N^ε,N^ε-Dimethyl-lysine cytochrome *c* as an NMR probe for lysine involvement in protein–protein complex formation

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The reductively dimethylated derivatives of horse and yeast iso-1-ferricytochromes *c* have been prepared and characterized for use as NMR probes of the complexes formed by cytochrome *c* with bovine liver cytochrome *b₅* and yeast cytochrome *c* peroxidase. The electrostatic properties and structures of the derivatized cytochromes are not significantly perturbed by the modifications; neither are the electrostatics of protein–protein complex formation or rates of interprotein electron transfer. Two-dimensional ¹H–¹³C NMR spectroscopy of the complexes formed by the derivatized cytochromes with cytochrome *b₅* and cytochrome *c* peroxidase has been used to investigate the number and identity of lysine residues of cytochrome *c* that are

involved in interprotein interactions of cytochrome *c*. The NMR data are incompatible with simple static models proposed previously for the complexes formed by these proteins, but are consistent with the presence of multiple, interconverting complexes of comparable stability, consistent with studies employing Brownian dynamics to model the complexes. The NMR characteristics of the *N^ε,N^ε*-dimethyl-lysine groups, their chemical shift dispersion, oxidation state and temperature dependences and the possibility of chemical exchange phenomena are discussed with relevance to the utility of *N^ε,N^ε*-dimethyl-lysine's being a generally useful derivative for characterizing protein–protein complexes.

INTRODUCTION

The characterization of protein–protein complexes formed between cytochrome *c* and its redox partners is central to a full description of the intermolecular electron-transfer processes in which they participate. Since the early modelling studies of Salemme [1] considerable progress towards this goal has been achieved with the cytochrome *c*–cytochrome *b₅* complex. Salemme's model predicted the structure of the cytochrome *c*–cytochrome *b₅* complex from the three-dimensional structures of the individual proteins and implicated four lysine residues on the surface of cytochrome *c* in the formation of this complex. Such predictive methods subsequently evolved to incorporate both complex electrostatic considerations and molecular dynamics calculations that, together with the results of kinetic and thermodynamic investigations of chemically modified derivatives of cytochrome *c*, and variants produced by site-directed mutagenesis, have led to considerable progress in the characterization of the cytochrome *c*–cytochrome *b₅* complex in solution (reviewed in [2]). Similar modelling [3–7], chemical modification [8,9] and mutagenesis [10–12] studies of cytochrome *c* interacting with cytochrome *c* peroxidase (CcP), together with X-ray crystallographic studies of several cytochrome *c*–CcP complexes [13], have produced high-resolution structural models for this system. However, as for the cytochrome *c*–cytochrome *b₅* complex [2], controversy persists concerning the structure of the cytochrome *c*–CcP complex in solution [14], particularly in connection with the identity of the key lysine residues of cytochrome *c* responsible for recognition and binding of its reaction partner.

One approach to investigating interprotein complexes of cytochrome *c* has been to use NMR spectroscopy [15–30].

However, neither one-dimensional (1D) ¹H nor two-dimensional (2D) ¹H–¹H NMR spectra provide sufficient resolution to allow small perturbations of the interprotein contact residues, particularly the lysine residues, to be monitored in such complexes. The large number of lysine residues (19 out of 104 for horse cytochrome *c*) further complicates matters. To help overcome this resolution problem, we have modified the lysine residues of horse heart cytochrome *c* to produce the fully *N^ε*-acetimidylated derivative [21] and the fully *N^ε,N^ε*-dimethylated derivative reported here. These modifications maintain the positive charge on the derivatized lysine residues and provide each derivatized residue with one (the *N^ε*-acetimidylated protein) or two (the *N^ε,N^ε*-dimethylated protein) methyl groups, whose resonances can be observed in 1D ¹H NMR spectra. This approach can be enhanced further through the use of ¹³C-enriched modifying reagents that label the derivatives with ¹³C and allow the acquisition of 2D ¹H–¹³C COSY or heteronuclear single quantum coherence (HSQC) spectra. Perturbations of the derivatized lysine methyl resonances resulting from complex formation between the modified cytochrome *c* and its reaction partners can then be used to identify the interprotein binding surface on cytochrome *c* in a manner similar to that of Dick et al. [31], who used reductive methylation of the lysine residues of the fd gene 5 DNA-binding protein to provide NMR probes for identifying its DNA-binding surface. In the present study we have focused on the *N^ε,N^ε*-dimethylated cytochrome *c* because this modification provides better NMR sensitivity, greater ease of ¹³C labelling and a more extensive literature on the characterization of proteins modified in this manner than does modification to the *N^ε*-acetimidylated cytochrome. Furthermore NMR studies of the ¹³C-enriched *N^ε,N^ε*-dimethylated derivatives of ribonuclease [32],

Abbreviations used: 1D, one-dimensional; 2D, two-dimensional; a.m.u., atomic mass units; CcP, cytochrome *c* peroxidase; EXSY, chemical exchange correlated spectroscopy; HSQC, heteronuclear single quantum coherence.

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lysozyme [33], fd gene 5 protein [31], ferredoxin [34], calmodulin [35] and calbindin [36] have been reported, and the consensus from these studies is that dimethylation is a relatively benign modification in terms of altering protein structures.

In the present paper we describe the preparation of the $N^{\epsilon},N^{\epsilon}$ -dimethylated derivative of ferricytochrome *c* and have studied the effects of this modification on a range of chemical properties of cytochrome *c*, including: its proton titration, the pH dependence of the stability of the cytochrome *c*-cytochrome b_5 complex, and the kinetics by which ferricytochrome *c* is reduced by ferrocyanochrome b_5 . Use of the fully dimethylated cytochrome as a spectroscopic probe for identification of surface lysine residues involved in the interaction of the cytochrome with cytochrome b_5 and cytochrome *c* peroxidase has been initiated through ^1H and ^{13}C NMR studies of the complexes formed by the native and modified cytochrome.

