Journal of Bioenergetics and Biomembranes, Vol. 27, No. 3, 1995

Experimental and Theoretical Analysis of the Interaction Between Cytochrome *c* and Cytochrome b_5

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Received March 2, 1995

Experimental and theoretical investigation of the interaction of cytochrome *c* and cytochrome b_5 performed over nearly twenty years has produced considerable insight into the manner in which these proteins recognize and bind to each other. The results of these studies and the experimental and theoretical strategies that have been developed to achieve these results have significant implications for understanding the behavior of similar complexes formed by more complex and less-well characterized electron transfer proteins. The current review provides a comprehensive summary and critical evaluation of the literature on which the current status of our understanding of the interaction of cytochrome *c* and cytochrome b_5 is based. The general issues related to the study of electron transfer complexes of this type are discussed and some new directions for future investigation of such systems are considered.

KEY WORDS: Cytochrome *c;* cytochrome b_5; protein–protein interaction; electron transfer; NMR spectroscopy; molecular modelling.

INTRODUCTION

The transfer of an electron from one protein to another is the simplest chemical reaction that can occur between two proteins. As a result, such reactions have attracted considerable experimental and theoretical attention. The appeal of such reactions is that they should, in principle, be amenable to detailed structural and mechanistic characterization. As with electron transfer reactions involving two substitutionally-inert coordination complexes, protein–protein electron transfer reactions can be described as a series of events that involve diffusion of the two reactant molecules to form an electron transfer precursor complex, followed by the electron transfer event and subsequent dissociation of the resulting successor complex (Scott *et al.*, 1985; Marcus and Sutin, 1985). The greater structural complexity of proteins, however, increases the complexity of each of these processes and their detailed characterization. In particular, the greater specificity with which proteins recognize and interact with each other compared to substitutionally inert coordination complexes has necessitated considerable attention to the evaluation of the structural and functional characterization of protein–protein complex formation independent of electron transfer. For these reasons, most attention has been focused on investigation of electron transfer reactions involving proteins for which three-dimensional structures have been determined by X-ray crystallography. In recent years, particular emphasis has been placed on investigation of systems that are also amenable to modification by site-directed mutagenesis.

The first protein–protein electron transfer reaction for which a detailed structural model of the precursor complex was proposed was the reaction between cytochrome b_5 and cytochrome *c* (Fig. 1; Salemme, 1976). This hypothetical model was based on the known three-dimensional structures of the two component proteins and was proposed largely on the supposition that an electrostatically stabilized complex should be formed

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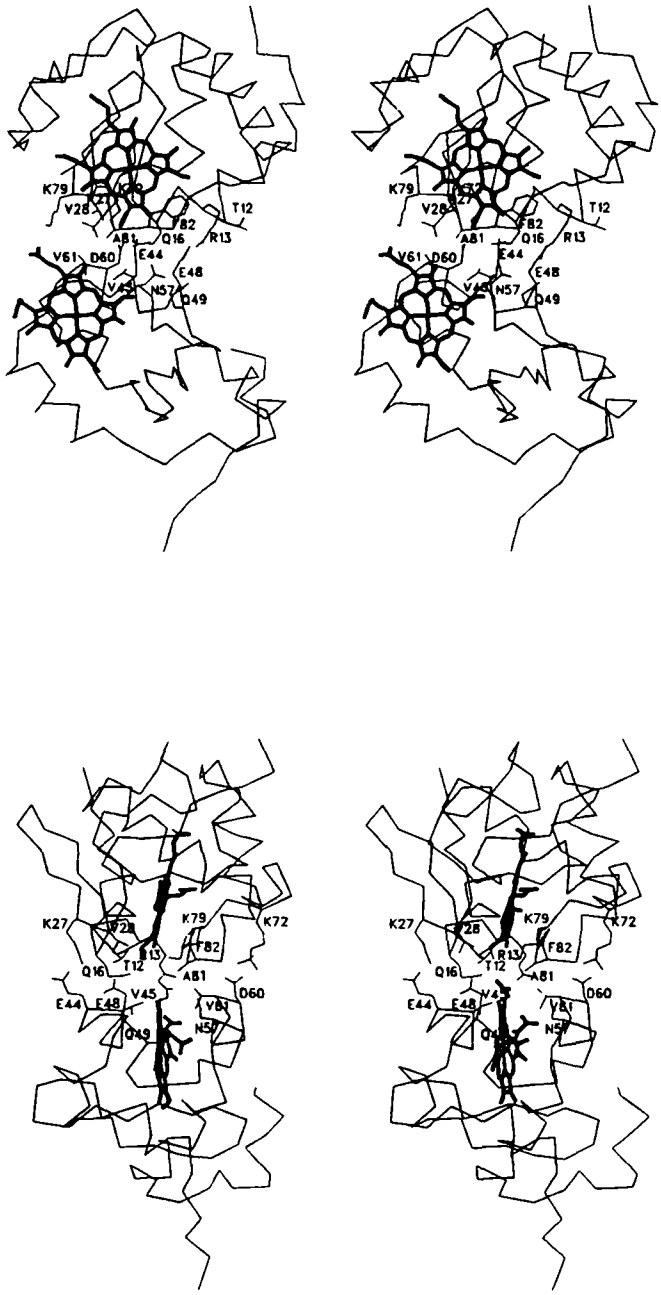

Fig. 1. Stereodiagrams of the energy-minimized, putative complex formed by yeast *iso*-1-cytochrome *c* and trypsin-solubilized bovine liver microsomal cytochrome b_5 (Guillemette *et al.*, 1994) based on the Salemme (1976) model for the hypothetical complex formed by tuna cytochrome *c* and lipase-solubilized bovine liver microsomal cytochrome b_5.

by the interaction of a strongly basic protein, cytochrome *c*, and a strongly acidic protein, cytochrome b_5. As the surface charge distribution of each of these two proteins is highly asymmetric and centered around the partially exposed edge of the heme prosthetic group in each case, computer graphics was used to produce a complex in which the electrostatic contacts of these proteins were optimized by a combination of visual inspection and least squares optimization to minimize the distance between the heme groups of the two proteins. The resulting model implied that the stoichiometry of complex formation was 1:1, that the planes of the heme groups were near parallel in orientation, and that complex formation results in displacement of several water molecules from the surface of each protein. The identification of specific surface residues in the recognition and binding process also implied the existence of a unique, well-defined complex. As such, this model provided a stimulating and productive basis for development of experimental strategies to evaluate the mechanism by which these two proteins interact with each other in solution. This model was remarkably prescient as there was no experimental evidence at that time for formation of a stable complex by these two proteins.

As a result of this model, however, attention became focused on the experimental characterization of the interaction of these proteins, and many of the features predicted by this model have subsequently been demonstrated. As experimental evaluation has continued, though, it has become apparent that the dynamics of interaction between electron transfer proteins is a fundamental characteristic of such complexes. Recent efforts have attempted to account for this property and the subtle structural and functional consequences that it produces. As the result of this work, the cytochrome *c*–cytochrome b_5 complex has become a paradigm for investigation of similar complexes formed by other pairs of electron transfer proteins. Although the cytochrome *c*–cytochrome b_5 complex has been considered briefly in previous reviews of electron transfer protein complexes (Marcus and Sutin, 1985; McLendon, 1988; Mathews, 1985; Kostic, 1991), a comprehensive summary of work concerning the cytochrome *c*–cytochrome b_5 complex is not presently available. The current review, therefore, surveys the literature available concerning the interaction of these two proteins and attempts to provide a cohesive assessment of our present understanding of it and to anticipate those directions in which future work will be directed. The emphasis of the discussion concerns experimental and theoretical characterization of the interaction of these two proteins because detailed understanding of this process is fundamental to a correspondingly detailed understanding of the electron

transfer reactions that these proteins undergo. A far more complete consideration of electron transfer reactions between cytochrome *c* and cytochrome b_5 is provided in another review in this issue (Durham *et al.*, this issue).

