutions were deoxygenated by gentle bubbling of argon and a blanket of this gas was maintained during the measurements. Concerned that the platinum labels might be displaced from the proteins by the mediator, we dialysed the samples after the electrochemical measurements and demonstrated, by the UV/VIS spectra, that the labels remained.

Results and Discussion

The products of reactions of native cyt c with *trans*-$[PtCl_2(NH_3)_2]$ at pH 5.5 and 7 for 48 h were chromatographed on a Sephadex G-75 gel filtration column, and the elution profiles are shown in Fig. 1. Two components (I and II) were obtained. Their molecular weights determined by SDS-PAGE are 12 500 and 25 000, respectively. So, we conclude that the complex can cross-link cytochrome c selectively. Cytochrome c dimers probably have *trans* configurations in view of the protein size, which is consistent with the conclusion obtained by Peerey and Kostic.[8] In addition, from Fig. 1, it can be seen that cyt c dimer derivatives are produced more easily at pH 5.5 than at pH 7.0.

Component I was then placed on a CM 52 cation-exchange column and the elution profiles are shown in Fig. 2. A few components (A–D) were separated and the Pt:Fe molar ratios of each component are listed in Table 1. It can be seen that component A is primarily the native cyt c, B and C have Pt:Fe molar ratios of 1:1 (corresponding to singly labelled cyt c derivatives) and D has a Pt:Fe molar ratio of 2:1 (corresponding to the doubly labelled cyt c derivative) and $II^{(a)}$ and $II^{(b)}$ are cyt c dimers containing five or more platinum atoms.

To elucidate the binding sites of platinum in the cyt c derivatives we examined the ^1H NMR spectrum of each component. The results indicate that the spectra of corresponding components (*i.e.* those having the same retention time on the CM 52 cation-exchange column) obtained from reactions under different conditions are similar. Some of the results are shown in Tables 2 and 3, and in Figs. 3 and 4. From Table 2 and Fig. 3 it can be seen that the resonances of the

Table 1 The Pt:Fe molar ratios of products from the reaction of native cyt c with *trans*-$[PtCl_2(NH_3)_2]$ under different pH conditions

	Pt:Fe Molar ratio					
pH	A	B	C	D	$II^{(a)}$	$II^{(b)}$
7.0	0.38:1	1.05:1	1.04:1	2.00:1	5.22:2	
5.5	0.23:1	1.00:1	1.06:1			7.24:2

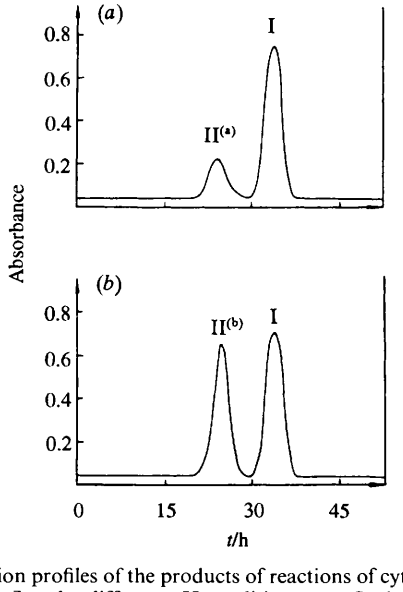

Fig. 1 Elution profiles of the products of reactions of cyt c with *trans*-$[PtCl_2(NH_3)_2]$ under different pH conditions on a Sephadex G-75 gel filtration column (1.6 × 100 cm) at 4 °C, detected at 280 nm. Flow rate: 5 cm^3 h^{-1}. pH 7.0 (*a*) and 5.5 (*b*)

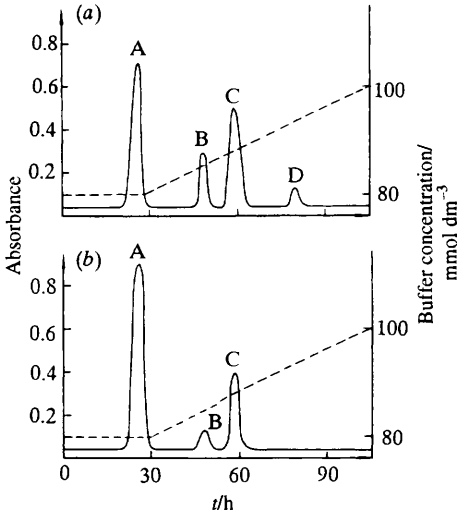

Fig. 2 Elution profiles of component I obtained under different pH conditions on a CM 52 cation-exchange column (2.6 × 70 cm) at 4 °C detected at 280 nm. Flow rate: 30 cm^3 h^{-1}. pH 7.0 (*a*) and 5.5 (*b*)