EXPERIMENTAL

Protein preparation, purification and modification

Recombinant yeast cytochrome *c* peroxidase was prepared as described previously [37,38] and concentrations determined on the basis of $\epsilon_{409} = 97000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [39]. The yeast iso-1-cytochromes *c* were prepared as described previously [40]. All yeast cytochrome *c* samples carried a Cys¹⁰²Thr substitution, which prevents intermolecular disulphide bond formation and greatly decreases the rate of autoreduction without affecting the structural or functional properties of the protein [41]. The Lys⁷³Ala, Lys⁷⁹Ala, Lys⁸⁶Ala and Lys⁸⁷Ala variants of yeast iso-1-cytochromes *c* used in the present work have been described elsewhere [42], and F. I. Rosell, J. C. Ferrer and A. G. Mauk, unpublished work). Recombinant trypsin-solubilized [44,45] and recombinant lipase-solubilized [44] bovine liver microsomal cytochromes b_5 expressed in *Escherichia coli* were prepared as described previously. Concentrations of ferricytochrome b_5 solutions were based on $\epsilon_{412.5} = 117000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [46]. Horse heart cytochrome *c* (Type VI; Sigma) was purified by cation-exchange chromatography to remove deamidated protein by the procedure of Brautigan et al. [47]. Concentrations of native and derivatized ferricytochrome *c* solutions were determined on the basis of $\epsilon_{409.5} = 106000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [48] and the assumption that the spectrum of the modified cytochrome is the same as that of the native protein.

Dimethylation of the lysine N^{ϵ} -amino groups of cytochrome *c* was performed by the method of Jentoft and Dearborn [49] as modified by Wallace and Cortésy [50]. Typically, to a solution of 50 mg of cytochrome *c* in 10 ml of 0.1 M sodium tetraborate buffer at pH 9 were added five 20 μl volumes of 37% aqueous formaldehyde at 2 min intervals and two 5 mg quantities of solid NaBH_4 at 4 min intervals after alternate additions of formaldehyde. After a total of 10 min, 10 ml of 0.1 M HCl was added to stop the reaction and the mixture was passed through a Sephadex G-25 column (45 cm $\times$ 1.5 cm) equilibrated with 0.05 M ammonium bicarbonate. The modified protein was then oxidized with $\text{K}_3[\text{Fe}(\text{CN})_6]$ and chromatographed on a CM-cellulose ion-exchange column (Whatman CM-52) equilibrated with 0.005 M sodium phosphate buffer at pH 7. The modified protein was eluted as a single sharp band with 0.5 M NaCl/0.005 M sodium phosphate (pH 7.5); the NaCl was removed by passage through a Sephadex G-25 column equilibrated with 0.05 M sodium phosphate, pH 7.5. ^{13}C -labelled dimethylcytochrome *c* was obtained by using 21.3% (v/v) ^{13}C formaldehyde (MSD Isotopes). Final purification of methylated ferricytochrome *c* for potentiometric experiments was achieved by FPLC (Pharmacia Mono-S HR 10/10 cation

exchange columns in 20 mM sodium phosphate buffer, pH 7.25, with a 70–150 mM NaCl gradient).

Electrospray mass spectrometry

Mass spectra were recorded with a VG BIO Q quadrupole mass spectrometer or with a triple quadrupole mass spectrometer constructed in-house [51] that was fitted with a pneumatically assisted electrospray interface. The sprayer and ion-sampling orifice voltages were maintained at 5.5 and 0.2 kV. Samples were introduced at 1 $\mu\text{l}/\text{min}$ at ambient temperature; mass spectra were obtained at a dwell time of 10 s per scan with 10 scans summed per spectrum and with a step size of 0.2 atomic mass units (a.m.u.). Protein samples (2–3 μM) were prepared in 10 mM ammonium acetate buffer, pH 7.0, by dilution directly into this buffer or by buffer exchange by centrifugal ultrafiltration (Centricon-10; Amicon).

Potentiometric titrations

Potentiometric titrations were performed with a Radiometer ABU93 Triburette operated under computer control as described previously [45,52]. Protein samples used in these measurements were deionized shortly before use by exhaustive exchange (at least 10^6 -fold) into 10 mM NaCl by centrifugal ultrafiltration (Centriprep-10; Amicon). For the protein–protein titrations [45,53] the titrand protein concentration was 45–55 μM and the titrant protein was 0.7–1.0 μM . For the continuous proton titrations [45], initial methylated ferricytochrome *c* concentrations were 40–50 μM and the initial ferricytochrome b_5 concentration was 0.7 μM . This ratio of cytochrome b_5 to cytochrome *c* was required to ensure that the proportion of cytochrome *c* bound to cytochrome b_5 varied minimally over the range of pH studied. The curves of q against pH obtained from the continuous proton titrations were integrated with the program Spectracalc (Galactic Industries, Northampton, MA, U.S.A.) to generate curves of $\log K_a$ against pH. The integration constants used in this process were evaluated from the $\log K_a$ values obtained from protein–protein titrations. L-Lysine dihydrochloride (L5751; Sigma), N^{ϵ} -methyl-L-lysine hydrochloride (M6004; Sigma), $N^{\epsilon},N^{\epsilon}$ -dimethyl-L-lysine hydrochloride (E1810; Bachem) and $N^{\epsilon},N^{\epsilon},N^{\epsilon}$ -trimethyl-L-lysine hydrochloride (T1660; Sigma) were purchased from the vendors indicated. Potentiometric titration curves obtained for the lysine derivatives were analysed in terms of two or three titratable groups as appropriate by a least-squares process with the program SCIENTIST (MicroMath, Salt Lake City, UT, U.S.A.). The pH titration curves of each protein were reproducible to ± 0.1 proton between pH 3 and 11. Difference titration curves derived by subtracting one such titration curve from another have an uncertainty of ± 0.14 proton over this pH range.

Anaerobic stopped-flow kinetics

Second-order rate constants for the bimolecular reduction of ferricytochrome *c* derivatives by ferrocyanochrome b_5 were determined under anaerobic conditions with a thermostatically controlled Durrum stopped-flow spectrophotometer housed in a glove box and interfaced to a microcomputer (Olis, Bogart, GA, U.S.A.) as described previously [54,55]. Homogeneous second-order reaction conditions (equal concentrations of reactants) were required because the reduction of ferricytochrome *c* by ferrocyanochrome b_5 is too rapid to be studied under pseudo-first-order conditions [54]. Rate data were analysed by least-squares fitting to a homogeneous second-order rate expression with the program MINSQ (MicroMath).

NMR spectroscopy

Samples were prepared for NMR spectroscopy by centrifugal ultrafiltration (Centricon-10 devices; Amicon). Four cycles of concentration and dilution with appropriate buffer in $^2\text{H}_2\text{O}$ were required to achieve an adequate exchange of H_2O for $^2\text{H}_2\text{O}$. The final protein solutions were diluted with buffer in $^2\text{H}_2\text{O}$ to give the desired protein concentrations, and the pH was adjusted with NaO^2H or ^2HCl . All reported pH values for $^2\text{H}_2\text{O}$ solutions, indicated by p^2H , are the direct meter readings uncorrected for any isotope effect. The electrolyte solution in the pH electrodes used for measurements with $^2\text{H}_2\text{O}$ solutions was not exchanged with the corresponding $^2\text{H}_2\text{O}$ solution.