Although the utility of studying the interaction of cytochrome *c* and cytochrome b_5 as a means of gaining insight into the mechanism of interaction between electron transfer proteins is demonstrated in the studies summarized here, the question of the physiological role of this reaction inevitably arises in such discussions and merits comment. With rare exception (i.e., Meyer *et al.*, 1993), the cytochrome b_5 used in the studies reviewed here is a solubilized form of the microsomal cytochrome in which the heme-binding domain has been separated by proteolysis from the hydrophobic, membrane-binding domain. Use of the heme-binding domain simplifies the logistics of experimental work and is justifiable because the functional properties of the two domains of the protein are known to be independent of each other. Nevertheless, cellular compartmentalization presumably minimizes the likelihood of reaction between microsomal cytochrome b_5 and cytochrome *c*, which is normally associated with the inner mitochondrial membrane. However, Bernardi and Azzone (1981) have presented evidence that cytochrome *c* can serve as an efficient electron shuttle between a form of cytochrome b_5 that is present on the outer mitochondrial membrane (Lederer *et al.*, 1983) and cytochrome *c* oxidase. The ~58% sequence identity of the mitochondrial form of cytochrome b_5 and the microsomal form of cytochrome b_5 from the same species suggest that the interaction of cytochrome *c* and cytochrome b_5 is a physiological event that is represented with some validity by the interaction of microsomal cytochrome b_5 with cytochrome *c*. In addition, cytochrome *c* is known to react physioglocially with cytochrome b_5-like domains (albeit of lower sequence identity) present in the mitochondrial enzyme sulfite oxidase (Cohen and Fridovich, 1971a,b) and yeast lactate dehydrogenase (reviewed in Lederer, 1994). For these reasons, detailed characterization of the interaction of cytochromes *c* and b_5 can be regarded as having biological as well as pedagogical validity.

THERMODYNAMICS, ELECTROSTATICS, AND SOLVATION

As indicated above, experimental characterization of interaction between cytochrome *c* and cytochrome b_5 has emphasized the stoichiometry of complex formation, the stability of complex formation, the identity of surface residues involved in protein–protein recognition, the contributions of electrostatics in complex formation, and the role of surface water molecules in complex formation. Most of this effort has involved the use of spectroscopic techniques although hydrodynamic, crosslinking, and potentiometric techniques have also been employed. A general feature of this experimental work is that the oxidation states of the proteins that can be used to study their interaction under equilibrium conditions cannot be the same as the oxidation states that are relevant to formation of the electron transfer precursor complex. As a result, most studies concern complex formation by the two ferricytochromes with the assumption that the effect of the charge state of the heme iron makes a relatively small contribution to the stability of complex formation. This assumption is substantiated, at least in part, by the fact that the structures (and particularly the surfaces) of the two cytochromes are virtually unaffected by the oxidation state of the iron (Argos and Mathews, 1975; Berghuis and Brayer, 1992). One means of addressing this issue experimentally is through the use of cytochrome derivatives in which the central metal is replaced by a redox-inert metal atom with charge properties similar to those of Fe(II). As these derivatives are generally fluorescent, they provide an additional experimental avenue by which interaction of the two proteins can be investigated. Nevertheless, these metal-substituted derivatives introduce additional difficulties, as discussed in greater detail later.

Early Kinetic Studies

The first experimental investigations of the cytochrome *c*–cytochrome b_5 system were kinetics studies of the reaction between cytochrome *c* and cytochrome b_5 that used either purified proteins or cytochrome *c* and a microsomal preparation that included cytochrome b_5 as a membrane-bound protein (Strittmatter, 1964; Strittmatter *et al.*, 1972). These reports predated the Salemme model and did not concern the issue of complex formation. Following the appearance of the Salemme model, the first studies of this system addressed several of the basic predictions that it provided. For example, Millett and co-workers used steady-state kinetics to demonstrate that the rate of ferricytochrome *c* reduction by microsomal prepara-

tions of cytochrome b_5 and its reductase is inversely proportional to ionic strength (Ng *et al.*, 1977; Stonehuerner *et al.*, 1979) and that chemical modification of specific lysyl residues on the surface of cytochrome *c*, identified by Salemme as critical to complex formation, decreased the rate of this reaction (Ng *et al.*, 1977; Smith *et al.*, 1980). These investigators also provided the first direct evidence for formation of a cytochrome *c*–cytochrome b_5 complex through the use of gel permeation chromatography and analytical ultracentrifugation (Stonehuerner *et al.*, 1979).

Early Spectroscopic Studies

The first detailed analysis of the stability of complex formation was subsequently provided by optical difference titrations that monitored a small change in the electronic absorption spectrum of the proteins that occurs when the two cytochromes are mixed under solution conditions that promote complex formation (Mauk *et al.*, 1982). This work demonstrated that complex formation is also acutely sensitive to ionic strength ($K_A = 8 \pm 3 \times 10^4$ M^{-1} at pH 7.0, $I = 10$ mM; $K_A = 4 \pm 3 \times 10^6$ M^{-1} at pH 7.0, $I = 1$ mM) and pH and that the stability of complex formation is independent of temperature within the limits of uncertainty of this method. This latter finding supported the prediction (Salemme, 1976) that the displacement of surface water molecules upon complexation is a major contribution to the stability of the resulting complex. Furthermore, optimal stability of complex formation was found to occur at a pH that is midway between the isoelectric pH values of the two proteins. Shortly thereafter, Eley and Moore (1983) reported NMR studies that demonstrated the existence of a 1:1 cytochrome *c*–cytochrome b_5 complex in solution, in contrast to the conclusions of Miura *et al.* (1980). These studies and other uses of NMR spectroscopy to study the interaction of these proteins are discussed in detail below.

The involvement of one of the heme propionate groups of cytochrome b_5 in formation of the complex with cytochrome *c* predicted by the Salemme model was studied by Mauk *et al.* (1986) through use of the dimethylesterheme-substituted derivative of cytochrome b_5. The effects of this modification on complex formation was again assessed by optical difference spectroscopy and led to the unexpected finding that the principal effect of propionate modification is a shift in the dependence of complex stability on pH. Electrostatic modelling of complex formation by native cytochrome *c* and native and modified cytochrome b_5 (*vide infra*) led to the first suggestion that more than one structure of this complex can occur in solution and that the structure of the complex is a function of pH (Mauk *et al.*, 1986). Subsequent kinetic studies in which the pH-dependent electron transfer reactions of the complexes formed by cytochrome *c* with the native and dimethylesterheme-substituted derivative of cytochrome b_5 with flavin semiquinones were evaluated were at least qualitatively consistent with this conclusion (Eltis *et al.*, 1988).

Other attempts to assess the involvement of the cytochrome b_5 heme propionate in complex formation involved investigation of the effects of $Cr(en)_3^{3+}$ on complex stability. As the three-dimensional structure of cytochrome b_5 suggested relatively specific binding of cations to the exposed heme propionate, this probe reagent was used by Chapman *et al.* (1983) in optical difference titrations to indicate inhibition of protein–protein complex formation by the chromium complex. Subsequent NMR studies, however, indicated that a ternary cytochrome *c*–cytochrome b_5-$Cr(en)_3^{3+}$ complex could form under these conditions (Hartshorn *et al.*, 1987) (*vide infra*). This result provided further evidence for lability in the solution structure of this complex.

Hyperbaric Spectroscopy

The investigation of involvement of specific residues on the surface of cytochrome b_5 in the interaction with cytochrome *c* has been greatly facilitated through the use of site-directed mutagenesis by Sligar and coworkers. This group has used two approaches to the use of these derivatives. One of their strategies (Rodgers *et al.*, 1988; Rodgers and Sligar, 1991) involved analysis of the interaction of cytochrome *c* with 14 variants of cytochrome b_5 in which specific surface acidic groups were converted to the corresponding amidated forms and with dimethylesterheme-substituted cytochrome b_5. In this work, complex dissociation at elevated pressure was monitored by hyperbaric visible electronic difference spectroscopy. In addition to verifying the critical contributions of certain of these groups to the stability of complex formation, this work (i) provided the direct information concerning the change in hydration that occurs with complex formation, (ii) was consistent with the presence of 4–5 salt bridges in the cytochrome *c*–cytochrome b_5 complex, and (iii) indi-

cated an interfacial area for protein–protein contact of ~800 Å^2. In related reports, Stayton *et al.*, (1988) used variants in which a surface cysteinyl residue was introduced at a site remote from the presumed recognition surface of the protein. This cysteinyl residue was used as the site of attachment for a fluorescent acrylodan probe, the fluorescence intensity of which was quenched upon binding of the protein to cytochrome *c*. The fluorescence quenching titrations permitted through use of these derivatives allowed greater sensitivity in detection of complex formation and greater precision in the determination of association constants.

