¹H-n.m.r. investigation of the interaction between cytochrome *c* and cytochrome *b₅*

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The interaction between eukaryotic cytochrome *c* and the tryptic fragment of bovine liver microsomal cytochrome *b₅* was studied by ¹H-n.m.r. spectroscopy, and a procedure was developed that may be generally applicable to the study of macromolecular interactions by n.m.r. At pH 6.3 (27°C, *I* approx. 0.04) the two ferricytochromes were found to form a 1:1 complex with an association constant of approx. 10³ M⁻¹. The protein–protein-interaction region was found to encompass the region of the surface of horse cytochrome *c* that includes Ile-81, Phe-82, Ala-83 and Ile-85, and Lys-13 and Lys-72 of horse cytochrome *c* were suggested to be involved in two important intermolecular interactions. Me₃Lys-72 of *Candida krusei* cytochrome *c* was shown to be involved in the interaction.

Two of the best-characterized classes of electron-transfer proteins are the eukaryotic cytochromes *c* and the cytochromes *b₅*. Eukaryotic cytochromes *c* are monohaem proteins of 103–114 amino acid residues with a redox potential of 260 ± 20 mV, whose main physiological function is to transport electrons from cytochrome reductase to cytochrome oxidase in the mitochondrial respiratory chain (Lemberg & Barrett, 1973). The cytochrome *b₅* class of proteins is characterized (Guiard & Lederer, 1979) by the presence of a monohaem globular domain of about 90 amino acid residues (cytochrome *b₅*) either attached to another globular domain, as in sulphite oxidase or *Saccharomyces* lactate dehydrogenase (Guiard & Lederer, 1976, 1979), or attached to a hydrophobic peptide that is responsible for binding cytochrome *b₅* to a membrane, as in microsomal cytochrome *b₅* or the cytochrome *b₅* located in the mitochondrial outer membrane (Ito & Sato, 1968; Spatz & Strittmatter, 1971; Fukushima & Sato, 1973; Nisimoto *et al.*, 1977). The redox potential of cytochrome *b₅* is in the range -5 to 20 mV (Iyanagi, 1977; Abe & Sugita, 1979; Reid *et al.*, 1982), and thus it is capable of acting as an electron donor to cytochrome *c*. Indeed, the reduction of ferricytochrome *c* by ferrocycytochrome *b₅* is a rapid reaction with a rate constant of approx. 10⁷ M⁻¹·s⁻¹ (Strittmatter, 1964; Stonehuerner *et al.*, 1979).

Physiologically important reactions between cytochrome *c* and cytochrome *b₅* occur in two well-documented cases: namely those of sulphite oxidase (Johnson & Rajagopalan, 1977, 1980), which is

located in the intermembrane space of animal mitochondria (Rajagopalan, 1980), and *Saccharomyces* L-lactate dehydrogenase (Lemberg & Barrett, 1973; Briquet *et al.*, 1982), which is also located in the intermembrane space (Daum *et al.*, 1982). In addition to these reactions, under some conditions cytochrome *c* may act as a shuttle between the mitochondrial outer-membrane cytochrome *b₅* and cytochrome oxidase, although the physiological significance of this reaction is not clear (Nicholls *et al.*, 1969; Matlib & O'Brien, 1976; Osheroff *et al.*, 1978; Bernadi & Azzone, 1981).

As a result of the studies mentioned above, the reaction of cytochrome *c* with cytochrome *b₅* is the ideal choice for the study of haemoprotein-mediated electron transfer. This requires the structures of the precursor and product complexes, namely ferricytochrome *c*–ferrocycytochrome *b₅* and ferrocycytochrome *c*–ferricytochrome *b₅*, respectively, to be determined. Such a determination is greatly assisted by knowledge of the structures of the two haemoproteins obtained by crystallographic methods (Mathews *et al.*, 1972; Mathews, 1980; Takano & Dickerson, 1981*a,b*). Indeed, Salemme (1976) has proposed a hypothetical structure for the complexes based on the X-ray-crystallographic structures, and kinetic studies of chemically modified cytochrome *c* reacting with cytochrome *b₅* have supported the general outline of Salemme's model (Ng *et al.*, 1977; Smith *et al.*, 1980). However, so far the complex has not been studied by a direct structural method. In the present paper we present a study of the complexes ferricytochrome *c*–ferricytochrome *b₅*,

and ferrocytochrome *c*–ferricytochrome *b₅* by high-resolution ^1H -n.m.r. spectroscopy, and we describe a procedure for studying interactions between macromolecules that may be generally applicable (Williams *et al.*, 1982).