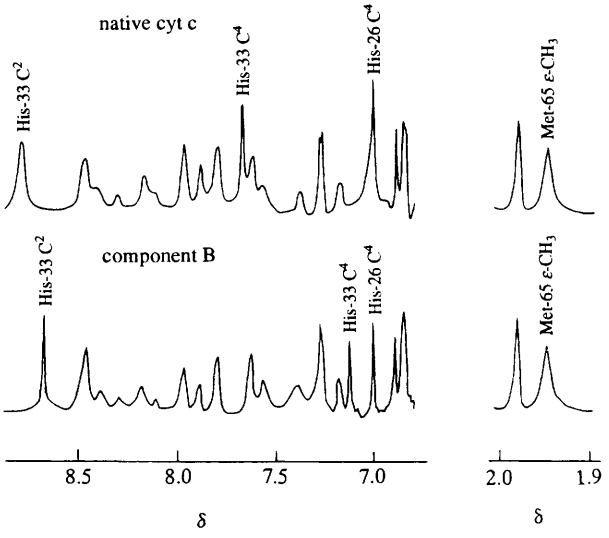

Fig. 3 Proton NMR spectra of native cyt c and component B in the aliphatic and aromatic regions

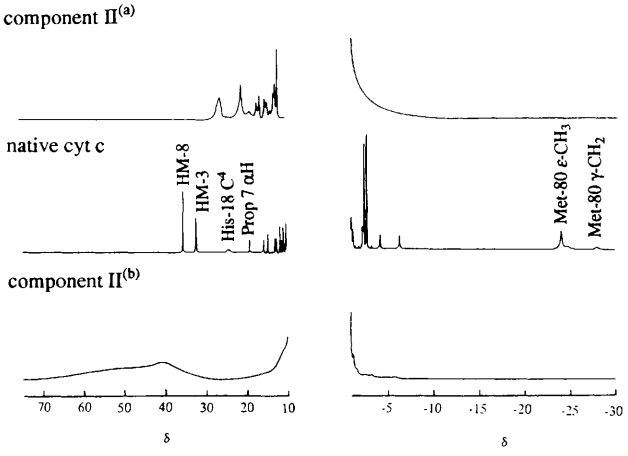

Fig. 4 Proton NMR spectra of native cyt c and components $II^{(a)}$ and $II^{(b)}$ in the hyperfine-shifted region

Table 2 Proton NMR chemical shifts (δ) of His-33, Met-65 and Met-80 residues of components B–D and native cyt c

	cyt c	B	C	D
His-33 C^2	8.79	8.68	8.78	8.66
His-33 C^4	7.67	7.14	7.67	7.12
His-26 C^2	7.67	7.67	7.65	7.66
His-26 C^4	7.00	7.00	7.00	7.00
Met-65 ε-CH_3	1.94	1.94	Absent	Absent
Met-80 ε-CH_3	−24.48	−24.57	−24.32	−24.38
Met-80 γ-CH_2	−28.31	−28.36	−28.21	−28.46

Table 3 Proton NMR resonances (δ) of components B and C and native cyt c in the hyperfine-shifted region

	cyt c	B	C
HM-8[a]	35.60	35.88	35.19
HM-3[b]	32.48	32.36	32.21
His-18 C^4	24.36	24.45	24.00
Prop 7βH[c]	19.18	18.90	19.11
His-18 H_β	14.61	14.75	14.54
His-18 C^2	12.70	12.69	12.67
Prop 7βH[c]	11.38	11.87	11.43
HM-5[d]	9.95	9.57	10.05
TE-4[e]	−2.50	−2.53	−2.38
TE-2[f]	−2.79	−2.73	−2.76
meso H[g]	−4.34	−4.34	−4.19
meso H[g]	−6.51	−6.68	−6.38
Met-80 ε-CH_3	−24.48	−24.57	−24.32
Met-80 γ-CH_2	−28.31	−28.36	−28.21

[a] CH_3 in haem ring IV. [b] CH_3 in haem ring II. [c] β-CH_2 of propionate in haem ring IV. [d] CH_3 in haem ring III. [e] $SCHCH_3$ in haem ring II. [f] $SCHCH_3$ in haem ring I. [g] *meso* H of haem.