Fluorescence Quenching Studies

Information concerning the distance between the heme groups in the cytochrome *c*–cytochrome b_5 complex has been sought through fluorescence quencing experiments involving fluorescent derivatives of cytochrome *c* in which the prosthetic group has been modified by replacement of the central iron atom with zinc (McLendon *et al.*, 1985) or by complete removal of the central metal atom (Eltis, 1989). The Fe–Fe distance estimated in both studies for the complex formed by the native cytochromes was ~18 Å and is consistent with the Salemme model. This apparent distance was found to increase to 20 Å upon esterification of the cytochrome b_5 heme propionate groups (Eltis, 1989), consistent with an alteration of docking geometry introduced by this modification. Such small effects must be regarded with caution, however, because approximations used for influence of the relative orientations of the donor and acceptor sites in these calculations may introduce an uncertainty as great as 20% (Eltis, 1989). Although metal-substituted heme and protoheme IX-substituted derivatives of cytochrome b_5 can be prepared (Vaz *et al.*, 1979; Ozols and Strittmatter, 1964), fluorescence quenching experiments involving fluorescent derivatives of cytochrome b_5 generated in this manner have not proved to be possible owing to inability to prepare these derivatives devoid of apocytochrome b_5 which could potentially compete in binding to cytochrome *c* (M. R. Mauk, unpublished). The binding of these metal-substituted heme derivatives to apocytochrome b_5 is sufficiently weak that they are partially removed during elution over gel filtration media.

Kornblatt *et al.* (1988) combined the use of fluorescent cytochrome *c* derivatives with the use of hyperbaric fluorescence quenching titrations to derive further information concerning the effects on complex formation on protein surface hydration. Their initial studies were consistent with involvement of 4–5 salt bridges in stabilization of the cytochrome *c*–cytochrome b_5 complex. However, these investigators observed only ~50% of the change in volume upon complex formation that was reported by Rodgers and *et al.* (1988). The discrepancy between these two studies was attributed by these authors to the use of different species and derivatives of proteins in the two studies. In more recent work, Kornblatt *et al.* (1993) extended this work to verify that both increased pressure and glycerol disrupt the cytochrome *c*–cytochrome b_5 complex by increasing hydration of the interacting protein surfaces and provided evidence that complex formation involves removal of ~3 molecules of water at the protein–protein interface.

Potentiometric Titrations

The pH-dependent nature of cytochrome *c*–cytochrome b_5 complex formation observed by electronic difference spectroscopy (Mauk *et al.*, 1982) established by definition that the formation of this complex should result in a change in proton binding that is produced by a change in pK_a of one or more titratable groups caused by interaction of the two proteins. This observation led to the use of potentiometric titrations to characterize the electrostatic properties of this complex in greater detail based on the pioneering work of Laskowski and co-workers (reviewed in Laskowski and Finkenstadt, 1972). Based on this fundamental work, Mauk *et al.* (1991) employed two complementary strategies to study the interaction of cytochrome *c* and cytochrome b_5 with far greater precision than could be achieved previously by the relatively insensitive method of electronic difference spectroscopy.

The first of these potentiometric methods involved the analysis of proton release or uptake that occurs upon addition of one cytochrome to the other. These measurements provided highly-precise values for the association constant and for the stoichiometry of proton liberation or uptake that is linked to complex formation, q. The dependence of q on pH is informative because it represents the difference in the titration curves of the proteins free in solution and in the complex. The second type of potentiometric experiment employed in this work involved determination of the complex formation-induced difference titration curve.

This titration curve was obtained by determining the titration curve of the complex and then subtracting the titration curve of the two individual proteins free in solution. As previously demonstrated (Laskowski and Finkenstadt, 1972), integration of the complex-induced difference titration curve produces a continuous plot of the dependence of K_a on pH. The integration constant required to place this K_a vs. pH plot on the ordinate was obtained by determination of the association constant at one or two values of pH. Based on this work, it is apparent that for those cases where the stability of complex formation is sufficient over a reasonable range of pH, potentiometric experiments can provide an efficient means of determining the pH-dependence of complex formation in a continuous fashion that cannot be achieved by other techniques.

A benefit of this approach is that it should permit detailed modelling calculations to assess in a more rigorous fashion the precise nature of the pK_a changes that occur with complex formation and assist in the identification of those functional groups that exhibit complexation-dependent pK_as. Initial attempts at simulating these results indicate that 10–12 titratable groups undergo pK_a shifts of this type, though the identity of the groups involved remains speculative (Mauk *et al.*, 1991). The large number of these groups presumably results at least in part from the existence of more than one structure of the complex and from a pH-dependent distribution of these structures.

Infrared Spectroscopy

A novel approach to investigating perturbation of the surface of cytochrome b_5 upon interaction with cytochrome *c* that has not been applied to the study of other protein–protein complexes involves the use of FTIR spectroscopy. By careful analysis of the infrared spectrum between 1500 and 1600 cm^{-1}, Hollaway and Mantsch (1988) observed a band at 1562 cm^{-1} in the spectrum of rabbit liver cytochrome b_5 that they attributed to the combined contributions of the 11 carboxylate groups present in this species of this cytochrome. Upon formation of the cytochrome *c*–cytochrome b_5 complex, the intensity of this band decreased ~28%, an observation that the authors attributed to the involvement of ~3 Glu residues on the surface of cytochrome b_5 in complex formation with cytochrome *c*. The uncertainty in this estimate is difficult to assess, but it may be significant owing to the difficulties inherent in determination of infrared molar absorptivities. Extension of this technique to the study of interaction of variant forms of cytochrome b_5 with cytochrome *c* or to evaluation of the influence of pH and other solution conditions on the structure of this complex could provide useful new information concerning the structural variability of this complex and could perhaps provide more critical validation of the interpretation of the results in this initial application of the technique.

Crosslinking

The presumed stabilization of the cytochrome *c*–cytochrome b_5 complex by electrostatic attraction resulting from the stereochemically complementary interaction of lysyl ϵ-amino groups on the surface of cytochrome *c* with appropriately positioned carboxylate groups on the surface of cytochrome b_5 makes this complex a logical candidate for chemical crosslinking with water-soluble carbodiimides. Carbodiimides catalyze the formation of amide bonds between carboxylate and amino groups that in the case of complexes such as this should result in the formation of one or more isopeptide bonds per binary complex. Complexes stabilized in this fashion should be stable to a wide range of solution conditions and, in principle, should be useful for a wide range of functional studies to test a variety of assumptions concerning protein–protein interaction and electron transfer.

There are two fundamental reasons why crosslinking of electron transfer proteins with carbodiimides have attracted a great deal of experimental attention. The dominant reason that this technique has been used is the widely held belief that this strategy can provide important information concerning the identities of specific surface residues involved in protein–protein recognition. An alternative, but less frequently invoked reason for such studies is the potential that this strategy promises for preparation of stable, homogeneous, structurally characterized (or at least characterizable) binary complexes of electron transfer proteins that can be used to study detailed mechanistic attributes of protein–protein electron transfer reactions. As reviewed previously, such rationales have resulted in the use of water-soluble carbodiimides to crosslink a large number of electron transfer proteins (Mauk and Mauk, 1989).

In the case of the cytochrome *c*–cytochrome b_5 complex, crosslinking with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) under solution condi-

tions where the electrostatically-stabilized complex formed by the two proteins is most stable (pH 7.25, μ = 1 mM; ~90% of the total protein should be in the cytochrome *c*–cytochrome b_5 complex under these conditions) produces a covalently-linked binary protein–protein complex in ~30% yield that can be separated from monomeric, unreacted cytochromes and from polymeric crosslinked material by gel filtration chromatography (Mauk and Mauk, 1989). Elution of the resulting covalently-linked binary complex over anion exchange columns (CM-cellulose or Mono-Q) resulted in isolation of at least six chromatographic species that could not be separated from each other with baseline resolution. Subsequent attempts to purify and characterize the sequences of crosslinked peptides from peptide maps of two of these crosslinked species prepared from various proteolytic digests in combination with cyanogen bromide treatment encountered considerable difficulty (Mauk and Mauk, 1989). In the end, these authors concluded that for protein–protein complexes crosslinked in this manner, the use of traditional proteolytic strategies in combination with peptide mapping were not capable of permitting detailed structural characterization of the sites and numbers of covalent attachments.