Mixtures of oxidized and reduced cytochromes were prepared for exchange spectroscopy and saturation transfer experiments by reducing the protein with an approx. 20% excess of sodium dithionite followed by removal of the excess reducing agent by centrifugal ultrafiltration. The reduced cytochrome was then combined with a previously prepared oxidized sample. Ratios of oxidized to reduced cytochrome were calculated from integrated intensities of the ^1H NMR signals of the Met^{80} methyl group of the reduced cytochrome and the Pro^{30} δ -methylene proton of the oxidized cytochrome. These groups have chemical shifts of -3.3 and -6.3 p.p.m. respectively [56,57].

NMR spectra were recorded with a Jeol GX-400 spectrometer. The intensity of the residual HO^2H peak was decreased by gated irradiation for 0.5 s immediately before application of the 90° pulse. Chemical shifts are quoted relative to the methyl resonance of 2,2-dimethylsilapentane-5-sulphonic acid at 0.000 p.p.m. ^1H - ^{13}C COSY was performed with the procedure described by Maudsley et al. [58]. Spectra were collected over a frequency range of 1000 Hz in t_2 with 256 transients in t_1 or 4000 Hz in t_2 with 512 transients in t_1 . The Jeol spectrometer used for this work was not configured for HSQC or heteronuclear multiple quantum coherence experiments of the type that has been found to be useful for similar experiments on calmodulins [36], but the COSY approach adopted, although requiring longer data acquisition times, provides equivalent information.

The bimolecular electron self-exchange rate of N^ϵ - N^ϵ -dimethylcytochrome *c* was measured with the NMR saturation transfer technique [59–61]. Ionic strength was varied by the addition of NaCl. T_1 values were obtained for the Met^{80} methyl resonance of native and modified horse heart cytochromes *c* by using an inversion recovery sequence. ^{13}C - ^{13}C chemical exchange correlated spectroscopy (EXSY) was performed with the NOESY pulse sequence [62,63]. Spectra were collected over a range of 1000 Hz in each dimension with 256 transients in t_1 . 2D data were processed with FELIX 2.30 (Biosym, Cambridge, U.K.). To compare linewidths in 2D spectra, data were processed with a skewed sinebell window function weighted to minimize distortions. Widths were estimated at half height with a 1D slice taken through the centre of the peak in each dimension. This method is adequate for the comparison of relative linewidths, although not for the measurement of absolute values. Titrations of cytochromes *c* with cytochrome *c* peroxidase were performed by adding small volumes of an approx. 3 mM cytochrome *c* peroxidase solution to approx. 1.4 mM cytochrome *c* solutions.

RESULTS

Chemical characterization of methylated horse and yeast cytochromes *c*

The observed masses of horse heart cytochrome *c* (12359 ± 3 a.m.u.) and the methylated derivative (12894 ± 3 a.m.u.)

Table 1 Parameters obtained from potentiometric titrations of horse heart ferricytochrome *c* derivatives

Protein	pH range	Number of titratable groups	pH of zero net charge
Native	3 to 11	28.6	10
Native	11 to 3	30.5	
Methylated	3 to 11	25.6	9.6
Methylated	11 to 3	24.8

Figure 1 Chemical modification-induced difference titration curves of horse heart ferricytochrome *c* [25 °C, $\mu = 0.1$ M (KCl)]

(A) The titration curve (from pH 11 to pH 3) of acetylmethylated ferricytochrome *c* minus the titration curve (from pH 11 to pH 3) of native ferricytochrome *c*. (B) The titration curve of methylated ferricytochrome *c* (from pH 11 to pH 3) minus the titration curve (from pH 11 to pH 3) of native ferricytochrome *c*.

were in good agreement with the values anticipated for the unmodified (12360 a.m.u.) and exhaustively dimethylated (12893 a.m.u.) proteins. The observed mass of the native yeast cytochrome (12708 ± 2 a.m.u.) was also in agreement with the calculated value (12709 a.m.u.), and analysis of the modified derivative provided a mass of 13130 ± 1 a.m.u. as expected for dimethylation of all 15 lysine residues (calculated mass 13130 a.m.u.).

Potentiometric titrations of dimethylated horse cytochrome *c*

Potentiometric titrations of native and dimethylated horse cytochromes *c* were performed to assess whether the modification had substantially altered the electrostatic properties of the cytochrome and to provide a control for the potentiometric studies of dimethylated cytochrome *c* interacting with cytochrome *b₅*. The parameters extracted from the titration curves obtained for native cytochrome *c* and its methylated derivative are summarized in Table 1. As seen from these results, native horse heart cytochrome *c* exhibited a small increase in the number of groups that titrated between pH 3 and 11 after the

Figure 2 Dependence of q and $\log K_A$ on pH ($\mu = 0.01$ M, 25 °C) for the association of native and dimethylated horse heart ferricytochrome c with trypsin- and lipase-solubilized cytochrome b_5

The uncertainties in q and pK_a are smaller than the size of the data points. (A) Continuous proton titrations of native ferricytochrome c (0.115 mmol) with tryptic ferricytochrome b_5 (1.95 mmol) in 4.178 ml (solid line) and with methylated ferricytochrome c (0.128 mmol) with tryptic ferricytochrome b_5 (1.81 mmol) in 3.969 ml (broken line). Data are also shown from protein–protein titrations with native ferricytochrome c and lipase-solubilized ferricytochrome b_5 (○) at pH 6.72 and 8.47; and with methylated ferricytochrome c and lipase-solubilized cytochrome b_5 (●) at pH 6.75 and 8.25. (B) The curves in (A) were integrated to yield the curves of $\log K_A$ against pH for complex formation between native ferricytochrome c and tryptic ferricytochrome b_5 (solid line) and between methylated ferricytochrome c and tryptic ferricytochrome b_5 (broken line). Representative $\log K_A$ values from protein–protein titrations are shown: native ferricytochrome c with tryptic cytochrome b_5 (□) or lipase-solubilized cytochrome b_5 (○) and methylated cytochrome c with tryptic cytochrome b_5 (■) and lipase-solubilized cytochrome b_5 (●).

protein was exposed to basic pH. Over the same pH range, three fewer groups titrated in dimethylated ferricytochrome c than observed for the native protein, although the pH of zero net charge was virtually unchanged. As observed for the native protein [52], the titration curve of the dimethylated cytochrome exhibited hysteresis, but in this case exposure to alkaline pH resulted in a slight decrease in the number of groups that titrated rather than an increase.