Materials and methods

$\text{K}_3[\text{Cr}(\text{oxalate})_3]$ was prepared by the method of Yost (1939). Horse cytochrome *c* (type VI) and *Candida krusei* cytochrome *c* (type VIII) were obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.), and samples were prepared for n.m.r. as previously described (Eley *et al.*, 1982*b*). Cytochrome *b₅* was prepared from bovine liver microsomal fraction by the procedure of Omura & Takesue (1970), which involved tryptic digestion of the microsomal fraction followed by chromatography on Sephadex G-100 (10 mM-sodium phosphate buffer, pH 7.5) and DEAE-Sephadex (50 mM-sodium phosphate buffer, pH 7.5), with the final elution obtained with a linear gradient of KCl (0–0.35 M in 50 mM-sodium phosphate buffer, pH 7.5). Gel electrophoresis, optical spectra and n.m.r. spectra were used to check the purity of the product. The optical purity index ($A_{355}^{\text{red.}}/A_{280}^{\text{ox.}}$) was 1.27, which compares with reported values of

1.28–1.32 (Omura & Takesue, 1970). The yield of cytochrome *b₅* was 26 mg from approx. 1 kg of liver. Samples were prepared for n.m.r. by the procedure described for cytochrome *c* (Eley *et al.*, 1982*b*). The cytochrome *b₅* was reclaimed after mixing with cytochrome *c* by chromatography on CM-cellulose (CM-23; 5 mM-sodium phosphate buffer; pH 7), which bound cytochrome *c* but did not bind cytochrome *b₅*.

The n.m.r. spectra were recorded either with a Bruker 300 MHz spectrometer or with the 470 MHz spectrometer developed by the Department of Biochemistry, University of Oxford. Paramagnetic difference spectra were obtained as previously described (Eley *et al.*, 1982*a*). 1,4-Dioxan was used as an internal standard, but all chemical shifts are quoted in p.p.m. downfield from the methyl resonance of 2,2-dimethyl-2-silapentane-5-sulphonate.

Results and discussion

N.m.r. of complexes formed between cytochrome c and cytochrome b₅

The low-field regions of the n.m.r. spectra of ferricytochrome *c*, ferricytochrome *b₅* and various mixtures of the two proteins are shown in Fig. 1. All

Fig. 1. Low-field regions of the 300 MHz ^1H -n.m.r. spectra of (a) 4 mM-ferricytochrome *c*, (b)–(e) 4 mM-ferricytochrome *c* and 0.57 mM-, 0.8 mM-, 1.33 mM- and 2 mM-ferricytochrome *b₅* respectively, and (f) 2 mM-ferricytochrome *b₅*. All the samples were unbuffered in $^2\text{H}_2\text{O}$ at pH 6.3 ± 0.1 and at 27°C . The resonances marked M are from haem methyl groups and those marked P are from haem propionate groups.

the resonances downfield of 11 p.p.m. come from the haem group and axial ligands of the iron (Redfield & Gupta, 1971; Keller *et al.*, 1976; Keller & Wüthrich, 1978; Burns & La Mar, 1979; La Mar *et al.*, 1981; Ångström *et al.*, 1982). Ferricytochrome *b₅* exists in two isomers that are related by the orientation of the haem group and that have different n.m.r. spectra (Keller *et al.*, 1976; Keller & Wüthrich, 1980; La Mar *et al.*, 1981). The major isomer, which corresponds to the X-ray-crystallographic structure (Mathews, 1980), forms approx. 90% of the cytochrome *b₅* studied in the present work.

The titration shown in Fig. 1 was performed at a constant concentration of ferricytochrome *c* at pH 6.3 ± 0.1 with no additional salts added. The ionic strength varied throughout the titration from 0.04 M to 0.055 M; these values were calculated by taking the net charge of horse ferricytochrome *c* to be 10+ (Margoliash *et al.*, 1961) and the net charge of ferricytochrome *b₅* to be 7– (Ozols & Strittmatter, 1969; Tsugita *et al.*, 1970). As the fraction of ferricytochrome *c* bound to ferricytochrome *b₅* increased, some of the haem-*c* resonances shifted (Fig. 1). These shifts result from complex-formation, since they are both similar to the shifts resulting from ferricytochrome *c* binding to cytochrome *c* peroxidase and to polyglutamate (Boswell *et al.*, 1980) and different from the pH-induced shifts (Burns & La Mar, 1979; Ångström *et al.*, 1982). The shifts of Fig. 1 indicate that cytochrome *c* is in fast exchange between the free and the bound forms. The largest of the shifts was that of the haem methyl-8 resonance at approx. 35 p.p.m., which was shifted upfield by 0.4 p.p.m. at the end of the titration. Analysis of this shift, assuming the formation of a 1:1 cytochrome *c*–cytochrome *b₅* complex, yielded a binding constant of $960 \pm 100 \text{ M}^{-1}$ (Fig. 2).