Nevertheless, recent advances in the development of electrospray and MALDI mass spectrometry and their applications to the study of proteins may provide a viable means of characterizing the structures of complex crosslinked peptides formed in such reactions with structurally characterized reactant proteins. However, it is not clear that comparable advances have been achieved in protein purification that would permit resolution of the complex mixtures of products seen following the crosslinking of cytochrome *c* and cytochrome b_5 with EDC (Mauk and Mauk, 1989). Even if these technical barriers to characterizing the covalently-stabilized complexes formed by carbodiimides are overcome, it is by no means clear that this strategy is necessarily appropriate for characterizing the surfaces at which interacting proteins of this type recognize and bind to each other. As noted previously (Mauk and Mauk, 1989), water-soluble carbodiimides must be added to aqueous solution as the hydrochloride to achieve solubility. The consequence of this fact is that the addition of the crosslinking agent to a solution containing the electrostatically-stabilized cytochrome *c*–cytochrome b_5 complex at low ionic strength necessarily involves an ionic strength jump that will result in disruption of the complex at the same time the crosslinking process is initiated. As a result, it is not clear that the crosslinked species produced in this manner represent species present at equilibrium in solution. This uncertainty arises in part from the tacit assumption in such work that the carbodiimide will activate all of the surface carboxyl groups at comparable rates. If this is not the case, then the nature of the crosslinked species observed (and the distribution of such species) will be influenced by kinetic factors related to the reactivity of individual carboxylate groups that are not currently understood. Based on these considerations, it appears that if the structures of crosslinked complexes prepared in this manner could be determined in detail unambiguously, such complexes could be of considerable use in the study of intramolecular electron transfer reactions and of less apparent utility in the characterization of binding sites for other electron transfer proteins.

Electrochemical Studies

The well established dependence of the rate of electron transfer on the thermodynamic driving force for the reaction (reviewed in Marcus and Sutin, 1985; Scott *et al.*, 1985; McLendon, 1988) makes the effect of complex formation on the midpoint potentials of the interacting proteins a matter of considerable practical and theoretical interest. Unambiguous evaluation of this effect, however, is a difficult experimental undertaking. The work of Bagby *et al.* (1990) illustrates aspects of this challenge. These investigators used direct electrochemistry at an edge-oriented graphite electrode and a gold electrode modified with $(Lys\text{-}Cys\text{-}Ome)_2$ to study the behavior of a mixture of horse heart cytochrome *c* and chicken liver trypsin-solubilized cytochrome b_5 [pH 7, HEPES buffer (1 mM), and KCl (20 mM)]. In this work they determined that cytochrome *c* promotes the electrochemistry of cytochrome b_5 at the graphite electrode surface, while cytochrome b_5 promotes cytochrome *c* electrochemistry at the modified gold electrode surface. Although they were able to observe small changes in midpoint reduction potentials of the two interacting proteins under these solution conditions, Bagby and co-workers concluded that the behavior of the proteins represented the effects of formation of a ternary complex in which the electrode surface is one of the three interacting species. As a result, they were not convinced that the small changes in potential that they observed for the two cytochromes represented the true behavior of the proteins in the binary complex.

In subsequent studies, Burrows *et al.* (1991) extended the application of direct electrochemistry at edge/plane graphite surfaces to evaluate the behavior of the Asn52Ile, Asn52Ala, Lys27Gln, and Lys27Asp variants of yeast cytochrome *c* (all in the Cys102Thr background) in the presence of trypsin-solubilized bovine liver cytochrome b_5 [1 mM HEPES buffer (pH 7.0), 10 mM KCl]. While the variant proteins exhibited somewhat altered electrochemical behavior in these experiments, the authors once again concluded that the presence of the electrode surface at the moment of electron transfer within the complex resulted in an effective ternary complex, the functional properties of which cannot be regarded as representative of the behavior of the two proteins in the binary complex.

Interaction of proteins with the electrode surface is an intrinsic requirement of direct electrochemical techniques. In spectroelectrochemical measurements, an alternative electrochemical approach, the oxidation–reduction equilibrium of the protein or binary protein complex is equilibrated with the potential of the working electrode through use of a mediator that has a reduction potential close to that of the protein system being studied. Potentiometric measurements of this type under the conditions of low ionic strength (5–10 mM) required to favor formation of the binary protein–protein complex are inherently difficult owing to the absence of a supporting electrolyte. Although with patience it may be possible to overcome the prolonged equilibration times required under these circumstances, it is not completely clear that the midpoint potentials observed represent the behavior of the metal centers in the complex. Alternatively, it seems equally plausible that these apparent potentials are the midpoint potentials of the monomeric proteins influenced by (presumably small) differential, oxidation-state dependent formation of the cytochrome *c*-cytochrome b_5 complex.

STRUCTURE, DYNAMICS, AND NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Before undertaking detailed consideration of the application of NMR spectroscopy to characterization of the cytochrome *c*–cytochrome b_5 complex, it is worthwhile recalling two key characteristics of NMR spectroscopy and the use of this technique in the study of the two cytochromes individually. First, NMR is an insensitive technique with inherently poor signal:noise ratio. As a result, relatively high protein concentrations (~1–2 mM) are required for such experiments. Assuming that protein aggregation does not occur, then this concentration of protein does not present major problems for structural studies, but it does present a problem for binding studies. Namely, how is ionic strength to be controlled? Note that this is not such a concern in spectrophotometric or potentiometric studies that are carried out at lower concentrations of protein (1–80 μM). We will return to this issue later. Second, NMR spectroscopy is an equilibrium method with a relatively long data acquisition time. This means both that the investigation of kinetic processes by NMR is not straightforward and that NMR spectra often reflect an average of different states of a protein rather than distinguishing between those states. This is a particularly important point for cytochromes. Electron self-exchange rates for cytochrome *c* and cytochrome b_5 are relatively slow on the NMR time scale so that mixtures of the oxidized and reduced forms of these proteins give spectra that are weighted sums of the spectra of the individual oxidation states. Association and dissociation of cytochrome *c* and cytochrome b_5 is relatively rapid on the NMR time scale so that complexation yields spectra that are the weighted averages of the individual spectra and the spectrum of the complex. If there is more than one form of the complex, this too is in fast exchange with the free proteins and other forms of the complex. Though rapid mixing and data acquisition methods can be used to monitor fast kinetic processes by NMR spectroscopy, all such studies of the cytochrome *c*–cytochrome b_5 complex known to the authors have been carried out at equilibrium. With these points in mind, we now turn to the literature concerning NMR studies of the interaction of cytochrome *c* and cytochrome b_5.

Binding Studies

The binding stoichiometry and thermodynamic stability of the cytochrome *c*–cytochrome b_5 complex has been investigated with the two proteins in their oxidized, paramagnetic, states. Some of the heme resonances of the two cytochromes are shifted by the paramagnetism into the region 10–36 ppm where they are well resolved. Protein complexation induces small shifts of some of these resonances because the two proteins are in fast exchange between their free and bound forms, and these can be monitored to give a saturation binding curve from which the stoichiometry

and affinity are obtained. In the study of Eley and Moore (1983), the cytochrome *c* concentration was held constant, and the concentration of cytochrome b_5 was varied at pH 6.3 with no additional salts added. However, the protein concentration was sufficiently high that there was an appreciable contribution to the ionic strength from this source. Eley and Moore calculated the approximate ionic strength contributions from the proteins by assuming that: (i) the total charge of cytochrome *c* and cytochrome b_5 was + 10 and −7, respectively; (ii) each protein molecule had a corresponding number of univalent counterions to achieve charge neutrality; (iii) the counterions were not bound to the proteins; (iv) only the counterions contributed to ionic strength. On this basis, the ionic strength for the binding curves reported by these authors varied from an initial 0.04 M to a final 0.055 M over the course of the titration. As a result, the binding constant reported from this work was only an apparent constant since it is known that the interaction of cytochrome *c* and cytochrome b_5 is strongly ionic strength dependent. The binding curve obtained indicated that the cytochrome *c*–cytochrome b_5 complex formed with a 1:1 binding stoichiometry and with an apparent K_a consistent with that reported by Mauk *et al.* (1982).

In a previous study by Miura *et al.* (1980) and a later study by Whitford *et al.* (1990), NMR data were reported that were interpreted to be consistent with a ternary complex comprised of two molecules of cytochrome *c* and a single molecule of cytochrome b_5. Neither study appeared to consider the importance of the ionic strength contribution from the proteins, and in the former case, the experimental design caused relatively large variations in this term that prevents a simple determination of binding stoichiometry from the data reported. Whitford *et al.* (1990) carried out some experiments at constant cytochrome b_5 concentration and variable cytochrome *c* conentration (pH 7.2), while monitoring resonances of cytochrome b_5, so the resulting binding curve should be amenable to analysis. Their key observation was that some of the binding curves obtained in this manner were biphasic, unlike the curves observed by Eley and Moore at constant cytochrome *c* and variable cytochrome b_5 concentrations.

