NMR Characterization of Surface Interactions in the Cytochrome b_5–Cytochrome c Complex

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The complex formed in solution by native and chemically modified cytochrome c with cytochrome b_5 has been studied by ^1H and ^{13}C nuclear magnetic resonance spectroscopy (NMR). Contrary to predictions of recent theoretical analysis, ^1H NMR spectroscopy indicates that there is no major movement of cytochrome c residue Phe^{82} on binding to cytochrome b_5. The greater resolution provided by ^{13}C NMR spectroscopy permits detection of small perturbations in the environments of cytochrome c residues Ile^{75} and Ile^{85} on binding with cytochrome b_5, a result that is in agreement with earlier model-building experiments. As individual cytochrome c lysyl residues are resolved in the ^1H NMR spectrum of N-acetimidylated cytochrome c, the interaction of this modified protein with cytochrome b_5 has been studied to evaluate the number of cytochrome c lysyl residues involved in binding to cytochrome b_5. The results of this experiment indicate that at least six lysyl residues are involved, two more than predicted by static model building, which indicates that cytochrome c and cytochrome b_5 form two or more structurally similar 1:1 complexes in solution.

PROTEIN-PROTEIN COMPLEXES ARE of central importance to many biological processes, so the structures of such complexes are necessarily of considerable interest (*1*). At present, however, the structures of only a few protein-protein complexes are known in detail. Specifically, the three-dimensional structures of some protease-polypeptide inhibitor complexes (*2*) and of certain antibody-protein antigen complexes (*3*) have been determined by x-ray diffraction. In each of these types of complex, the interaction between proteins exhibits considerable thermodynamic stability and a combination of hydrophobic and electrostatic interactions. Attempts to apply crystallographic techniques to other classes of protein-protein complexes for which the thermodynamic stability is not as great and in which interactions are dominated by electrostatic contacts have not been successful to date. The difficulty in the latter case appears to be that the standard methods used for protein crystallization (such as salting out with ammonium sulfate) result in dissociation of the complex. Poulos *et al.*, however, report use of an alternative crystallization strategy to produce co-crystals of cytochrome c (cyt c) and cyt c peroxidase at relatively low ionic strength (*4*). Although these crystals produced acceptable electron density maps for the cyt c peroxidase present, the structure of the complex could not be determined because the cyt c failed to diffract well, possibly owing to orientational disorder. This report describes complementary nuclear magnetic resonance (NMR) methods that provide structural information concerning another electrostatically stabilized complex, the cyt c–cytochrome b_5 (cyt b_5) complex, that may be generally applicable to other interacting systems that entail extensive electrostatic interactions.

Cyt c is an electron-transfer heme protein (molecular weight ~12,500) that operates in the mitochondrial respiratory chain (*5*). Cytochrome b_5 is a heme protein that operates in a variety of redox systems as an electron-transfer agent (*6,7*). Cyt b_5–like domains exist as part of water-soluble enzymes [such as sulfite oxidase (*8*) and nitrate reductase (*9*)], as a small water-soluble protein [for example, as part of the methemoglobin reductase system in erythrocytes (*10*)], or as a membrane-bound protein with a carboxyl-terminal hydrophobic peptide extension [as in mitochondria (*11*) and hepatic microsomes (*6, 7*)]. In all cases, the cyt b_5 heme-binding core domain consists of a single b-heme and ~90 to 100 amino acids that may be released from the holoprotein by the action of proteases.

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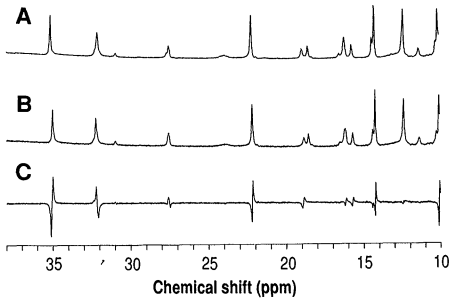

Fig. 1. High-frequency regions of the 400-MHz ^1H NMR spectra of ferricyts c and b_5 in 50-m*M* phosphate (*p*H 7.6) at 25°C. (**A**) Sum of spectra of individual proteins; (**B**) spectrum of an equimolar mixture of the two proteins; and (**C**) the difference (B) − (A).