His-33 C^2 and C^4 protons in component B shift upfield compared with the corresponding resonances of native cytochrome c, and that the resonances of the His-26 C^2 and C^4 protons and Met-65 ε-CH_3 were not affected by modification. All these results indicate that in component B the Pt^{II} co-ordinates to His-33 through the nitrogen atom of the imidazole ring. As shown in Table 2, the chemical shifts of the imidazole C^2 and C^4 protons of the His-33 and His-26 residues in component C are similar to those of corresponding resonances of native cyt c, but no Met-65 ε-CH_3 resonance at δ 1.94 for native cyt c is observed for component C, which shows that the binding site of platinum in singly labelled component C is the thioether side chain of the Met-65 residue. For the doubly labelled component D, the chemical shifts of the His-33 C^2 and C^4 protons are similar to those of corresponding resonances of component B and move upfield compared with those of corresponding resonances of native cyt c. The resonance of Met-65 ε-CH_3 is not observed at δ 1.94, as for component C. The resonances of the His-26 C^2 and C^4 protons do not shift on modification. All the foregoing results indicate that *trans*-$[PtCl_2(NH_3)_2]$ binds to the His-33 and Met-65 residues of cytochrome c in component D.

From Table 3 it can be seen that the resonances in the hyperfine-shifted region for components B and C do not shift greatly upon binding of the Pt atom to His-33 and Met-65 residues, which indicates that the co-ordination of the haem periphery remains unaltered and there is no significant conformational change. This conclusion is consistent with the results from X-ray crystallographic analysis, *i.e.* the Met-65 and His-33 residues are all on or near the surface of the protein.[15]

Since the proton resonances in the ^1H NMR spectra of cyt c dimer components $II^{(a)}$ (Pt : Fe molar ratio 5 : 2) and $II^{(b)}$ (Pt : Fe molar ratio 7 : 2) are broader than those observed for the native protein,[16,17] it is difficult to confirm the platinum binding sites of these components from the shift of resonances in the aliphatic and aromatic regions. However, as shown in Fig. 4, it is apparent that the resonances of components $II^{(a)}$ and $II^{(b)}$ in the hyperfine-shifted region move much more compared with those of native cyt c in the corresponding region, and no Met-80 ε-CH_3 and γ-CH_2 resonances of components $II^{(a)}$ and $II^{(b)}$ are observed in this region. All these results indicate that the structure of the protein is flexible in solution and the small molecule *trans*-$[PtCl_2(NH_3)_2]$ can reach into the hydrophobic pocket of cytochrome c and bind to the axial ligand Met-80. The binding of platinum complex to the Met-80 residue of components $II^{(a)}$ and $II^{(b)}$ causes the Fe–S bond of ferricytochrome c to be broken and the chemical shift increment due to Fermi-contact interaction between a nuclear spin and unpaired electron spin to be absent, and so the Met-80 ε-CH_3 and γ-CH_2 resonances move from the hyperfine-shifted region to the region of methyl and methylidyne resonances.