The interpretation of these results provided by Whitford *et al.* (1990) in terms of a ternary complex comprises the only evidence in favor of such a complex of which we are aware. Consequently, this raises the question as to why there is such a major disgreement between this work and that of other investigators. One possibility is that the experimental design employed by Whitford *et al.* is significantly different from that of all other workers, so that the latter have been unable to observe ternary complex formation. This is the suggestion favored by Whitford *et al.* However, another possibility is that tenary complex formation does not occur and that the reported biphasic plots have an alternative origin. We cannot choose between these two possibilities without further data, although we offer the following observations that provide an alternative interpretation to the one offered by Whitford and coworkers. Namely, that these investigators observed a binary complex the nature of which varied throughout the experiment.

The ionic strength employed in the NMR experiments reported by Whitford *et al.* (1990) varied through the course of the experiment, but without knowledge of the history of the samples, it is not possible to determine by how much. Using the assumptions made by Eley and Moore (1983) over the range of [cytochrome *c*]/[cytochrome b_5] = 0–2, for which biphasic behavior was observed, and with the other conditions specified (Fig. 2 in Whitford *et al.*, 1990) the ionic strength varied from 0.0485 to 0.0585 M or from 0.0335 to 0.0435 M. Thus, even for their high-affinity binary complex, these investigators can obtain only an apparent binding constant. As the value for this association constant is ~10-fold greater than that obtained from spectrophotometric titrations (Mauk *et al.*, 1982) (pH 7.2 and μ = 0.015 M), it is clear that interpretation of the NMR data in terms of simple complex formation is incorrect.

In contrast to previous NMR experiments (Eley and Moore, 1983), the experiments of Whitford *et al.* were conducted with a background of relatively high phosphate concentration and with variable cytochrome *c* concentration. As a result, it is likely that the degree of competition between cytochrome b_5 and phosphate for cytochrome *c* varied throughout the titration. Thus, the more recent NMR experiments are complicated by both a general ionic strength effect and a specific ion effect. Note that the spectrophotometric studies of Mauk *et al.* (1982, 1986), though also carried out in phosphate buffer, do not suffer from the same complications because the protein concentrations are so low that their variation does not significantly disturb either the ionic strength or the occupation of the phosphate binding sites on the cytochrome *c*. This contention is supported by the agreement between the results of the spectrophotometric titrations and subsequent potentio-

metric titrations that were conducted in the absence of buffer (Mauk *et al.*, 1991).

Finally, assuming that only a binary complex was formed but that the precise alignment of the two proteins in that complex depends on the ionic strength and composition of the solution as implied by electrostatic modelling studies (Mauk *et al.*, 1986), then the biphasic pattern observed by Whitford might result from a rearrangement of the cytochromes in the complex as the solution conditions changed. It may be that Eley and Moore (1983) did not observe biphasic behavior both because their solutions did not contain phosphate, and because they monitored changes in resonances assigned to cytochrome *c*. Since the heme 6-propionate of cytochrome b_5 is not necessarily required for intermolecular interaction in some forms of the cytochrome *c*–cytochrome b_5 complex (Mauk *et al.*, 1986) and since Whitford and co-workers monitored heme resonances of cytochrome b_5, their experiments will be extremely sensitive to the population of cytochrome b_5 complexes present. Whitford *et al.* (1991) also reported that *Saccharomyces cerevisiae* iso-1-cytochrome *c* forms a ternary complex with trypsin-solubilized bovine liver cytochrome b_5. However, their reported plots of changes in chemical shifts with [cytochrome *c*]/[cytochrome b_5] ratios are also consistent with the formation of a binary complex in which there is some alteration in the nature of the complex formed at high [cytochrome *c*].

Whitford *et al.* (1990, 1991) suggest that linewidth changes for heme resonances of cytochrome b_5 are indicative of ternary complex formation, but without a more complete investigation of resonance linewidths, this conclusion is not valid. Linewidths will increase not only because the tumbling times of the molecules decrease as complexation occurs, thus leading to enhanced dipolar broadening, but also because interchange between different forms of the complex may cause exchange broadening. Moreover, at the high field strengths used in these studies (600 MHz) there is a possibility that Curie spin relaxation could become important for some states of the complex. This phenomenon, which arises from the paramagnetism of the cytochromes, does not affect the free proteins. However, as this spin relaxation term is very sensitive to protein tumbling times, it could become important in some forms of the complex. As with the chemical shift data, changes in the alignment of cytochrome *c* and cytochrome b_5 in a binary complex might cause the observed linewidth increases.

Thus, in the absence of supporting data, we believe the case for ternary complex formation is unproven and suggest that the observations of Whitford *et al.* (1990, 1991) 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 NMR spectrum of cytochrome *c*.

Kinetic Studies

NMR is widely used to determine self-exchange rates of cytochromes and, indeed, following from the work of Gupta *et al.* (1972), NMR is often the method of choice for such experiments. The simplest case addressed by these initial investigators concerned a protein in slow self-exchange, and the procedure they adopted was to label a resonance of one of the oxidation states (usually the oxidized form) magnetically and to monitor the transfer of this label to the other oxidation state. The degree of transfer depends on the rate of electron exchange, which is the only process by which such transfer can occur. Nevertheless, electron transfer competes with relaxation phenomena that reduce the amount of label available for exchange. Thus, to use this approach, the relevant relaxation parameters must be known. If they are not known, then the electron-exchange rate calculated from the NMR data is likely to underestimate the true electron-exchange rate. For self-exchange rates, this is generally a straightforward correction, and both cytochrome *c* (Gupta and Redfield, 1970; Gupta *et al.*, 1972; Kowalsky, 1965, in fact, provided the first estimate of this self-exchange rate constant on the basis of linewidth analysis) and cytochrome b_5 (Dixon *et al.*, 1990) have been thoroughly characterized in this regard. For cross-exchange rates involving interprotein complexes such as the cytochrome *c*–cytochrome b_5 complex, these corrections are not so simple. Nevertheless, Concar *et al.* (1991) and Whitford *et al.* (1991) have attempted to measure the cross-exchange electron transfer rates for the cytochrome *c*–cytochrome b_5 complex by NMR. In their experiments, they measured the reverse electron transfer from ferrocytochrome *c* to ferricytochrome b_5 and suggested that this reaction exhibits a rate constant of $\sim$0.3–1 s^{-1}. However, this value appears to be too low in comparison with the forward rate constants for transfer from ferrocytochrome b_5 to ferricytochrome *c* within the preformed complex reported by Meyer *et al.* (1993) and Willie *et al.* (1993) when an equilibrium

constant calculated from the relative reduction potentials of the two protein couples is considered. Willie *et al.* (1993) suggested that this discrepancy results from a population of the complex in the NMR experiment that is in fast exchange and that does contribute to the magnetic labelling effect. However, this rationale is unlikely to have been the problem because the spectra reported by Concar *et al.* (1991) exhibit no evidence of fast or even intermediate exchange behavior. A more likely explanation for the discrepancy is that the leakage of the magnetic labelling effect to relaxation was not fully taken into account. Indeed, the effect of ferrocytochrome b_5 on relaxation properties of ferrocytochrome *c* was used as a control to correct for this effect (Concar *et al.*, 1991; Whitford *et al.*, 1991). Among other points, this correction assumes that the paramagnetism of ferricytochrome b_5 does not contribute to the relaxation of the appropriate ferrocytochrome *c* resonances and that dynamic properties of the ferrocytochrome *c*–ferricytochrome b_5 complex that contribute to relaxation are the same as those of the ferrocytochrome *c*–ferrocytochrome b_5 complex.

The most significant contributions of NMR spectroscopy to our understanding of the cytochrome *c*–cytochrome b_5 complex has come from definition of structural characteristics of the complex. It is pertinent to recall at this point that owing to the fast exchange of bound and unbound forms of cytochromes *c* and b_5, NMR spectroscopy monitors an average of the forms present, weighted for their relative populations. Also, NMR detects those forms of the proteins present at equilibrium. Thus, where modelling indicates that protein association causes transient conformational changes, e.g., movement of Phe82 (Wendoloski *et al.*, 1987), these will not be detected by NMR spectroscopy. Only conformational perturbations that persist into the equilibrium complex will be detected. The NMR investigations of Eley and Moore were particularly important because they were among the first experimental structural data to show Salemme's model existed in solution, and because they were the first experimental indication that dynamic characteristics of the cytochrome *c*–cytochrome b_5 complex might be important. Indeed, these authors noted that "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."