Ferricytochrome c (ferricyt c) is reduced by ferrocytochrome b_5 (ferrocyt b_5) in a rapid reaction that proceeds through a bimolecular protein complex (*12*). This complex has been studied extensively by a variety of experimental and theoretical means (*12–17*), although its precise structure in solution remains unknown. A structural model of the cyt c–cyt b_5 complex has been proposed by computer graphics docking of the three-dimensional structures of tuna ferricyt c and lipase-solubilized bovine liver cyt b_5 (*18*). Subsequent electrostatic (*16*) and dynamics calculations (*19*) of this complex have led to suggested refinements in the details of the structure of this complex with changes in *p*H and with time. These model-building studies were based on a three-dimensional structure for cyt b_5 that was produced by fitting an amino acid sequence now known to possess three errors (*20*) to the electron density map. By using molecular genetic techniques, we have produced a form of cyt b_5 that has the sequence of bovine liver microsomal lipase-solubilized cyt b_5 that has been used in previous model-building experiments (*16, 18, 19, 21*). We have found that this form of the protein interacts with horse heart cyt c in a manner that is quantitatively different from that of cyt b_5 having the correct wild-type sequence (*15, 22*). Therefore, we have now studied the complex formed by cyt c with the form of cyt b_5 having the incorrect sequence by NMR spectroscopy in an attempt to simulate better the assumptions inherent in previous structural modeling efforts.

In the downfield region of the ^1H NMR spectrum of the ferricyt c–ferricyt b_5 complex (Fig. 1), the resonance shifts that occur on complex formation are similar to those reported to occur on complex formation between authentic bovine liver microsomal tryptic cyt b_5 with horse ferricyt c (*17*). The effect of complex formation at a relatively high ionic strength on the aromatic region of the ^1H NMR spectrum is shown in Fig.

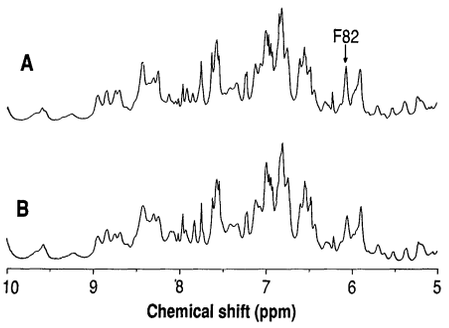

Fig. 2. Aromatic regions of the ^1H NMR spectra of ferricyts c and b_5. Conditions as for Fig. 1. (**A**) Sum of spectra of individual proteins; and (**B**) spectrum of an equimolar mixture of the two proteins.

2. There are slight changes in this region of the spectrum, although they are difficult to interpret fully because of the resonance overlap. However, the resonances of Phe^{82} of cyt c (*23*) are only marginally affected. This is consistent with the environment of Phe^{82} changing as a result of complex formation but not to the extent that there is a substantial alteration in the relative alignment of Phe^{82} with respect to the heme. Thus, the Phe^{82} ring atomic positions do not change by more than 1 Å on complexation.

The aliphatic region of the ^1H NMR spectrum of the complex shown in Fig. 3A supports the general conclusion that complex formation occurs without significantly distorting the structure of cyt c in the vicinity of Phe^{82}. Any major movement of Phe^{82} would lead to sizable shifts of resonances of Leu^{64}, Leu^{68}, and Ile^{85}, and these were not observed. A change does occur in the region from 0.8 to 1.3 ppm, but this strongly overlapping region is difficult to analyze. Because of this, we have used ^{13}C NMR to monitor the same complex (*24*); ^{13}C NMR spectroscopy has the advantage over ^1H NMR of a greater chemical shift dispersion and thus a reduced overlap problem. Furthermore, ^{13}C NMR shifts are more sensitive to minor structural perturbations. Part of the methyl regions of the spectra corresponding to those in Fig. 3A is shown in Fig. 3B. The improved resolution of the ^{13}C NMR spectra allows groups to be monitored directly. The environment of Ile^{81} of horse cyt c is only slightly affected by complexation, and the environments of Ile^{75} and Ile^{85} are, at most, also only slightly perturbed. The shift of the resonance at 16.9 ppm, which is the chemical shift of the γ-CH_3 of Ile^{85} (*25*), indicates that it is probably the δ-CH_3 resonance of Ile^{85} at 11.9 ppm that is perturbed by complexation. Further analysis of the ^{13}C NMR spectra requires a full assignment of the cyt b_5 spectrum.

The overall impression gained from the ^1H and ^{13}C NMR data in Figs. 1 through 3 is that the general location of the cyt b_5 binding site on cyt c proposed by Salemme (*18*) is correct; namely, a region encompassing Ile^{81}, Phe^{82}, and Ile^{85} is involved. This finding is consistent with NMR studies of the native cyt c–cyt b_5 complex (*17*). However, the data we present are not consistent with models of the interaction in which there is a substantial change in the cyt c structure. In particular, the proposal of Wendoloski *et al.* (*19*) that Phe^{82} undergoes a conformational change that leads to its relocation close to the cyt b_5 heme is not supported by the present work. There are a number of possible reasons for this discrepancy. The major source may be that the NMR studies are probing the collision complex, whereas the molecular dynamics calculations are investigating the subsequent activated complex.