The modification-induced difference titration curve obtained for the fully methylated ferricytochrome is shown in Figure 1. The titration curves of native and methylated ferricytochrome c were identical between pH 4 and 8.5. Beyond this range, the electrostatic properties of the modified protein began to deviate significantly from those of the native protein. Importantly, the shape of the curve between pH 9 and pH 11 indicates that the modification had resulted in a decrease in the pK_a values of many of the groups ionizing at alkaline pH. This finding is consistent with N^ϵ, N^ϵ -dimethylation of the lysine residues and is inconsistent with their monomethylation or trimethylation, as shown by the side chain pK_a values determined from titration curves [25 °C, 0.1 M KCl (results not shown)] of lysine (11.01 ± 0.02),

Table 2 pH dependence of proton release and complex stability for interaction of various forms of ferricytochrome c with ferricytochrome b_5

The uncertainty in q values is approx. ± 0.01 , and the uncertainty in pK_a values is approx. ± 0.05 .

Proteins	pH	q	pK_a
Native cytochrome c –tryptic cytochrome b_5	6.75	0.39	5.94
	8.45	−0.84	5.34
Methylated cytochrome c –tryptic cytochrome b_5	6.73	0.59	4.93
	8.25	−0.76	5.29
Native cytochrome c –lipase cytochrome b_5	6.72	0.38	5.75
	8.47	−1.15	4.90
Methylated cytochrome c –lipase cytochrome b_5	6.75	0.45	4.89
	8.25	−0.89	4.95

N^ϵ -monomethyl-lysine (11.34 ± 0.02), N^ϵ, N^ϵ -dimethyl-lysine (10.70 ± 0.02); the quaternary amine group of $N^\epsilon, N^\epsilon, N^\epsilon$ -trimethyl-lysine did not ionize over the pH range 3–12. These pK_a values are in good agreement with those reported by Jentoft and Dearborn [49].

NMR characterization of the conformations of dimethyl-lysine horse and yeast cytochromes c

Comparison of the modified and unmodified ferrocyclochromes c and ferricytochromes c by 1D and 2D NMR methods showed that resonances of amino acid residues not directly attached to the haem were either unaffected by the modification or were shifted by no more than 0.05 p.p.m. ([21,64], and M. C. Cox, D. Crowe and G. R. Moore, unpublished work). Some of the haem resonances were shifted by substantially more than this, most notably haem resonances of the ferricytochromes, but this effect, which has been observed with other modifications of cytochrome c [17,65,66], reflects small changes in the distribution of unpaired electrons within the haem and not a major structural change. Thus we conclude that the conformation of cytochrome c is not altered significantly by the modification. The dynamic properties of the structure, as manifested by the mobilities of aromatic amino acids [67,68] and a haem-linked chemical exchange [69], also seem not to have been perturbed [21,23,64].

Potentiometric characterization of the interaction of cytochrome b_5 with horse N^ϵ, N^ϵ -dimethylcytochrome c

Continuous potentiometric titration of the complexes formed by ferricytochrome b_5 with native and dimethylferricytochrome c are shown in Figure 2(A). In addition, q values obtained from the titration of native and dimethylated cytochrome c with lipase- and trypsin-solubilized cytochrome b_5 at two values of pH are shown. The parameters derived from titrations performed at acidic and basic pH (Table 2) permit a quantitative comparison of the electrostatic character of the complexes formed by lipase- and trypsin-solubilized cytochrome b_5 with the native and modified cytochrome c . From the results summarized in Figure 2 and Table 2, the electrostatic properties of all four protein–protein complexes were virtually identical. Integration of the continuous titration curves of the complexes (Figure 2A) to determine the dependence of binding affinity ($\log pK_a$) on pH [45] reveals that the shapes of the pH-dependence curves obtained for

Figure 3 Dependence of the second-order rate constant for reduction of native (□) and *N,N'*-dimethyl-lysine ferricytochrome *c* (○) by tryptic ferrocycytochrome *b₅* on ionic strength (25 °C, sodium phosphate buffer)

The uncertainties in the $\ln k_{12}$ values are smaller than the size of the data points.

the native and modified cytochrome *c* were virtually indistinguishable. Nevertheless, the modified cytochrome *c* exhibited a significantly lower affinity for cytochrome *b₅* between pH 5.5 and 9. We conclude from these results that the lipase- and trypsin-solubilized cytochromes, which differ by the presence of a two residue extension at the N-terminus of the lipase-solubilized cytochrome, are, to a first approximation, sufficiently similar in their interaction with the native and modified cytochrome *c* that they could be used interchangeably in these studies.

Bimolecular electron transfer kinetics of horse *N,N'*-dimethylcytochrome *c*

The electron self-exchange rate of *N,N'*-dimethyl-lysine cytochrome *c* was $240 \text{ M}^{-1} \cdot \text{s}^{-1}$ at zero added ionic strength, a value only marginally lower than the $260 \text{ M}^{-1} \cdot \text{s}^{-1}$ obtained for the native cytochrome (results not shown). Both rate constants were markedly, and similarly, dependent on ionic strength, as observed previously for cytochrome *c* [60,61].

The ionic strength dependence of the second-order rate constants for the reduction of native and dimethylated ferricytochrome *c* by ferrocycytochrome *b₅* is shown in Figure 3. Under all conditions studied, the native ferricytochrome *c* was reduced more rapidly than the modified cytochrome, consistent with their respective self-exchange rates. The curves fitted to these results in Figure 3 were based on the analytical procedure of van Leeuwen [70] and employed estimates for the radii and dipole moments of horse heart cytochrome *c* [16 Å, 284 D ($0.947 \times 10^{-27} \text{ C} \cdot \text{m}$)] and cytochrome *b₅* [17 Å, 640 D ($2.14 \times 10^{-27} \text{ C} \cdot \text{m}$)]. The resulting fits indicated in the figure are consistent with values for the logarithm of the rate constant at infinite ionic strength ($\ln k_{\text{inf}}$) and the apparent charge products of $12.9 \pm 0.2 \text{ M}^{-1} \cdot \text{s}^{-1}$, -240 ± 90 for the reaction of the two native proteins and $12.4 \pm 0.2 \text{ M}^{-1} \cdot \text{s}^{-1}$, -70 ± 50 for the reduction of modified cytochrome *c* by ferrocycytochrome *b₅*. Although the mechanistic significance of these values is debatable, we note that the rate constants for the two reactions are more similar at low ionic strength, where electrostatic factors dominate, and diverge at higher ionic strength, where shielding effects diminish the contribution of electrostatics. The inequivalent curvatures of the two fits (Figure 3) might indicate that the nature of the rate-determining step changes with ionic strength in a manner that is inequivalent for the reactions of the native and modified cytochromes. This is consistent with the ionic strength dependence of the rate constant for intramolecular electron transfer in the cytochrome *c*-cytochrome *b₅* and cytochrome *c*-CcP complexes noted previously [71,72].