The concentration of cytochrome *b₅* varied throughout the course of the titration, and thus the intensity of its resonances decreased. Nevertheless it is clear from Fig. 1 that many of the cytochrome *b₅* resonances shifted throughout the titration. The most marked shifts were to the propionate βCH resonances at approx. 16.3 p.p.m. and approx. 16.0 p.p.m., but other resonances also shifted. These shifts are similar to those that occur with decreasing pH (Keller *et al.*, 1976), and thus they may be caused by a small fall in pH (<0.05 pH unit).

The spectra of Fig. 1 do not give any detailed information on the location of the binding site, because the paramagnetically shifted resonances are so sensitive to their environment. Indeed, the cytochrome *c* resonance most affected by complex-formation comes from haem methyl-8, which is buried within the protein (Takano & Dickerson, 1981*a,b*). In order to obtain information about the binding site, spectra of the amino acid resonances were obtained. Fig. 3 shows the spectra between

Fig. 2. Graph showing the inverse of the induced shift of the haem methyl-8 resonance of horse ferricytochrome *c* as a function of the inverse of the ferricytochrome *b₅* concentration

The data were obtained from the spectra of Fig. 1 and analysed for 1:1 complex-formation with the equation:

$$\frac{1}{\delta_i} = \frac{1}{\Delta_i} + \frac{1}{K\Delta_i[\text{Cyt. } b_5]}$$

where δ_i is the induced shift, Δ_i is the shift in the fully bound complex and K is the association constant. This equation is valid under conditions where the fraction of cytochrome *b₅* (Cyt. *b₅*) bound is negligible compared with the fraction unbound. From the graph: $\Delta_i = 0.57 \text{ p.p.m.}$ and $K = 960 \pm 100 \text{ M}^{-1}$. The inset shows the ionic-strength-dependence of the association constant determined by Mauk *et al.* (1982) extrapolated to the ionic strength used in the present work; ●, Mauk *et al.* (1982); △, present work.

10 p.p.m. and -0.5 p.p.m. of horse ferricytochrome *c*, ferricytochrome *b₅* and an equimolar mixture of the two. The spectrum of the mixture appears to be a superposition of the spectra of the individual proteins but considerably broadened.

The broadening results from the increased rotational correlation time (τ_R) for the complex compared with that for the individual proteins. It is important to determine the correlation time of the complex, though at present only a rough estimate is possible. τ_R for a globular protein can be estimated from the Stokes–Einstein equation by assuming the protein to be spherical (Dwek, 1973). The calculation for cytochrome *c* yields a value of $4 \times 10^{-9} \text{ s}$ compared with the experimentally determined value of $6 \times$

Fig. 3. 470 MHz ^1H -n.m.r. spectra of (a) 4 mM-ferricytochrome *c*, (b) 2 mM-ferricytochrome *c* and 2 mM-ferricytochrome *b*₅, and (c) 4 mM-ferricytochrome *b*₅, all in $^2\text{H}_2\text{O}$ at pH 7.2 and at 25°C

Resonances marked H are histidine resonances, and the resonance marked N is the *N*-acetyl methyl resonance. The sharp peak at 8.5 p.p.m. in (a) and (b) is from an impurity.

10^{-9} s (Andersson *et al.*, 1979). However, for a markedly non-spherical system the Stokes-Einstein equation is not applicable (Cantor & Schimmel, 1980). Approximating the complex of cytochromes to an ellipsoid means that a minimum of three correlation times have to be determined to describe its motion: a time for rotation about the long ellipsoidal axis, a time for rotation about the short ellipsoidal axis, and a time for the independent motion of one protein within the complex. These rotational correlation times cannot be calculated from the data in the present paper, although an upper limit for the overall correlation time for complexed cytochrome *c* can be estimated. The maximum τ_R can be obtained by assuming the complex is a sphere of 3.22 nm radius (radius of cytochrome *c* = 1.65 nm; radius of cytochrome *b*₅ = 1.57 nm). Then, $\tau_R = 3 \times 10^{-8}$ s.