The perturbations to heme resonances caused by complex formation do not provide any detailed structural information because these perturbations arise from disturbances to the distribution of the unpaired electron in the heme that results largely from perturbations to the surface electric field accompanying complexation (Eley and Moore, 1983, Moore *et al.*, 1995). Information concerning the protein structure has come from observation of the amino acid resonances of the proteins under conditions in which bound and unbound forms of the proteins co-exist in solution. For both horse and yeast ferricytochromes *c* interacting with trypsin and lipase-solubilized bovine ferricytochrome b_5, there is no evidence of any substantial conformational differences to the proteins resulting from complex formation (Eley and Moore, 1983, Burch *et al.*, 1990; Whitford *et al.*, 1990). In fact, specific spectroscopic perturbations are so small that the binding surfaces of the interacting cytochromes cannot be delineated in a straightforward way. The only clear indications of residues at the intercytochrome interaction region from NMR of a cytochrome *c*–cytochrome b_5 complex came from the observation that the CH_3 ^1H NMR signal of trimethyllysine72 (TML72) of *Candida krusei* cytochrome *c* was strongly affected by complexation with cytochrome b_5, because the motion of TML72 was hindered in the complex (Eley and Moore, 1983), and from shifts in the ^{13}C spectrum of cytochrome *c* induced by the binding of cytochrome b_5 (Burch *et al.*, 1990). ^{13}C NMR was chosen instead of ^1H NMR because ^{13}C NMR chemical shifts are more sensitive to chemical environments than ^1H NMR. In the study reported by Burch *et al.* (1990) resonances of Ile81 and Ile85 of cytochrome *c* were affected by complexation consistent with the Salemme (1976) model.

To investigate the lysines of cytochrome *c* further, Burch *et al.* (1990) and Moore *et al.* (1995) modified them to produce the fully N^{ϵ}-acetimidylated and fully N^{ϵ}-dimethylated cytochrome *c* derivatives. The modified groups in these derivatives retained their positive charges, and hence reacted with cytochrome b_5 with similar kinetic and thermodynamic parameters to those of the native cytochrome, and had methyl substituents that were readily detected by NMR. Work with the dimethylated derivative is most advanced. Figure 2 (Moore *et al.*, 1995) shows that in the ^{13}C-^1H COSY spectrum of the fully modified horse ferricytochrome *c*, 15 of the 19 lysines are resolved and that the addition

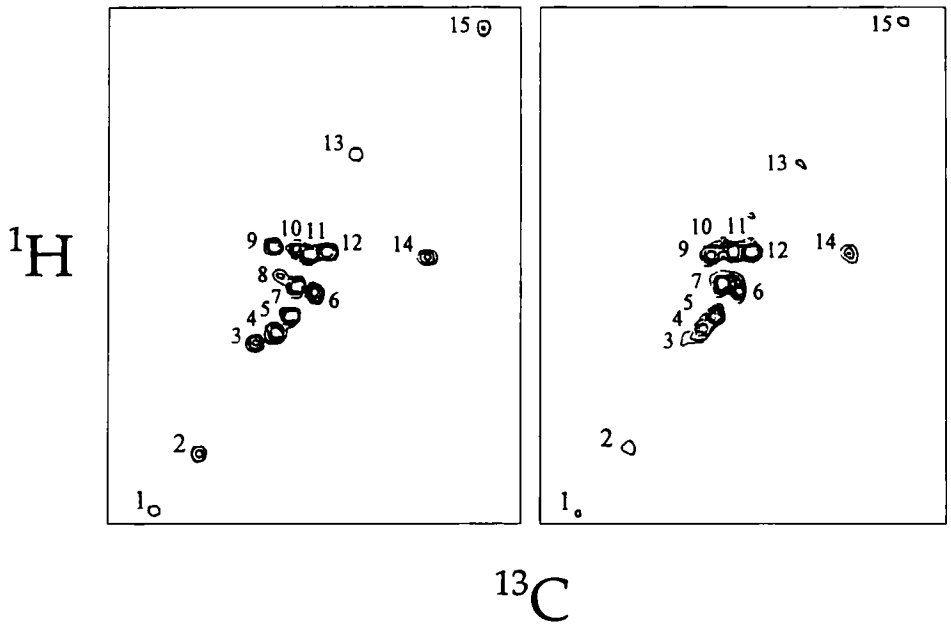

Fig. 2. Two-dimensional ^{1}H-^{13}C COSY spectra (400 MHz) of ^{13}C-labelled N^{ε} dimethylferricytochrome *c* (3 mM) in the absence (left) and presence (right) of recombinant lipase-solubilized ferricytochrome b_5 (3 mM) (5 mM sodium phosphate buffer, pH 7.0, 25°C) (from Moore *et al.*, 1995).

of ferricytochrome b_5 shifts at least 6 of these. Binding curves for the most affected resonances (13 and 9) mirror the binding curve for the cytochrome *c* heme methyl substituents, indicating that the normal cytochrome *c*–cytochrome b_5 complex is formed in these experiments. Specific assignments of the peaks in the spectrum of Fig. 2 are required to identify which lysines are involved in complexation. These assignments are currently being obtained with single-site variants of yeast iso-1-cytochrome *c* in which lyines have been replaced by alanines. However, for now we limit ourselves to two points concerning these data. First, the observation that at least six lysyl residues are affected by complex formation is different from predictions based on computer-graphics molecular-modelling experiments of static complexes, all of which predict four or five lysines to be involved in an individual complex. Second, the chemical shift perturbations on complex formation are extremely small. This was unexpected, but it may be explicable for the reason given below. The chemical shift perturbations will be a weighted average of their values in the different complexed and uncomplexed states, and it may well be that in a dynamic complex important lysine residues are not always involved in intermolecular interactions, even though the cytochrome *c* remains bound to cytochrome b_5 for most of the time. In effect, the expected chemical shift perturbations are spread over more lysine residues than are involved in intermolecular interactions at any moment of time. This would be consistent with the observation that more lysines are seen to be perturbed than expected.

Another NMR approach to determining interprotein interaction regions is to use the competitive paramagnetic difference spectroscopy method introduced by Eley and Moore (1983). This method employs redox-inactive, paramagnetic reagents to perturb the NMR spectra of the uncomplexed proteins and then compares these perturbations with those produced by the reagents under conditions when the interprotein complex has formed. For cytochrome b_5 binding to cytochrome *c*, $[Cr(ox)_3]_3^{3-}$ and $[Cr(CN)_6]^{3-}$ are suitable competitors (Eley and Moore, 1983), and for cytochrome *c* binding to cytochrome b_5 $[Cr(NH_3)_6]^{3+}$ and $[Cr(en)_3]^{3+}$ are suitable competitors (Hartshorn *et al.*, 1987; Moore *et al.*, 1995). The $[Cr(ox)_3]_3^{3-}$ and $[Cr(CN)_6]^{3-}$ experiments in particular have been useful in delineating the interaction region on cytochrome *c* for cytochrome b_5. At pH 7.2, the main interaction surface on cytochrome *c* for cytochrome b_5 was shown to include Lys72, Ile81, Ala83, Phe82, and Ile85, consistent with the Salemme model. Weaker interaction was observed at a site involving Thr19. $[Cr(en)_3]^{3+}$ competition with cytochrome *c* for cytochrome b_5

showed that within the cytochrome *c*–cytochrome b_5 complex the solvent-exposed propionate-bearing edge of cytochrome b_5 remains accessible to $[Cr(en)_3]^{3+}$ (Hartshorn *et al.*, 1987). Thus, a ternary complex was formed. Taken together with other observations, this result is consistent with the dynamic nature of the cytochrome *c*–cytochrome b_5 complex, with the effect of $[Cr(en)_3]^{3+}$ binding being to change the relative stabilities of different protein alignments with comparable thermodynamic stabilities (Mauk *et al.*, 1986). Whitford (1992) repeated the experiments of Hartshorn *et al.* (1987) using 2D NMR spectroscopy and found that $[Cr(en)_3]^{3+}$ binds to at least three sites on cytochrome b_5, only one of which is close to the heme prosthetic group. This latter site, which was indicated as a potential cytochrome *c* binding site by the modelling studies of Mauk *et al.* (1986), was partially protected from $[Cr(en)_3]^{3+}$ by cytochrome *c* binding. Thus, NMR investigations indicate that the cytochrome *c*–cytochrome b_5 complex is a dynamic structure in solution and are consistent with the formation of the structures suggested by modelling calculations (Salemme, 1976; Mauk *et al.*, 1986; Northrup *et al.*, 1993).