Figure 4 ^1H - ^{13}C COSY spectra of 3 mM ^{13}C -labelled *N,N'*-dimethyl-lysine ferricytochrome *c* in the absence (left) and the presence (right) of 3 mM ferrocycytochrome *b₅* (5 mM phosphate at p^2H 7, 25 °C)

The acquisition parameters for the COSY spectrum were: spectral widths of 4000 and 1000 Hz respectively for the ^{13}C and ^1H dimensions; 512 t_1 increments of 2048 data points each; and 16 scans per increment.

Figure 5 ^1H – ^{13}C COSY spectra of ^{13}C -labelled N^ϵ,N^ϵ -dimethyl-lysine yeast iso-1-ferri cytochrome *c* variants in 50 mM sodium phosphate buffer, p^2H 7.0

(A) Wild type (Cys¹⁰²Thr) at 25 °C, (B) Lys⁷³Ala at 25 °C, (C) Lys⁷⁹Ala at 25 °C, (D) wild type (Cys¹⁰²Thr) at 35 °C, (E) Lys⁸⁶Ala at 35 °C, (F) Lys⁸⁷Ala at 35 °C. The arrows in (B), (C) and (E) indicate the locations of the peaks of Lys⁷³, Lys⁷⁹ and Lys⁸⁶ respectively.

The kinetic results, in combination with results of the potentiometric titrations described above, lead us to conclude that the electrostatic properties of the cytochrome *c*–cytochrome *b₅* complex are unaffected by the dimethylation of surface lysine residues of cytochrome *c* and that differences in complex stability and reactivity involve primarily non-electrostatic factors. As a result, dimethylated cytochrome *c* is a good NMR probe for characterizing complexes formed by cytochrome *c*.

Characterization of the NMR resonances of dimethyl-lysine horse and yeast cytochromes *c*

The ^{13}C NMR signals of the lysine ϵ -methyl groups appear in the chemical shift range expected for dimethylated species [73], with no signals in the 30–35 or 55–60 p.p.m. ranges expected for mono- or trimethylated lysine residues respectively (results not

shown), consistent with the mass spectrometric and potentiometric determination of the reaction product as dimethylated.

The ^1H – ^{13}C COSY spectrum of dimethyl horse ferri cytochrome *c* (Figure 4) contains cross-peaks from 15 of the 19 dimethyl-lysine groups. Two further resonances are present in the 1D ^1H spectrum, at chemical shifts of 2.67 and 2.72 p.p.m. respectively [23], but these do not appear in the 2D spectrum because they have relatively short relaxation times, perhaps associated with chemical exchange phenomena. Such effects seem to have a significant role in the appearance of the ^{13}C spectrum. Resonances 1, 2 and 15 (Figure 4) exhibit a significant decrease in their linewidths on increasing the temperature to 45 °C, consistent with a decrease in the contribution of a chemical exchange process to the linewidth as the rate of the process increased (results not shown). Two kinds of exchange process could be responsible for this behaviour. If rapid rotation about the ϵC –N bond of the dimethyl-lysine residue were prevented, the two methyl groups could become inequivalent and have different chemical shifts in either or both the ^1H and ^{13}C dimensions, as has been observed with calmodulin [74] and the fd gene 5 protein [31]. Alternatively, because lysine side chains are relatively mobile, they could move between two or more positions in each of which salt-bridging or hydrogen-bonding interactions with carboxylate groups could occur. Such behaviour has previously been suggested for Lys¹³ and Lys⁷⁹ of horse cytochrome *c* [75].

Unambiguous assignment of the dimethyl-lysine resonances in Figure 4 has not been possible. ^1H – ^1H TOCSY and NOESY spectra have not provided connectivities [64], and mutants of horse cytochrome *c* in which lysine residues have been selectively replaced are not available. Tentative assignments for some of the resonances have been obtained from the effect of $[\text{Cr}(\text{CN})_6]^{3-}$ on resonance linewidths [23,64] and the location of $[\text{Cr}(\text{CN})_6]^{3-}$ binding sites on the surface of the cytochrome [76] (Table 3).

The chemical shift dispersion of the dimethyl-lysine resonances (Figures 4 and 5) is partly a result of the presence of a paramagnetic haem causing pseudocontact shifts; the corresponding resonances of the diamagnetic reduced cytochromes overlap to a greater extent [23,64]. 2D ^{13}C – ^{13}C EXSY spectra of mixtures of dimethylated oxidized and reduced cytochrome *c* have allowed the ferri cytochrome *c* counterparts to some of the resolved ferri cytochrome *c* resonances to be identified, and all but one are in a heavily overlapped region with ^{13}C chemical shifts between 44.9 and 45.4 p.p.m. (results not shown). Consistent with this, many of the resonances of the modified ferri cytochrome have temperature-dependent chemical shifts that move towards the ferri cytochrome *c* chemical shift as temperature increases [64] (results not shown). Peak 15, with a ^{13}C chemical shift of 44.7 p.p.m. (Figure 4), is the only well-resolved resonance that has a negligible temperature dependence, and because there is a peak at the same position in the spectrum of the ferri cytochrome *c* this resonance must arise from a group far from the haem, consistent with the tentative assignment obtained from the effect of $[\text{Cr}(\text{CN})_6]^{3-}$ binding (Table 3) [23].

As with horse cytochrome *c*, the ^1H – ^{13}C COSY spectrum of dimethyl yeast cytochrome *c* (Figure 5) improves on the significant degree of overlap in the 1D spectra as well as showing the chemical shift correlations. Eleven of the expected 15 dimethyl-lysine signals are resolved at 35 °C, with resonances being significantly sharpened by the slightly elevated temperature. Chemical exchange leading to rapid relaxation in either or both of the ^1H and ^{13}C dimensions might cause the other resonance to be missing.