Although the broadening affects most of the resonances of the complex, some resonances remain sharp. Most notably, the singlet resonance of ferricytochrome *c* at 1.95 p.p.m. marked N, and the singlet resonances of ferricytochrome *b*₅ at approx. 7.9 p.p.m. and approx. 7.6 p.p.m. marked H (Fig. 3). Resonance N comes from the *N*-terminal *N*-acetyl group of cytochrome *c* (Boswell *et al.*, 1981). This resonance is particularly sharp in the spectrum of ferricytochrome *c* because its linewidth is principally determined not by the τ_R of the protein but rather by independent motion of the *N*-terminus of the protein chain. Even in the crystalline state the *N*-terminus of

the protein is mobile (Takano & Dickerson, 1981*a,b*). Since complex-formation does not restrict the motion of the *N*-acetyl group, it is unlikely to lie in the protein-protein-interaction region. The sharp ferricytochrome *b*₅ resonances (resonances H) have not been assigned, but they must also correspond to nuclei that are not in the protein-protein-interaction region. These resonances are pH-independent over the range pH 6.3–7.2, but nevertheless they must arise from one or more of the non-ligated histidine residues at positions 15, 26 and 80, although it is not clear from which.

Candida krusei cytochrome *c* contains Me₃Lys-72 (Narita & Titani, 1968; Lederer, 1972), and its trimethyl resonance is exceedingly sharp (Boswell *et al.*, 1982): its linewidth at half peak height is approx. 10 Hz. This indicates that the trimethyl group undergoes rapid motion independent of the rotation of the protein. Spectra of mixtures of *C. krusei* ferricytochrome *c* and ferricytochrome *b*₅ are markedly broadened as a result of the complex formation, and it is particularly significant that the sharp Me₃Lys-72 resonance at 3.44 p.p.m. is also considerably broadened. This indicates that the motion of Me₃Lys-72 is restricted within the complex, probably because it forms part of the protein-protein-interaction region.

The interaction of horse ferrocytochrome *c* with ferricytochrome *b*₅ was studied in the same manner as were the complexes of ferric proteins. However, since ferrocytochrome *c* is diamagnetic, there were

no paramagnetically shifted resonances that could be satisfactorily used to obtain an association constant. Complex-formation was again accompanied by extensive broadening of the spectrum, and again resonances N and H remained sharp. None of the resolved resonances of ferrocytochrome *c* were shifted by the complex-formation.

N.m.r. competition experiments with paramagnetic reagents

The problems introduced by non-specific broadening can be partly overcome by the use of paramagnetic reagents to amplify specific effects resulting from complex-formation by two macromolecules. Fig. 4 illustrates the idea behind the experiment. In Fig. 4(i) three resonances of protein I are non-specifically broadened as a result of the formation of a complex between proteins I and II. The resulting difference spectrum resembles a convolution difference spectrum (Campbell *et al.*, 1973). The specific effects of a small relaxation reagent (such as Gd^{3+} , Mn^{2+} and Cr^{3+}) bound to protein I in the absence of protein II are illustrated in Fig. 4(ii). One resonance (α) is unaffected, one (β) is weakly affected, and one (γ) is strongly affected. The effect of the relaxation reagent on the resonances of protein I in the complex formed between proteins I and II is illustrated in Fig. 4(iii). Only resonance β is affected. A comparison of the difference spectra (*f*) and (*i*) reveals which resonances are protected from the paramagnetic reagent by complex-formation. In this case only resonance γ is protected. The protected resonances come from groups of protein I that are shielded by the bound protein II, and thus

specific information is obtained about the interaction between the two proteins.

There are a number of complications to this experiment, but they should be open to complete analysis. Firstly, in a solution of a mixture of two proteins and a small reagent, a number of different complexed species may exist, with the amounts of each depending on the initial concentrations of the uncomplexed species and their relative association constants. This creates a problem: if the presence of protein II results in the loss of a resonance of protein I from a paramagnetic difference spectrum [such as resonance γ from Fig. 4(*i*)] it could be because protein II sequesters the reagent, or because protein II and the reagent compete for the same binding site on protein I. In general, it is not possible to distinguish between these two mechanisms unless the appropriate association constants are known. However, when there are several binding sites for the small reagent on protein I, a test of which mechanism operates is possible. It is necessary to determine if all the resonances of protein I are protected by protein II or if only resonances from one of the small reagent sites are protected. If the latter, it is necessary to know if resonances from a strong binding site or a weak binding site are protected. When resonances from a strong binding site are protected and resonances from a weak binding site are unprotected, protein II must be shielding particular regions of protein I. This is the situation illustrated in Fig. 4.