One important aspect of the complexation for which the experiments represented by Figs. 1 through 3 provide no direct information on is the number of cyt c Lys residues involved in binding cyt b_5. This deficiency arises because the 19 Lys proton resonances of cyt c are complex multiplets, and both the ^1H and ^{13}C resonances occur in

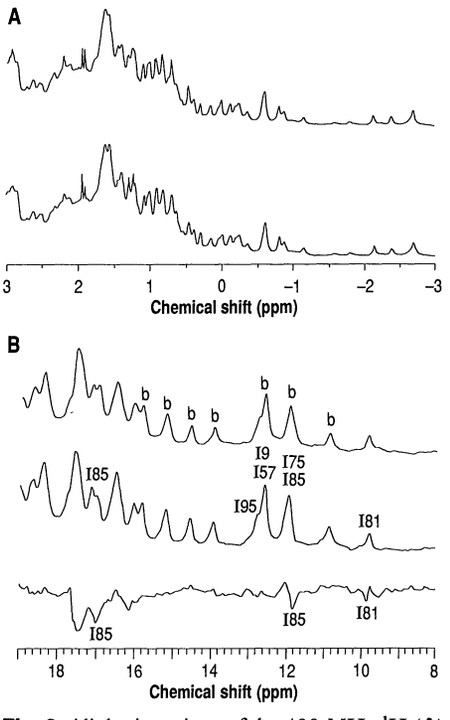

Fig. 3. Aliphatic regions of the 400-MHz ^1H (**A**) and 100-MHz ^{13}C (**B**) NMR spectra of ferricyts c and b_5. Conditions as for Fig. 1. (A) Top, summed spectrum; and bottom, mixture spectrum. (B) Top, mixture spectrum; middle, summed spectrum; and bottom, difference spectrum (summed − mixture). Resonances labeled "b" are resonances of cyt b_5 that have not yet been assigned.

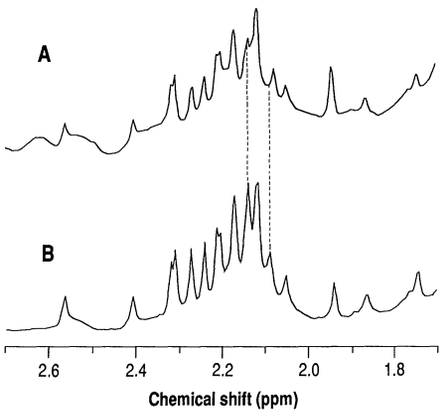

Fig. 4. The *N*-acetimidyl methyl region of the 400-MHz ^1H NMR spectrum of *N*-acetimidyl–ferricyt c and ferricyt b_5. Conditions as in Fig. 1. (**A**) and (**B**) are the summed spectrum and mixture spectrum, respectively. The broken lines indicate perturbed resonances.

crowded regions of the spectrum. Thus, these Lys residues are, in effect, NMR invisible. To make them visible in NMR, we chemically modified them to produce the N-acetimidylated derivative (*26*). This modification retains the structure of native cyt c (*27*) and key functional properties (*26*).

The modification and the relevant part of the ^1H NMR spectrum of the derivative bound to cyt b_5 are shown in Fig. 4. All 19 of the *N*-acetimidyl methyl peaks are singlets with chemical shifts in the range from 2.0 to 2.6 ppm. At least six of these peaks are shifted on complexation with cyt b_5. These resonance shifts result from the formation of salt bridges between the *N*-acetimidylated Lys residues and carboxylate groups (*28*). Thus, the implication of this result is that at least six of the Lys residues are directly involved in salt bridges with groups on cyt b_5. This finding contrasts with the Salemme model (*19*), which only includes four Lys groups in salt bridges, and with the modified Salemme model of Rodgers *et al.* (*14*), which puts five lysyl residues in salt bridges. These static models and the NMR data may be rationalized by a dynamic model akin to that proposed by Eley and Moore (*17*) and investigated theoretically by Wendoloski *et al.* (*19*). This dynamic model requires that the proteins retain some independent motion within the complex so that the two interacting surfaces move against one another, that is, a "rolling ball" model. This is not to say that the two proteins migrate freely over each other's surface. It seems more likely that the two proteins are instead restricted to movement about specific regions of their respective complementarily charged surfaces. These interaction domains would necessarily be larger than those indicated by the static modeling studies.