The ^1H – ^{13}C COSY spectra of dimethylated site-directed variants, Lys⁷³Ala, Lys⁷⁹Ala, Lys⁸⁶Ala and Lys⁸⁷Ala, of the yeast cytochrome *c* are shown in Figure 5. Although the assignments

Table 3 Effect of cytochrome *b*₅ and cytochrome *c* peroxidase on the dimethyl-lysine resonances of horse heart *N,N'*-dimethyl-lysine ferricytochrome *c*

The tentative lysine assignments were obtained from the effect of [Cr(CN)₆]³⁻ binding on NMR spectra [23]. The digital resolution for peak width measurements is 1 Hz per point in the ¹³C dimension and 1 Hz per point in the ¹H dimension. Abbreviations: u, unaffected by cytochrome *b*₅ or by CcP, ?, affected by cytochrome *b*₅ but resolution insufficient to quantify the effect, or linewidth could not be estimated owing to overlap.

Lysine resonance	Tentative lysine assignment	Maximum $\Delta(\delta)$ on binding cytochrome <i>b</i> ₅ (p.p.m.)		$\Delta(\delta)$ on binding CcP (p.p.m.)		Increase in peak width on binding CcP (Hz)	
		¹³ C	¹ H	¹³ C	¹ H	¹³ C	¹ H
1	7/25/27	0.03	0.02	0.03	0.02	2	3
2	13/72/86	u	u	0.13	0.06	8	u
3		0.02	0.01	u	u	?	u
4	7/25/27	u	u	u	u	u	2
5		0.03	0.02	u	u	u	5
6		u	u	u	u	u	u
7		u	u	u	u	u	?
8		?	?	u	0.01	?	?
9	13/72/86	0.05	0.03	0.03	0.04	u	3
10		?	?	-0.02	u	?	2
11		0.02	u	u	u	u	u
12	7/25/27	u	u	u	0.02	u	u
13	13/72/86	0.09	0.03	u	0.03	3	6
14	39/53/60	u	u	u	u	u	u
15	39/53/60	u	u	-0.04	-0.02	7	3

of the signals for dimethyl-lysine residues 73 and 79 are immediately apparent at 25 °C, those for lysine residues 86 and 87 are not. Increasing the temperature to 35 °C caused a narrowing of the signals and allowed the identification of the dimethyl-lysine 86 signal in the crowded region in the centre of the spectrum. The spectrum of the Lys⁸⁷Ala mutant contained all the signals resolved in the wild-type derivative, suggesting that the dimethyl-lysine 87 peak is one of those not appearing in the spectrum. The assignments for lysine residues 73 and 86 are not apparent from 1D spectra owing to spectral overlap.

NMR characterization of the interaction of cytochrome *b*₅ with *N,N'*-dimethyl-lysine horse cytochrome *c*

The addition of ferricytochrome *b*₅ to dimethylferricytochrome *c* causes ¹H NMR peaks of the cytochrome *c* haem to shift (results not shown). The most substantially affected were the haem methyl-3 and haem methyl-8 resonances (Figure 6). Similar perturbations have been observed for the interaction of ferricytochrome *b*₅ with both native cytochrome *c* and *N*-acetimidylated ferricytochrome *c* [16,18,21,22]; and they are indicative of the formation of a binary cytochrome *c*-cytochrome *b*₅ complex. Whitford et al. [22] have proposed that the biphasic plots resulting from similar experiments in which ferricytochrome *b*₅ is held constant and ferricytochrome *c* varied indicate the formation of ternary complexes. However, in the absence of supporting data we believe that the case for ternary complex formation is unproved and suggest that the observations of Whitford et al. are associated with a change in the binary cytochrome *c*-cytochrome *b*₅ complex that perturbs the cytochrome *b*₅ NMR spectrum but does not significantly affect the cytochrome *c* NMR spectrum [2].

The addition of ferricytochrome *b*₅ to dimethylferricytochrome *c* also causes small changes in the chemical shift of some of the *N*-CH₃ resonances. However, the 1D ¹H NMR spectrum is not sufficiently well resolved for these perturbations to be monitored easily [23]. To achieve adequate resolution we used ¹H-¹³C COSY spectra of the ¹³C-labelled dimethylferricytochrome *c*

Figure 6 Change in ¹³C chemical shifts of two *N,N'*-dimethyl-lysine ferricytochrome *c* *N*-CH₃ peaks (●, peak 9; ○, peak 13) and the comparable ¹H chemical shift of its haem methyl-8 resonance (□) on the addition of lipase-solubilized cytochrome *b*₅ to *N,N'*-dimethyl-lysine ferricytochrome *c*

(Figure 5). The incremental addition of small amounts of lipase-solubilized bovine microsomal cytochrome *b*₅ to a sample of ¹³C-labelled dimethylcytochrome *c* at low ionic strength (5 mM

Figure 7 ^1H – ^{13}C COSY spectra (50 mM sodium phosphate buffer, p^2H 7.0, 25 °C) of a 1:1 mixture of CcP and N,N' -dimethyl-lysine horse heart ferricytochrome *c* (A) and N,N' -dimethyl-lysine horse heart ferricytochrome *c* (B)

sodium phosphate buffer) resulted in a gradual increase in the cytochrome b_5 to cytochrome *c* ratio that was accompanied by a corresponding shift in the positions of the haem methyl resonances of cytochrome *c* (Figure 6). Some of the dimethyl-lysine ^1H – ^{13}C COSY peaks also shifted throughout the titration. The ^1H – ^{13}C COSY spectrum of a 1:1 mixture of these two proteins obtained at low ionic strength is shown in Figure 4, and the change in ^{13}C chemical shifts of peaks 9 and 13 as a function of the ratio of cytochrome b_5 to cytochrome *c* is shown in Figure 6. In all, at least six of the ^1H – ^{13}C methyl resonances were shifted by the cytochrome b_5 . Increasing the buffer concentration to 50 mM significantly attenuated the ability to detect complex formation by NMR spectroscopy, with only peaks 9 and 13 shifting slightly, consistent with the acute inverse dependence of the stability of the complex on ionic strength [77].

The interaction between horse N,N' -dimethylated cytochrome *c* and cytochrome *c* peroxidase

In similar experiments to those described above, the effect of CcP complexation on N,N' -dimethyl-lysine horse heart cytochrome *c* was monitored. The addition of CcP caused a shift for haem methyl resonances and for some of the lysine N-CH_3 signals. The ^1H – ^{13}C COSY spectrum of a 1:1 mixture of N,N' -dimethylated horse heart cytochrome *c* and CcP is given in Figure 7. Seven dimethyl-lysine N-CH_3 resonances were shifted by at least 0.02 p.p.m. in at least one dimension by interaction with CcP and, as with the yeast cytochrome *c*, some of the resonances were also broadened (Table 3). Linewidths for the unbound cytochrome *c* were 12–13 Hz in the proton dimension and 3–9 Hz in the carbon dimension, with five of the seven shifted resonances being broadened by at least 3 Hz on complexation. Only one resonance, peak 5, was broadened without a simultaneous chemical shift change.