Secondly, it is assumed that when proteins I and II form a complex they do not undergo a large conformation change that disrupts a reagent-binding

Fig. 4. Simulated spectra illustrating the effects of (i) correlation time broadening, (ii) paramagnetic broadening and (iii) a combination of the two effects

(i) (a) Three resonances in the spectrum of a macromolecule (I) with linewidths at half peak height of 25 Hz. (b) The effect of binding I to a second macromolecule (II) is to broaden the resonances non-specifically; the linewidths are 50 Hz. (c) The difference spectrum (a)–(b). (ii) (d) The spectrum of I. (e) The effect of adding a specific small paramagnetic relaxation reagent. Resonance α is unaffected, but the linewidths of resonances β and γ increase to 50 Hz and 100 Hz respectively. (f) The difference spectrum (d)–(e). (iii) (g) The spectrum of I bound to II. (h) The effect of adding the relaxation reagent. Only resonance β is affected, its linewidth increases to 75 Hz. (i) The difference spectrum (g)–(h).

site distant from the protein-protein-interaction site.

Thirdly, the different rotational correlation times of the protein complex compared with those of the individual proteins may affect the degree of paramagnetic relaxation in two ways. If τ_R for the complex is greatly increased so that the electron-spin relaxation time of the paramagnetic reagent is $>\tau_R$, the relaxation reagent will not produce significant dipolar relaxation (Dwek, 1973). Also, if τ_R increases, Curie-spin relaxation may become important (Gueron, 1975). In the case of ferricytochrome b_5 competing with $[\text{Cr(oxalate)}_3]^{3-}$ for ferricytochrome c under the conditions employed in the present work, the increase in τ_R is not large enough to produce the problems described above. In addition to this, complex-formation is not accompanied by a conformation change to the interior of cytochrome c . This is evident from the observation that the chemical shifts of conformationally sensitive resolved resonances of cytochrome c (i.e. resonances of groups that experience large conformation-dependent shifts) are unaffected (to within ± 0.03 p.p.m.) by the complex-formation. These resonances belong to the following groups: Thr-19, Leu-32, Ile-57, Leu-64, Leu-68, Met-80, Phe-82, Ile-85 and Leu-94. Finally, it is shown below that ferricytochrome b_5 shields a specific region of ferricytochrome c .

It may be useful at this stage to point out some of the factors that influence the appearance of resonances in paramagnetic difference spectra [such as Fig. 4(f)]. The most important factors are: the distance between the bound paramagnetic reagent and the nucleus under observation; the natural resonance linewidth of the nucleus under observation; the occupancy of the reagent-binding site. The basis of the competition experiment described above is that the occupancy of the reagent-binding site will be affected by the presence of protein II. The importance of the natural linewidth and the distance between the nucleus and bound reagent can be seen from the following hypothetical example.

Consider the appearance of two resonances in a paramagnetic difference spectrum; one resonance, A, has a natural linewidth of 10 Hz, and the second resonance, B, has a natural linewidth of 20 Hz. If both are equally broadened by the paramagnetic reagent, then A will be more intense in the difference spectrum than B at low reagent-binding-site occupancies. Now, consider the case when resonances A and B have the same natural linewidths but nucleus A is closer to the binding site than is nucleus B. In this case too resonance A will be more intense in the difference spectrum. These factors are important in any quantitative use of paramagnetic relaxation (Dwek, 1973) when they have to be assessed from measurements of natural linewidths

and from a detailed analysis of resonance intensities in paramagnetic difference spectra as a function of the mole-fraction of reagent. Such measurements have been made for the binding of $[\text{Cr(CN)}_6]^{3-}$ and $[\text{Cr(oxalate)}_3]^{3-}$ to cytochrome c (Eley, 1982; Eley *et al.*, 1982a). These complications do not affect the interpretations given in the present paper for the competition between cytochrome b_5 and $[\text{Cr(oxalate)}_3]^{3-}$ for cytochrome c .