SIMULATION AND MODELLING OF RECOGNITION AND DOCKING

A variety of computational methods have been used to simulate the mechanisms of interaction involved in complex formation between cytochromes. As discussed above, the early study of Salemme (1976) proposed a hypothetical structural complex of cytochrome *c* and cytochrome b_5 based on the crystallographic coordinates for each protein and a least-squares fitting procedure to establish charge complementarity between highly conserved charged groups surrounding the heme crevices. Similar computer-aided docking studies have been reported for other complexes (early examples include: cytochrome *c*–cytochrome *c* peroxidase: Poulos and Kraut, 1980; cytochrome *c*–flavodoxin: Simondsen *et al.* 1982; cytochrome b_5–methemoglobin: Poulos and Mauk, 1983). An extensive analysis of the computed electrostatic field of cytochrome *c* in solution and its likely effect on reaction with cytochrome b_5 was reported by Mauk *et al.* (1986). In a recent analysis, residue substitutions of 95 cytochrome *c* sequences have been categorized as either conservative or radical, color-coded for instant visual recognition, and mapped onto the three-dimensional structure of yeast cytochrome *c* (Meyer *et al.*, 1994). These studies have been useful in establishing the role of the conserved positively charged amino acids (lysines 13, 27, 72, 86, and 87) surrounding the heme crevice in the interactions of cytochrome *c* with other electron transfer proteins.

Mechanistic Issues Addressed by Modelling Studies

Initial modelling studies considered static structures for the cytochrome *c*–cytochrome b_5 complex exclusively and focussed on the construction and analysis of a single putative binary complex. Such a strategy neglected three important considerations in the mechanism of complex formation that require more rigorous simulation methods. The first of these considerations is the influence of protein flexibility and internal dynamics on an association complex. Molecular dynamics simulations of a complex of cytochrome *c* and cytochrome b_5 (Wendoloski *et al.*, 1987) indicate that this complex is better described as a flexible association that is able to sample alternative interheme geometries. For example, through dynamical relaxation the complex was able to achieve a more intimate conformation in which the inter-iron distance decreased by as much as 2 Å after >5 ps of simulation. Substantial conformational changes were also predicted by this simulation in which Phe82 of cytochrome *c* moved from its crystallographic location, with the phenyl sidechain packed near the heme, to a position where it could conceivably bridge the two heme group π-orbital systems. However, as described in the previous section, such conformational changes do not occur in the equilibrium complexes, even if they occur in the activated complexes. The flexibility observed within the complex could explain the relative lack of recognition specificity between electron transfer proteins and could explain why some nonphysiological electron transfer partners react at rates exhibited by proteins that react with each other physiologically.

A second consideration is the influence of protein structure on the diffusion processes leading to formation of a productive electron transfer complex. This factor was first addressed in studies of the reaction of cytochrome *c* with cytochrome *c* peroxidase (Northrup *et al.* 1988), and subsequently for the reduction of ferricytochrome *c* by ferrocytochrome b_5 (Eltis *et al.*, 1991; Northrup *et al.*, 1993). As indicated previously, bimolecular electron transfer reactions require the ini-

tial transport of reactants through solution by a diffusional mechanism, and in some cases the diffusional encounter of species limits the overall rate (Berg and von Hippel, 1985). Because the portion of the cytochrome surface that is involved in recognition and interaction with other electron transfer proteins is small relative to the overall size of the cytochrome, strict orientational criteria for the reactions between such proteins exist. Electron transfer proteins have overcome this obstacle by exploiting the "reduction in dimensionality" principle proposed by Adam and Delbrück (1968). Such species diffuse in three dimensions and initially associate in unreactive configurations by nonspecific forces of attraction that are predominantly electrostatic. This association is followed by a rotational diffusional search on a lower-dimensional configurational surface of associated molecules until ultimate production of a properly oriented pair is achieved. The magnitude of forces promoting the initial nonspecific association must be finely tuned to a range allowing particles to remain in juxtaposition for time scales required for the rotational diffusive search, but not so strong that encountered particles are locked into unproductive orientations. Additionally, for highly asymmetrically charged species, a "steering" effect may operate which selectively preorients the diffusing particles into productive configurations even prior to initial encounter.

A third important consideration in the mechanism of interaction of the cytochromes is the possible existence of an ensemble of near-optimal docking geometries rather than a single complex through which electron transfer might occur. This is a feature apart from the internal and surface group flexibility available within a given complex described above. On the basis of electrostatic stability alone, theoretical simulations do not predict a dominant protein–protein interaction but do predict a mixture of complexes the distribution between which is a function of pH and ionic strength. This phenomenon has been shown to be plausible through Brownian simulation of cytochrome *c* and cytochrome b_5 complex formation (Northrup *et al.*, 1993).

Principles of Brownian Dynamics Modelling

The most powerful and useful computational strategy beyond a simple static docking study has been the Brownian dynamics (BD) approach (Ermak and McCammon 1978; Northrup *et al.*, 1984). In BD, the Brownian motion of interacting macromolecules in a solvent is simulated stochastically by a series of small displacements chosen from a distribution that is equivalent to the short-time solution of the Smoluchowski diffusion equation with forces. A connection formula exists which permits extraction of bimolecular rate constants from trajectory fate statistics for comparison with kinetic experiments (Northrup *et al.*, 1984). Simulational modelling of proteins by BD begins with high-resolution, three-dimensional structures determined by X-ray crystallography. Models are constructed of the protein shapes and placement of charges on the reactants at varying levels of complexity, ranging from simple spherical models where each sphere has an imbedded monopole and dipolar charges, to rigorous studies in which every atom is modelled as a partial charge in its crystallographic position and the irregular surface topography is treated. The protonation state of each titratable amino acid residue is typically estimated by performing a Tanford–Kirkwood calculation with static-accessibility modification (Matthew, 1985; Matthew *et al.*, 1985). The BD method is able to compute a representative set of dynamical trajectories of bimolecular diffusion and association in the complicated electrostatic force and torque field arising from the actual charge distribution of the cytochromes. The atomic-resolution surface topography of the molecules is taken into account in computing excluded volume interactions, and the mutual geometric dispositions of the heme groups are monitored and incorporated into various electron transfer reaction criteria. Extensive analysis is possible of the electrostatic contacts involved in a large number of generated stable complexes which meet the electron transfer geometric criteria. Brownian simulations of a series of species having modified charged residues has allowed a quantitative assessment of the influence of amino acid composition on rate of attainment of productive complexes. The BD method is complementary to the molecular dynamics method in that the latter is able to simulate in detail a single given intimate complex on a short time scale (tens of picoseconds), while BD is capable of generating a large representative set of docked complexes for molecular dynamical simulation.

Electrostatic treatment has employed the numerical solution of the linearized Poisson–Boltzmann (LPB) equation on a cubic lattice by the method of Warwicker and Watson (1982). Details of this procedure are more adequately described elsewhere (Northrup *et al.*, 1987). The use of the LPB equation rather than the simple Coulomb/Debye treatment

allows one to account for (i) the atomic scale rough topography of the protein surfaces, (ii) the screening influence of diffusible ions in solvent medium which cannot penetrate the protein, and (iii) the discontinuity in the dielectric constant across protein surfaces.

The effect of site-specific mutations and species variations on the rates of bimolecular reactions can be estimated in BD simulations by first performing "computer mutagenesis" on the initial crystallographic structures in which substituted sidechains are placed into the coordinate set using standard residue coordinates (Northrup *et al.*, 1987). The electron transfer event is incoporated into BD through an intrinsic spatially-dependent electron transfer rate constant k_{et} (r_{Fe}) given as a function of inter-iron distance below:

$$k_{et}(r_{Fe}) = k_{et}^{\circ}\exp(-\beta(r_{Fe} - r_0)) \qquad (1)$$

The exponential equation (1) is reminiscent of the Siders, Cave, and Marcus theory (1984) for orientation and distance dependence of electron transfer between porphyrins. Here k_{et}° is the electron transfer rate constant when porphyrins are in direct contact edge-on. The value of this parameter was assumed to be 10^{11} s^{-1} in simulation of the reaction of horse heart cytochrome *c* with cytochrome b_5 (Eltis *et al.*, 1991). In simulation of the reaction of yeast cytochrome *c* variants with cytochrome b_5 (Northrup *et al.*, 1993), this parameter was calibrated to agree with experiment for the wild-type proteins and then applied to the reactions involving cytochrome *c* variants. The electron transfer distance-dependent factor β is viewed as an adjustable parameter that can be varied to fit the experimental data. Thus, an estimate of this important factor can be obtained from BD.