Although the Fe–S bonds of ferricytochrome c in components $II^{(a)}$ and $II^{(b)}$ are all broken, the ^1H NMR spectrum of component $II^{(a)}$ in the hyperfine-shifted region is different from that of component $II^{(b)}$. In the spectrum of component $II^{(a)}$ two haem peripheral methyl resonances at δ 35.60 and 32.48 for native cyt c move to the spectral region between δ 20 and 30 and no Met-80 ε-CH_3 or γ-CH_2 resonances are observed in the hyperfine-shifted region, which is similar to the spectrum of cyt c at pH 10.2.[18] Based on a comparison of NMR spectrum of the high-pH (11 > pH > 9) form of cytochrome c in the hyperfine-shifted region with that of chemically modified ferricytochrome c in the corresponding region, Morishima *et al.*[19] proposed that at high pH the Met-80 ligand to the iron in the native protein is replaced by the lysyl residue (Lys-79) and the cytochrome c remains low spin, which is consistent with results from electron spin resonance and electronic spectra.[20,21] All the foregoing results suggest that in component $II^{(a)}$ the sixth haem iron co-ordination position may be occupied by the ε-amino group of lysyl residue 79, which in the X-ray crystallographic model of the iron(III) protein can be made to rotate around its α-carbon atom to take up a position appropriate for such co-ordination.[21]

In the ^1H NMR spectrum of component $II^{(b)}$ the haem peripheral methyl resonance and the Met-80 ε-CH_3 and γ-CH_2 resonances are not present in the hyperfine-shifted region in which the above resonances for native cyt c should appear, and at the same time a new and very broad peak occurs in the region δ 35–60, which is similar to the spectrum of the iron(III) high-spin complex produced by binding of external fluoride to the haem iron atom of cytochrome c at 4 > pH > 2.5[19] and is characteristic of high-spin cytochrome c. When the reaction pH is decreased from 7.0 to 5.5, the ε-amino group of lysyl residue 79 (pK_a 8.95) is protonated and it is impossible for Lys-79 to co-ordinate to the haem iron atom, and so the Met-80 residue is replaced by a weak-field ligand such as H_2O supplied by the solvent,[19] which causes component $II^{(b)}$ to convert from low to high spin.

A large increase in the magnetic susceptibility of component $II^{(b)}$ was also observed by the NMR method. A magnetic moment of about 7.5 μ_B was calculated. This approximates to the value expected for a high-spin iron(III) haem dimer (8.4 μ_B). Our studies indicate that iron(III) cytochrome c has the structural flexibility to accommodate spin-state changes readily, similar to what was found by Otiko and Sadler.[6]

Further to confirm the spin-state change and co-ordination of the haem iron atom, the UV/VIS spectra of components $II^{(a)}$ and $II^{(b)}$ were examined. From Fig. 5 it can be seen that electronic spectrum of component $II^{(a)}$ is virtually identical with that of native cytochrome c in the region 200–600 nm: Soret band at 408 nm, α,β band at 528 nm, although the weak charge-transfer band in the near infrared at 695 nm, which is present only where there is an Fe–S bond,[22] is absent. These results further confirm that although the Fe–S bond of cyt c is broken, component $II^{(a)}$ remains low spin. In the UV/VIS spectrum of component $II^{(b)}$ (Fig. 5), the Soret band moves from 408 to 396

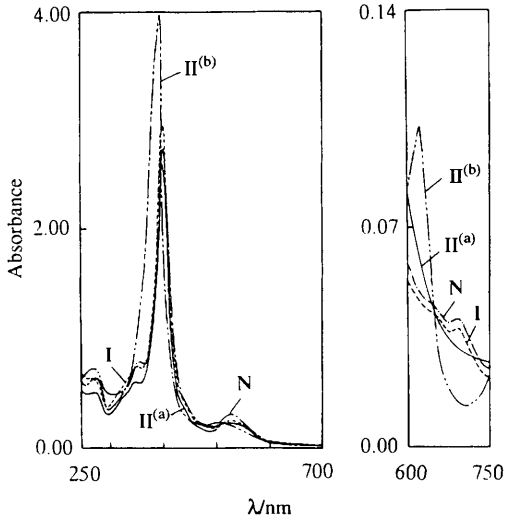

Fig. 5 The UV/VIS spectra of native cyt c (N) and of components I, $II^{(a)}$ and $II^{(b)}$

Table 4 Reduction potentials

Compound	$E°$/mV *vs.* NHE
Cyt c	262
B	261
C	265
D	262
$II^{(a)}$	43
$II^{(b)}$	−83

nm and a new band at 620 nm appears with that at 695 nm disappearing, which is characteristic of high-spin cyt c. The above results confirm that the strong-field Met-80 residue in native cytochrome c is replaced by a weak-field ligand presumably supplied by the solvent[5,19] and cyt c is converted from low to high spin in component $II^{(b)}$. However, components $II^{(a)}$ and $II^{(b)}$ are compatible products obtained at different pH, and can be interconverted by changes in pH, which is confirmed by the UV/VIS spectra. When a solution of component $II^{(b)}$ is raised from pH 5.5 to 7.5 the Soret band moves from 396 to 408 nm and the band at 620 nm disappears, but that at 695 nm does not reappear, which is characteristic of low-spin cyt c dimer $II^{(a)}$.