Competition between ferricytochrome b_5 and $[\text{Cr(oxalate)}_3]^{3-}$ for ferricytochrome c

Binding of the relaxation reagent $[\text{Cr(oxalate)}_3]^{3-}$ to ferricytochrome c has been extensively investigated by n.m.r. (Williams *et al.*, 1977; Eley *et al.*, 1982a; Eley, 1982). There are three sites close to the exposed haem edge at which $[\text{Cr(oxalate)}_3]^{3-}$ binds, sites I–III. Important binding groups in these sites are Lys-86 and Lys-87 (site I), Lys-25 and Lys-27 (site II), and Lys-13 and Lys-27 (site III). The individual association constants have not been determined, but they are extremely sensitive to ionic strength. Under the conditions of the present experiments ($I \sim 0.05$), the association constant for site III is approx. $800 \pm 200 \text{ M}^{-1}$. The relative association constants are site III $>$ site II $\approx$ site I.

Binding of $[\text{Cr(oxalate)}_3]^{3-}$ by ferricytochrome b_5 has not been previously studied. Addition of $[\text{Cr(oxalate)}_3]^{3-}$ caused only weak specific perturbations to the n.m.r. spectrum of ferricytochrome b_5 . Even at 95 mol of $[\text{Cr(oxalate)}_3]^{3-}$ /100 mol of cytochrome b_5 there were few resonances that were clearly specifically affected (Fig. 5). Resonances that were specifically affected included the histidine resonances at approx. 7.6 and approx. 7.9 p.p.m., lysine resonances at approx. 3 p.p.m. and aliphatic resonances, probably from methyl groups, between 1.6 and 0.6 p.p.m. These results indicate weak binding, and they are in keeping with the structure of cytochrome b_5 : the molecule contains only ten lysine and arginine residues, which are not clustered at a particular site on the protein, and it has a pI approx. 4.3 (Ozols & Strittmatter, 1969; Tsugita *et al.*, 1970; Mathews *et al.*, 1972).

Addition of $[\text{Cr(oxalate)}_3]^{3-}$ to a mixture of 4 mM-ferricytochrome c and 4 mM-ferricytochrome b_5 in $^2\text{H}_2\text{O}$ at pH 7.2 caused resonances of both ferricytochrome c and ferricytochrome b_5 to appear in the paramagnetic difference spectrum. Fig. 6 compares the paramagnetic difference spectra for (a) ferricytochrome c alone, (b) the mixture of cytochromes and (c) ferricytochrome b_5 alone. Resonances of ferricytochrome c (Fig. 6a) and the binding sites that affect them are: Ile-85 γ , Ala-83, Phe-82 and Ile-81 (site III); Thr-19 (site II); Ile-9 and Thr-89 (site I); Thr-28 is affected by binding at both sites II and IV, and Ile-85 δ is affected by binding at

Fig. 5. 470 MHz ^1H -n.m.r. spectra of 2 mM-ferricytochrome b_3 in $^2\text{H}_2\text{O}$ at pH 6.2 and at 25°C (a) Is the normal spectrum, (b) is the spectrum obtained after addition of 95 mol of $[\text{Cr}(\text{oxalate})_3]^{3-}$ /100 mol of cytochrome b_3 and (c) is the difference spectrum (a)–(b). Histidine resonances are marked by H.

both sites I and III (Eley *et al.*, 1982a; Eley, 1982; Williams *et al.*, 1982). Resonances of ferricytochrome b_3 (Fig. 6c) have not been assigned.

Many of the ferricytochrome *c* resonances in Fig. 6(a) are missing from Fig. 6(b). The most notable of these are resonances of Thr-19 α , Ile-81, Ala-83 and Ile-85 γ . Methyl resonances of Thr-19, Thr-28 and Thr-89 are unaffected by the complex-formation but resonances of Phe-82 and Ile-85 δ are considerably diminished in intensity. It is not clear whether the peak at approx. 0.4 p.p.m. in Fig. 6(b) is the Ile-9 resonance of ferricytochrome *c*, a resonance of ferricytochrome b_3 or a combination of both. The resonances missing from Fig. 6(b) correspond to those affected by $[\text{Cr}(\text{oxalate})_3]^{3-}$ -binding at site III, the strongest of the binding sites. Although the resonance of Thr-19 α is missing from Fig. 6(b), site II remains occupied by $[\text{Cr}(\text{oxalate})_3]^{3-}$, because methyl resonances of Thr-19 and Thr-28 are present. Site I also remains occupied in the complex by $[\text{Cr}(\text{oxalate})_3]^{3-}$. The marked effects involving resonances of Ala-83 and Ile-81 clearly demonstrate that their nuclei are shielded from $[\text{Cr}(\text{oxalate})_3]^{3-}$. These results indicate that ferri-

cytochrome b_3 binds ferricytochrome *c* in such a way as to protect site III strongly and possibly to protect site II weakly. Site I remains unprotected. Thus Lys-13 and Lys-72 are important in binding ferricytochrome b_3 . Further studies are required to evaluate the role of Lys-25 and Lys-27.