REFERENCES AND NOTES

1. C. Frieden and L. W. Nichol, Eds., *Protein-Protein Interactions* (Wiley, New York, 1981).
2. For example, W. Bode, E. Papamokos, D. Musil, U. Seemueller, H. Fritz, *EMBO J.* **5**, 813 (1986).
3. R. Huber, *Science* **233**, 702 (1986); A. G. Amit, R. A. Mariuzza, S. E. V. Phillips, R. J. Poljak, *ibid.*, p. 747; D. R. Davies, S. Sheriff, E. A. Padlan, *J. Biol. Chem.* **263**, 10541 (1988).
4. T. L. Poulos, S. Sheriff, A. J. Howard, *J. Biol. Chem.* **262**, 13881 (1987).
5. G. W. Pettigrew and G. R. Moore, *Cytochromes c: Biological Aspects* (Springer-Verlag, New York, 1987).
6. B. Guiard and F. Lederer, *J. Mol. Biol.* **135**, 639 (1979).
7. F. S. Mathews and E. W. Czerwinski, in *Enzymes of Biological Membranes*, A. N. Martonosi, Ed. (Plenum, New York, 1976), vol. 4, pp. 143–197; F. S. Mathews, *Prog. Biophys. Mol. Biol.* **45**, 1 (1985).
8. B. Guiard and F. Lederer, *Eur. J. Biochem.* **100**, 441 (1979).
9. K. H. D. Le and F. Lederer, *EMBO J.* **2**, 1909 (1983).
10. D. E. Hultquist and P. G. Passon, *Nature New Biol.* **229**, 252 (1972); L. J. Sannes and D. E. Hultquist, *Biochim. Biophys. Acta* **544**, 547 (1978).
11. A. Ito, *J. Biochem. (Tokyo)* **87**, 63 (1980); *ibid.*, p. 73; F. Lederer, R. Ghrir, B. Guiard, S. Cortial, A. Ito, *Eur. J. Biochem.* **132**, 95 (1983).
12. S. Ng, M. B. Smith, H. T. Smith, F. Millett, *Biochemistry* **16**, 4975 (1977); J. Stonehuerner, J. B. Williams, F. Millett, *ibid.* **18**, 5422 (1979).
13. P. W. Holloway and H. H. Mantsch, *ibid.* **27**, 7991 (1988).
14. K. K. Rodgers *et al.*, *Science* **240**, 1657 (1988).
15. M. R. Mauk, L. S. Reid, A. G. Mauk, *Biochemistry* **21**, 1843 (1982).
16. M. R. Mauk, A. G. Mauk, P. C. Weber, J. M. Matthew, *ibid.* **25**, 6381 (1986).
17. C. G. S. Eley and G. R. Moore, *Biochem. J.* **215**, 11 (1983).
18. F. R. Salemme, *J. Mol. Biol.* **106**, 563 (1976).
19. J. J. Wendoloski, J. B. Matthew, P. C. Weber, F. R. Salemme, *Science* **238**, 794 (1987).
20. R. J. Cristiano and A. W. Steggles [*Nucleic Acids Res.* **17**, 799 (1989)] reported the sequence of a full-length cDNA clone of bovine liver microsomal cyt b_5 that establishes the identity of residue 11 as Glu, residue 13 as Gln, and residue 57 as Asn [by using the crystallographic numbering system of Mathews and co-workers (7)]. Tryptic peptide sequence analysis of trypsin-solubilized bovine liver microsomal cyt b_5 has recently confirmed the identity of these residues (W. D. Funk, M. R. Mauk, R. T. A. MacGillivray, A. G. Mauk, unpublished results).
21. Model building has made use of both the tuna (*18, 19*) and simulated horse heart cyt c (*16*) structures. The close similarity in behavior and structure of these two proteins renders them effectively identical to each other for this purpose.
22. Recent potentiometric titrations demonstrate that the stability of the complex formed by horse heart cyt c is greater for the complex formed with the triple mutant of cyt b_5 than for the complex formed with the true wild-type cyt b_5 (*p*H 7.2, 25°C, ionic strength = 0.01*M* (M. R. Mauk, P. D. Barker, A. G. Mauk, unpublished results). Crystallographic analysis of the triple mutant has recently established that it does not exhibit any significant distortion of the main chain conformation relative to wild-type cyt b_5; the principal differences between the two structures involve surface hydrogen-bonding changes in the vicinity of the amino acid substitutions (W. D. Funk *et al.*, in preparation).
23. A. P. Boswell, G. R. Moore, R. J. P. Williams, J. C. W. Chien, I. C. Dickinson, *J. Inorg. Biochem.* **13**, 347 (1980).
24. A. M. Burch *et al.*, *Biochem. Soc. Trans.* **16**, 844 (1988).
25. H. Santos and D. L. Turner, *FEBS Lett.* **194**, 73 (1986).
26. C. J. A. Wallace and D. E. Harris, *Biochem. J.* **217**, 589 (1984); C. J. A. Wallace, *ibid.*, p. 595; G. W. Pettigrew, I. Aviram, A. Schejter, *Biochem. Biophys. Res. Commun.* **68**, 807 (1976).
27. A. P. Boswell *et al.*, *Biochem. J.* **213**, 679 (1983).
28. We discount the possibility that the shifts in the *N*-acetimidyl methyl peaks result from protein conformational changes induced by complex formation primarily because the shifts of these peaks are larger than for any other amino acid ^{1}H NMR perturbations induced by complex formation. However, shifts produced by a change in conformation of modified Lys residues that result from the presence of these residues within the protein complex interface region but not involved in salt bridges cannot be excluded on the basis of the NMR data alone. It seems unlikely that a Lys would be in such a position and not be involved in local electrostatic interactions, and, indeed, none of the computer graphics models place a Lys in such a position.
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Painting the Phase Space Portrait of an Integrable Dynamical System