Interaction between yeast N,N' -dimethylated iso-1-cytochrome *c* and cytochrome *c* peroxidase

A titration was performed in which small volumes of a CcP solution were added to a sample of ^{13}C -labelled dimethyl yeast cytochrome *c*, thus gradually increasing the ratio of CcP to cytochrome *c* during the experiment. The addition of CcP to dimethyl iso-1-ferricytochrome *c* caused ^1H NMR peaks of the

Figure 8 Aliphatic region of the ^1H NMR spectrum of N,N' -dimethyl-lysine yeast iso-1-ferricytochrome *c* in the presence and the absence of CcP, showing the N-CH_3 resonance of trimethyl-lysine 72 (indicated by T) (50 mM phosphate buffer, p^2H 7.0)

Bottom spectrum, cytochrome *c* in the absence of CcP; middle spectrum, a 0.5:1.0 mixture of CcP and modified cytochrome; top spectrum, a 1.4:1.0 mixture of CcP and modified cytochrome *c*.

ferricytochrome to shift (results not shown), as has been observed previously for cytochrome *c*–CcP complex formation [4,20,27]. Satterlee et al. [20] observed a change in chemical shift of +0.7 p.p.m. in the haem methyl-3 resonance of horse cytochrome *c* in a solution containing equimolar concentrations of cytochrome *c* and CcP in 0.01 M KNO_3 , p^2H 6.7, in comparison with +0.8 p.p.m. in the present work. These authors also observed [20] a –0.5 p.p.m. shift for the haem methyl-8 resonance, which moved less than 0.05 p.p.m. in our experiments. This difference

Table 4 Chemical shift changes in the ^1H – ^{13}C COSY spectrum of ^{13}C -labelled *N*^ε,*N*^ε-dimethyl-lysine yeast iso-1-ferricytochrome *c* on binding CcP

The digital resolution for line width measurements is 0.6 Hz per point in the ^{13}C dimension and 1 Hz per point in the ^1H dimension. Abbreviation: ?, the signal was affected, but broadening or shifting has obscured the signal in the bound protein, or a line width could not be estimated owing to overlap.

Dimethyl-lysine chemical shift in <i>N</i> ^ε , <i>N</i> ^ε -dimethyl-lysine yeast iso-1-ferricytochrome <i>c</i> (p.p.m.)			$\Delta\delta$ with CcP (p.p.m.)		Increase in line width on binding CcP (Hz)	
^{13}C	^1H	Dimethyl-lysine assignment	^{13}C	^1H	^{13}C	^1H
45.46	3.00	73	0.01	u	2	2
45.33	2.92		0.04	–0.01	u	4
45.27	2.85		–0.02	–0.02	u	u
45.23	2.81	86	–0.01	–0.01	?	u
45.19	2.82		?	?	?	?
45.19	2.75		u	u	u	u
44.88	2.79	79	–0.03	–0.01	7	u
43.84	2.18		u	–0.04	7	2
43.59	2.79		0.02	0.02	2	u

between results might be due to the different buffer conditions in the two studies. In the titration performed by Moench et al. [27], in which CcP concentration was gradually increased in a solution of yeast iso-1-cytochrome *c*, the haem methyl-3 resonance was observed to split into bound and unbound signals at low protein concentration in 10 mM KNO_3 , p^2H 6.4, demonstrating that the equilibrium was slow on the NMR timescale. In our study, lowering the temperature of the cytochrome *c*/CcP mixture to 4 °C did not split the resonance, indicating that under our conditions of ionic strength the complex remained in fast chemical exchange.

The addition of CcP to yeast iso-1-ferricytochrome *c* also caused small changes in the chemical shifts of some of the lysine *N*- CH_3 resonances. The methyl resonance of trimethyl-lysine 72, which was present in the unmodified protein and was well resolved at 3.4 p.p.m. in the ^1H spectrum, was shifted and broadened by the addition of CcP (Figure 8). A similar effect is seen for the addition of ferricytochrome *b₅* to *Candida krusei* ferricytochrome *c* [18]. The ^{13}C -labelled dimethyl-lysine peaks were not sufficiently well resolved in 1D spectra to monitor complexation-induced perturbations, so ^1H – ^{13}C COSY spectra were used (results not shown). In all, seven of the nine resolved peaks in the spectrum were shifted by the addition of CcP by 0.01 p.p.m. or more in at least one dimension (Table 4). These perturbations in chemical shift are significant in comparison with the digital resolution (0.006 p.p.m. per point and 0.0025 p.p.m. per point in the ^{13}C and ^1H dimensions respectively) but not all are significant in comparison with the effect of cytochrome *b₅*'s binding to *N*^ε,*N*^ε-dimethylated horse heart cytochrome *c*. In this case 6 out of 15 resonances shifted in one dimension by at least 0.02 p.p.m. The resonance of *N*^ε,*N*^ε-dimethylated yeast iso-1-cytochrome *c* corresponding to dimethyl-lysine 79 shifted by 0.04 p.p.m. in the proton dimension, whereas those corresponding to lysine residues 73 and 86 did not shift significantly on the addition of CcP. In addition to shifting, some of the *N*- CH_3 resonances of the yeast cytochrome *c* broadened on the addition of CcP. Linewidths in the magnitude mode ^1H – ^{13}C COSY spectrum of the unbound yeast cytochrome *c* were in the range 9–14 Hz in the proton dimension and 4–11 Hz in the carbon dimension and, as indicated in Table 4, the three peaks undergoing the largest chemical shift changes, in at least one dimension, on binding CcP also broadened by 4 Hz or more in one or other dimension. This result provides compelling evidence that these lysine residues are directly involved in the complex with their

conformational flexibility, which causes them to have a relatively sharp line in the unbound cytochrome, being restrained somewhat by the complex formation. Taken together, the chemical shift and linewidth changes (Table 4) indicate that at least six lysine residues are involved in intermolecular contacts with CcP.

DISCUSSION

NMR is most useful for investigating protein structures when resonances have been unambiguously assigned, but even though some of the cytochrome *c* lysine residues cannot be monitored by the approach adopted in this paper (4 of the 19 lysine residues of horse cytochrome *c* and 4 of the 11 lysine residues of yeast cytochrome *c*), and unambiguous assignments are available for only three lysine residues of the yeast protein, important information about interprotein complexes can be obtained. In the most extreme case with the unobserved lysine residues involved in interprotein contacts, the argument made below for the existence of dynamic complexes in which the proteins adopt a multitude of different orientations is considerably strengthened.