Most of the ferricytochrome b_3 resonances in Fig. 6(c) are present in Fig. 6(b). The appearance of the histidine resonance at approx. 7.9 p.p.m. is consistent with the residue being on the surface of the protein, and not part of the protein–protein-interaction region. Some of the aliphatic resonances present in Fig. 6(c) are missing from Fig. 6(b), but without further study it is not clear whether they arise from groups in the interaction region. Competition between cationic paramagnetic reagents and cytochrome *c* for cytochrome b_3 should enable the interaction sites on cytochrome b_3 to be mapped.

Comparison with previous studies

Previous investigations of the interactions between cytochrome *c* and liver microsomal cytochrome b_3 have centred around determining the stoichiometry

Fig. 6. 470 MHz ^1H -n.m.r. paramagnetic difference spectra of (a) 4 mM-ferricytochrome *c* + 34 mol of $[\text{Cr}(\text{oxalate})_3]^{3-}/100$ mol of cytochrome *b*₅ in $^2\text{H}_2\text{O}$ at pH 7.2 at 25°C, (b) 4 mM-ferricytochrome *c*, 4 mM-ferricytochrome *b*₅ + 34 mol of $[\text{Cr}(\text{oxalate})_3]^{3-}/100$ mol in $^2\text{H}_2\text{O}$ at pH 7.2 at 25°C, and (c) 2 mM-ferricytochrome *b*₅ + 95 mol of $[\text{Cr}(\text{oxalate})_3]^{3-}/\text{mol}$ of cytochrome *b*₅ in $^2\text{H}_2\text{O}$ at pH 6.2 at 25°C

The pattern of NH resonances between 8 p.p.m. and 10 p.p.m. differs, since the spectra were obtained with different samples in which the extent of NH exchange varied. The resonance labels are: ferricytochrome *b*₅, H (histidine) and * (unassigned aliphatics); ferricytochrome *c*, 9 (Ile-9), 19 (Thr-19), 28 (Thr-28), 81 (Ile-81), 82 (Phe-82), 83 (Ala-83), 85 (Ile-85) and 89 (Thr-89).

of binding, the association constants, the protein-protein-interaction regions and the rates of reaction. The first three topics were investigated in the present paper.

Mauk *et al.* (1982) have made a detailed study of the interaction by optical spectroscopy, and they have demonstrated that a 1:1 complex is formed with an association constant of $8 \times 10^3 \text{ M}^{-1}$ at pH 7 (25°C, *I* 0.01). Extrapolation of their ionic-strength-dependence to the ionic strength used in the n.m.r. experiments described above (*I* approx. 0.05) gives a

value for the association constant of approx. 600 M^{-1} , which is in reasonable agreement with the value obtained of approx. 10^3 M^{-1} (Fig. 2, inset), bearing in mind the markedly different protein concentrations used in the optical studies (approx. $1 \mu\text{M}$) and n.m.r. studies (approx. 1 mM). Miura *et al.* (1980) have performed some n.m.r. experiments with mixtures of ferricytochrome *c* and ferricytochrome *b*₅, although they concerned themselves solely with the low-field paramagnetically shifted resonances. It is difficult to compare their data with ours, since in

their titration both cytochrome *c* and cytochrome *b₅* concentrations were varied, with a consequent greater variation in ionic strength than for our experiments. Miura *et al.* (1980) concluded that a 2:1 cytochrome *c*–cytochrome *b₅* complex was formed, but they did not support this by determining association constants. Since the shifts on resonances of ferricytochrome *c* observed by them were similar to those observed by us (Fig. 1), and since both our data and those of Mauk *et al.* (1982) are consistent with 1:1 binding, the 2:1 complex postulated by Miura *et al.* (1980) probably does not exist.