Reduction potentials of components $II^{(a)}$ and $II^{(b)}$ were determined by differential-pulse voltammetry. Results are given in Table 4 in comparison to native cytochrome c. The reduction potential of cyt c dimer is *ca.* 220–340 mV lower than that of the native protein, which suggests that the co-ordination of the haem periphery of cyt c alters and significant conformational changes may occur in the cyt c dimer. This conclusion is consistent with the results obtained from NMR and UV/VIS spectra.

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References

1 Y. Lu, D. R. Casimiro, K. L. Bern, J. H. Richards and H. B. Gray, *Proc. Natl. Acad. Sci. USA*, 1993, **90**, 11456.
2 H. Theorell and A. Akesson, *J. Am. Chem. Soc.*, 1941, **63**, 1804.
3 J. C. Ferrer, J. G. Guillemette, R. Bogumil, S. C. Inglis, M. Smith and A. G. Mauk, *J. Am. Chem. Soc.*, 1993, **115**, 7507.
4 P. Hildebrandt, T. Heimburg, D. Marsh and G. L. Powell, *Biochemistry*, 1990, **29**, 1661.
5 G. A. Elöve, A. K. Bhuyan and H. Roder, *Biochemistry*, 1994, **33**, 6925.
6 G. Otiko and P. J. Sadler, *FEBS Lett.*, 1980, **116**, 227.
7 L. J. Jiang, Y. M. Yao, D. X. Zhu and W. X. Tang, *J. Inorg. Biochem.*, 1995, **58**, 21.
8 L. M. Peerey and N. M. Kostic, *Inorg. Chem.*, 1987, **26**, 2079.
9 D. L. Brautigan, S. Ferguson-Miller and E. Margoliash, *Methods Enzymol.*, 1978, **53**, 129.
10 G. H. W. Milburn and M. R. Truter, *J. Chem. Soc. A*, 1966, 1609.
11 M. Wyckoff, D. Rodbard and A. Chambach, *Anal. Biochem.*, 1977, **28**, 459.
12 L. H. Botelho and F. R. N. Gurd, *Biochemistry*, 1978, **17**, 5188.
13 D. F. Evans, *J. Chem. Soc.*, 1959, 2003.
14 M. M. Eddowes and H. A. O. Hill, *J. Am. Chem. Soc.*, 1979, **101**, 4461.
15 R. E. Dickerson, T. T. David Eisenberg, O. B. Kallat, L. Samson, A. Cooper and E. Margoliash, *J. Biol. Chem.*, 1971, **246**, 1511.
16 K. M. Yocon, J. B. Shelton, J. R. Shelton, W. A. Schroeder, G. Worosila, S. S. Isied, E. Bordignon and H. B. Gray, *Proc. Natl. Acad. Sci. USA*, 1982, **79**, 7052.
17 A. P. Boswell, G. R. Moore, R. J. P. Williams, C. J. A. Wallace, P. J. Boon, R. J. F. Nivard and G. I. Tesser, *Biochem J.*, 1981, **193**, 493.
18 R. K. Gupta and S. H. Koenig, *Biochem. Biophys. Res. Commun.*, 1971, **45**, 1134.
19 I. Morishima, S. Ogawa and T. Yonezawa, *Biochim. Biophys. Acta*, 1977, **495**, 287.
20 L. L. Pearce, A. L. Gartner, M. Smith and A. G. Mauk, *Biochemistry*, 1989, **28**, 3152.
21 D. L. Brautigan, B. A. Feinberg, B. M. Hoffman and E. Margoliash, *J. Biol. Chem.*, 1977, **252**, 574.
22 C. C. McDonald and W. D. Phillips, *Biochemistry*, 1973, **12**, 3170.

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