The ^1H – ^{13}C COSY spectra of ferricytochrome *b₅* binding to *N*^ε,*N*^ε-dimethyl-lysine ferricytochrome *c* (Figure 4) reveal that of the 15 dimethyl-lysine residues observed, six are perturbed by complex formation (Table 3). The most affected are the peak 9 and 13 lysine residues, which titrate with increasing ferricytochrome *b₅* in a similar fashion to the haem methyl resonances (Figure 6). These lysine residues are associated with a $[\text{Cr}(\text{CN})_6]^{3-}$ -binding site close to lysine residues 13, 72 or 86 [23], although not all the lysine residues associated with this site are perturbed by ferricytochrome *b₅* as peak 2 is affected by $[\text{Cr}(\text{CN})_6]^{3-}$ but not by the cytochrome (Table 3). Also, one of the lysine residues associated with a second $[\text{Cr}(\text{CN})_6]^{3-}$ -binding site, lysine 7, 25 or 27, is affected by ferricytochrome *b₅* but the other two lysine residues are not. Both of these $[\text{Cr}(\text{CN})_6]^{3-}$ -binding sites are close to the exposed haem edge and thus it seems that ferricytochrome *b₅* binds to the region of cytochrome *c* that includes its exposed haem edge, interacting with a minimum of six lysine residues as it does so. The variable magnitude of the chemical shift perturbations indicates that the six lysine residues are not affected equally in NMR terms.

Chemical shifts are highly sensitive to the local magnetic environments of nuclei and these might be altered when two proteins form a complex because the nuclei being detected are located at the interprotein binding region on the surface of one

of the partners, or because the environments of nuclei not located at the interprotein interface region are altered by a conformational change induced by complex formation. All available evidence points to the formation of a complex by cytochromes *c* and *b₅* without conformational changes [2], so the chemical shift perturbations (Table 3) are because the *N*-CH₃ groups are located in the interface region. There are no reported studies directly related to ours, but in comparison with complexation-induced shifts for peptide ¹H-¹⁵N resonances used to map intermolecular interaction regions for a number of protein-protein complexes in which major conformational changes did not accompany complexation [78–82], the chemical shift perturbations we observed are small. This is because we were looking at relatively weakly associated proteins for which the perturbations were a weighted average of their values in the complexed and uncomplexed states. It also seems likely that the expected perturbations in chemical shift are spread over more lysine residues than are involved in intermolecular interactions at any moment as a consequence of the dynamic nature of the complex. This is consistent with the observation that more lysine residues are seen to be perturbed than expected from molecular modelling studies of static complexes [1,83,84], and is consistent with Brownian dynamics studies [55] and other NMR investigations with different approaches [18,24]. A similar observation of smaller perturbations in chemical shift than expected for the complex of cytochrome *c* with plastocyanin has also been accounted for by the assumption that the proteins adopt a multitude of different orientations within the complex [30].

The crystal structures of CcP complexed with yeast iso-1-cytochrome *c* and horse cytochrome *c* [13] have provided a focus for discussions about these complexes in solution. Whereas the crystallographic data provide static structures for the complexes, the existence of alternative, more dynamic, structures has been a topic of continuing debate [14], with much evidence pointing to the formation of 2:1 cytochrome *c*-CcP complexes under a wide range of solution conditions [85–88]. A further indication of the complexity of the solution state interaction comes from energy transfer measurements [89] and ¹H NMR studies of the shielding of amide ¹H-²H exchange of cytochrome *c* by complex formation with CcP [28]. These suggest there are several closely related dynamically interconverting binding conformers, consistent with Brownian dynamics simulations [90].

In this context of multiple interconverting binding conformers our NMR findings are revealing in that they support a model in which there is more than one binding orientation, with some interfacial movement of the two proteins within the complex. A significant observation from our work (Tables 3 and 4) is the number of cytochrome *c* lysine methyl resonances shifting on complexation with CcP: seven for the horse heart cytochrome and six for the yeast cytochrome. The crystal structures of the cytochrome *c*-CcP complexes [13] show only three hydrogen bonds with lysine residues (8, 72 and 87) for the horse protein and two potential hydrogen bonds with lysine residues (73 and 87) of the yeast protein, with lysine 86 involved in an additional intermolecular van der Waals interaction. Neither interaction scheme is consistent with the NMR data. As with the cytochrome *c*-cytochrome *b₅* complex, the small magnitude of the perturbations in chemical shift (Tables 3 and 4) is consistent with the existence of several different complexes, each of which involves only a few of the lysine residues. This observation is fully consistent with the results of Brownian dynamics simulations of Northrup et al. [5], who concluded that several sites might be visited at each encounter between the proteins. During the relatively long-lived encounter, several plausible electron-transfer orientations were observed rather than a single dominant com-

plex. Northrup et al. [5] listed six lysine residues on horse cytochrome *c* as being most frequently involved in ionic contacts with CcP: residues 13, 25, 27, 72, 79 and 86. Interestingly, all three of the NMR signals tentatively assigned to lysine residues 13, 72 and 86 are broadened and shifted by the binding interaction (Table 3). The perturbation to the NMR signals of trimethyl-lysine 72 and dimethyl-lysine 79 of the yeast cytochrome *c* demonstrates the involvement of these two groups in intermolecular contacts, but the signal of dimethyl-lysine 73 is only minimally affected by complex formation (Table 4). Overall, the yeast cytochrome *c* NMR data are more in keeping with the theoretical predictions of Northrup et al. [5] for the horse cytochrome *c*-CcP complex than with the X-ray structure of the yeast cytochrome *c*-CcP complex.

Conclusions

Dimethylation of lysine residues provides an NMR spectroscopy probe of intermolecular interactions. For cytochrome *c* interacting with CcP and with cytochrome *b₅*, NMR studies are consistent with a model of the interprotein interactions that is dynamic, with several sites on cytochrome *c* being visited at each encounter between the proteins. 2D ¹H-¹³C NMR has been shown to be useful for monitoring the dimethyl-lysine groups but relatively short relaxation times prevented all the resonances from being observed. It is only the paramagnetism of the ferrihaem of cytochrome *c* that permits the chemical shift dispersion observed in this work; for diamagnetic proteins containing a large number of lysine residues, resolution might be a limiting factor.

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