Kinetic experiments with chemically modified cytochromes *c* have been used to map the cytochrome *b₅*-reaction site on cytochrome *c* (Ng *et al.*, 1977; Smith *et al.*, 1980). Cytochrome *c* derivatives in which one specific lysine residue was modified were used. The following order was found for the effect on the reaction: Lys-13 $\approx$ Lys-72 > Lys-25 $\approx$ Lys-79 $\approx$ Lys-27 > Lys-87 $\approx$ Lys-8. Modification of Lys-22, Lys-55, Lys-88, Lys-99 and Lys-100 had a small or no effect on the reaction rate. This reactivity pattern is illustrated in Fig. 7, along

Fig. 7. Ribbon diagram with a comparison of n.m.r. and lysine-modification data (Ng *et al.*, 1977; Smith *et al.*, 1980) for residues of cytochrome *c* that illustrates the binding site for cytochrome *b₅*.

The circles represent the lysine ϵNH_3^+ groups that have been shown by chemical modification to be involved in the interaction of cytochromes *b₅* and *c*; degree of implication in binding: ● > ⊗ > ⋯. ○ indicates no involvement. The ribbon represents the peptide backbone; groups shown by n.m.r. to be in the interaction region are indicated by ■, indicates groups not shielded by cytochrome *b₅*.

with the distribution of residues whose n.m.r. signals are affected or shielded by cytochrome *b₅*. There is reasonable agreement between the two sets of data, particularly in the dominance given to the top left side of the protein. However, the kinetic experiments also give a reasonably prominent role to Lys-25 and Lys-27, which contrasts with the n.m.r. data. Further work is required to resolve this difference.

Both the kinetic data and the n.m.r. data are in reasonable agreement with the hypothetical model of the complex proposed by Salemme (1976). This model was based solely on the X-ray-crystallographic structures, and the principal interactions were proposed to involve four carboxylate groups of cytochrome *b₅* and Lys-13, Lys-27, Lys-72 and Lys-79 of cytochrome *c*. The haem groups were co-planar in the hypothetical model, with the closest interatomic distance being 0.84 nm. Only a minimum inter-haem distance can be calculated from the n.m.r. data. This is based on the observation that none of the resolved haem resonances of ferrocytochrome *c* is shifted when the protein is complexed to ferricytochrome *b₅*. From the equations governing transition-metal pseudo-contact shifts, and from the estimate that the pseudo-contact shift is opposed by a ring-current shift that decreases the net shift by approx. 25% (see Wüthrich, 1976, and references cited therein), the minimum inter-haem distance was calculated to be 0.5 nm.

Two aspects of the interaction of cytochrome *c* with its oxidoreductases that have not been previously discussed and that may be of importance are: what happens to the reactivity of cytochrome *c* when complex-formation occurs?; and does the bound protein move around on the surface of cytochrome *c*?

All the available evidence suggests that the internal structure of cytochrome *c* is not greatly affected by complex-formation. However, Osheroff *et al.* (1980) have demonstrated that Lys-13 and Lys-79 are involved in interactions on the surface of cytochrome *c* that are critically important in stabilizing the protein; Lys-13 forms a salt-link with Glu-90, and Lys-79 forms a hydrogen bond with Ser/Thr-47. When these interactions are disrupted by chemical modifications, the protein is more readily denatured and its Fe–S bond broken (Osheroff *et al.*, 1980). Presumably when complex-formation occurs both of these interactions are disrupted, and, although the structure of cytochrome *c* may not be greatly perturbed, its reactivity may be affected. Lys-72 has not been implicated in any stabilizing interactions and indeed, unlike Lys-13 and Lys-79, its side chain is not clearly visible in X-ray-diffraction electron-density maps (Takano & Dickerson, 1981*a,b*), indicating that it is a mobile residue.

Current discussions about the interactions of cytochrome *c* with its redox partners have stressed that there is a fit between two complementary binding surfaces that is controlled by electrostatic interactions (Salemme, 1976; Koppenol & Margoliash, 1982; Matthew *et al.*, 1983), and that this leads to a complex of immobilized proteins (Matthew *et al.*, 1983). Since electron transfer requires a degree of orbital overlap, the binding surfaces of immobilized proteins would have to fit together precisely to avoid formation of unproductive complexes. However, if the proteins retained some independent motion within the complex, the two interacting surfaces could move against one another and so increase the probability of the formation of a productive complex. It is hoped that further study of the rotational correlation times of complexes of cytochrome *c* may shed some light on this proposed motion.

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