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For an integrable dynamical system with one degree of freedom, "painting" the integral over the phase space proves to be very effective for uncovering the global flow down to minute details. Applied to the main problem in artificial satellite theory, for instance, the technique reveals an intricate configuration of equilibria and bifurcations when the polar component of the angular momentum approaches zero.

SEVERAL DEVELOPMENTS UNDERLIE the present revival in classical mechanics, including computerized algebraic processors and color graphics. Dynamical processes are very hard to detect beneath the surface of the differential equations. It takes a fair amount of tedious algebra to extract, by averaging or by normalization, the essence of the mechanism from the background of short-term fluctuations and unremarkable perturbations. But to the benefit of nonlinear mechanics, computer algebra systems are becoming increasingly sophisticated. Once over the hurdle of tedious calculations, one faces the obstacle of obtaining a global picture for the long-term trends in the system. Color graphics proves invaluable in visualizing the global behavior of the system and in discovering minutiae of local behavior hidden beneath the mass of calculations. Pseudocoloring a function over a domain, a widespread technique in applied mathematics, has produced stunning pictures; they have opened the eyes of mathematicians to hitherto unsuspected phenomena in the dynamics of nonlinear maps (*1*). Extension to classical mechanics forces a search for refinements in the technique such as the automatic selection of colors to ensure enough contrast around isolated but close singularities.

Here is an example of basic facts discovered by color graphics. It is borrowed from a problem that has been vexing astronomers and space engineers since Orlov (*2*) uncovered the singularity of the "critical inclination." Take a point mass in the gravity field of the earth; ignore for the moment all other bodies in the vicinity, the nongravitational forces (drag and radiation pressure), and even those parts of the earth's gravity field that are dependent on the longitude. Furthermore, in the remaining, ignore all zonal harmonics except the one caused by the equatorial bulge. Thus, the system is represented by the Hamiltonian

$$\mathcal{H} = \frac{1}{2}(\mathbf{X} \cdot \mathbf{X}) - \frac{\mu}{r}\left[1 - J_2\left(\frac{\alpha}{r}\right)^2 P_2\left(\frac{\mathbf{k} \cdot \mathbf{x}}{r}\right)\right] \quad (1)$$

where the vectors $\mathbf{x}$ and $\mathbf{X}$ stand for the position and velocity of the spacecraft, respectively; $r = \|\mathbf{x}\|$ is the geocentric distance. The parameters of the system are as follows: μ, the product of the gravitational constant and the mass of the earth; α, the equatorial radius of the earth; $\mathbf{k}$, the direction of the polar axis of the earth; and a dimensionless quantity J_2. The function P_2 is the Legendre polynomial of degree 2. Hamiltonian Eq. 1 defines the main problem in artificial satellite theory. This dynamical system admits two integrals: (i) the energy $\mathcal{H}$ per unit of mass, because $\mathcal{H}$ is time-invariant, and (ii) the projection $H = \mathbf{k} \cdot \mathbf{G}$ of the angular momentum $\mathbf{G} = \mathbf{x} \times \mathbf{X}$ per unit of mass on the polar axis, because the group of rotations about $\mathbf{k}$ leaves $\mathcal{H}$ invariant. For lack of a third integral in involution with H, the consensus among experts is that the main

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