Bimolecular Rate Constants for the Reaction of Wild-Type and Variant Forms of Cytochrome *c* with Cytochrome b_5

The reduction of wild type yeast iso-1-ferricytochrome *c* and several of its variants by trypsin-solubilized bovine liver ferrocytochrome b_5 has been studied under solution conditions where electron transfer reaction is bimolecular (Northrup, *et al.*, 1993). The BD theory gives an excellent representation of the ionic strength dependence, indicating that this model provides a consistent representation of the electrostatic effects on reaction rate and a reasonable treatment of the coupling between diffusion and instrinsic electron transfer dynamics. The effect of electrostatic charge modifications and steric changes on the kinetics has been determined by experimental and theoretical observations of the electron transfer rates of cytochrome *c* variants Lys79Ala, Tm172Ala, Lys79Ala/Tm172/Ala, and Arg38Ala. The rigorous Brownian dynamics method that simulates diffusional docking and electron transfer was employed to predict the effect of the mutations on the second-order rate constants. The electron transfer event was treated by the exponential distance-dependent reactivity model as previously described. Not only did the rigorous BD method quantitatively predict rate constants over a considerable range of ionic strength, but quantitative prediction of rate constants for reduction of cytochrome *c* variants was also made possible by accounting for the perturbing influence of the mutations on the reduction potentials of the variants and the electrostatics of docking. Using these variations in E°, the Marcus equation, and the assumption of a reorganization energy of 0.7 eV, it was estimated that the variation in the intrinsic electron transfer rate constant factor k_{et}°. BD simulations were performed with these varying values of k_{et}° (Table I) for select variants. Both the experimentally observed rate constants and those predicted by BD descend in the order as follows: wild-type >Lys79Ala > Tml72Ala >Lys79Ala/Tml72Ala.

Models for Encounter Complexes Predicted by Brownian Dynamics

Docking dynamics and profiles were studied extensively through Brownian simulation (Northrup *et al.*, 1993). From this work, it appears that the two proteins approach one another for efficacious electron transfer through essentially a single domain rather than a single conformation and that the distance of closest approach of the heme groups of the two cytochromes in rigid body docking is typically ~12 Å. Within this domain are found basically two types of mutual orientations of the proteins which could contribute to electron transfer. One of these domains is identical to that proposed by Salemme (1976) in which the following groups on the surfaces of the two proteins form hydrogen bonding interactions with each other (cytochrome b_5–cytochrome *c*): Glu44-Lys27, Glu48-Lys13, Asp60-Tm172, and heme 6-propionate-Lys79. The electrostatic binding energy of this complex is −6.4 kcal/mol. Another complex is predicted by the BD calculations to have an average electrostatic energy of

Table I. Theoretical and Experimental Bimolecular Rate Constants for the Oxidation of Cytochrome b_5 by Wild-Type and Four Variants of Yeast *iso*-1-Cytochrome *c* (Northrup *et al.*, 1993)[a]

Protein	I(M)	Experimental	BD,[b] distance cutoff model	BD,[c] exponential model, fixed $k_{et}°$	BD,[d] exponential driving-force-corrected $k_{et}°$	$k_{et}°(ps^{-})$
Will-type	0.19	136 ± 3	172 ± 63	136 ± 17	136 ± 17	0.130
Lys79Ala	0.19	68 ± 3	62 ± 40	110 ± 10	112 ± 9	0.133
TML72Ala	0.19	37 ± 3	55 ± 15	63 ± 3	59 ± 6	0.121
Lys79Ala/TML72Ala	0.19	30 ± 2	15 ± 23	47 ± 6	46 ± 6	0.127
Wild-type	0.163	290 ± 31	266 ± 49	281 ± 30	281 ± 30	0.130
Arg38Ala	0.163	89	225 ± 77	238 ± 20	126 ± 15	0.069

[a] All rate constants are given in units of 10^6 $M^{-1}s^{-1}$. [b] Reaction criterion-exponential reactivity model with β = 1.0 $Å^{-1}$, $k_{et}°$ driving-force-corrected, varying according to Eq. (1). Value of $k_{et}°$ given in last column. [c] Reaction criterion = exponential reactivity model with β = 1.0 $Å^{-1}$, fixed $k_{et}° = 1.3 \times 10^{11}$ s^{-1}. [d] Reaction criterion = distance cutoff model with d_{rx} = 12 Å, ψ = 30°, pH7.0, T = 25°C.

−10 kcal/mol (Fig. 3). This electrostatically favored or "dominant" complex involves hydrogen bonding interactions of the following residues (cytochrome b_5–cytochrome *c*): Glu48-Lys13, Glu56-Lys87, Asp60-Lys86, and heme 6-propionate-Tml72.

Subsequent refinement of the complexes generated by the BD study have been performed in which these two complexes have been subjected to energy minimization and compared in greater detail (Guillemette *et al.*, 1994). The energy minimization calculations reported in this work are an attempt to produce models that reflect more accurately the structures of these complexes that are expected to occur as the initial encounter process evolves to produce a putative transition state complex. Examination of the two minimized complexes reveals several similarities with the crystallographic structure reported for the cytochrome *c*–cytochrome *c* peroxidase complex (Pelletier and Kraut, 1992). For example, all three complexes exhibit formation of salt bridges between the interacting proteins as well as the presence of hydrophobic residues at the protein–protein interface. Nevertheless, the number of salt bridges predicted for the cytochrome *c*–cytochrome b_5 complex is greater than that found in the structure of the cytochrome *c*–cytochrome *c* peroxidase complex. In addition, Phe82 of cytochrome *c* occupies a central position between the donor and acceptor centers in all three complexes that suggests at the least a structural or functional role for this residue in electron transfer between the two proteins. Finally, it is notable that the angles formed by the average plane of the heme groups in the crystallographically determined structure of the cytochrome *c*–cytochrome *c* peroxidase complex and the minimized models for the cytochrome *c*–cytochrome b_5 complex are all >20° in contrast to the original models proposed for these complexes (Salemme, 1976; Poulos and Kraut, 1980) that placed the heme groups in parallel planes.

REFLECTIONS AND PROJECTIONS

The fundamental issues addressed in the studies reviewed here and elsewhere in this issue (Durham, Millett, *et al.*, 1995) concern, in part, (i) the structures of the complexes formed by cytochrome *c* and cytochrome b_5 in solution, (ii) the manner in which electrostatic, hydrophobic, and solvation properties of the two proteins influence these structures and their thermodynamic stability, and (iii) the effect of the structures of these complexes on the kinetics of electron transfer between heme centers. Traditionally, X-ray crystallography has been the method of choice for defining three-dimensional macromolecular structures in biology. However, the relative instability of interactions between proteins such as cytochrome *c* and cytochrome b_5 and the presumed multiplicity of binary complexes formed by these proteins in solution make it less clear that crystallography can provide an unambiguous structural answer for such systems. Although such a structure for the cytochrome *c*–cytochrome b_5 complex would represent a significant experimental achievement, the question arises regarding the extent to which crystal lattice forces may selectively stabilize (or distort and then stabilize) one of several such structures present in solution. For this reason, dynamic techniques such as NMR spectroscopy may be intrinsically better suited to investigation of such complexes. However, as discussed above, the relative insensitivity of NMR and the small size of protein–protein com-

Fig. 3. Stereodiagrams of the energy-minimized, putative complex formed by yeast *iso*-1-cytochrome *c* and trypsin-solubilized bovine liver microsomal cytochrome b_5 (Guillemette *et al.*, 1994) based on the electrostatically "dominant" complex predicted by Brownian dynamics simulations (Northrup *et al.*, 1993).

plexes that can be studied by this method are serious limitations of this technique.

Although the relatively low thermodynamic stability of this complex in aqueous solution represents the principal experimental challenge to its characterization, other challenges presented by this system must also be overcome. For example, relatively low ionic strength (1–10 mM) must be used to achieve a sufficient degree of complex formation for physical studies of the complex. As a result, experiments must be

designed that can be performed at low ionic strength and that do not result in variation of ionic strength during the experiment. Care must also be taken in the design of experiments that employ cytochrome variants in which the surface charge properties of the proteins are altered. While there is little reason to expect that such amino acid substitutions generally alter the structures of cytochromes *c* and b_5 significantly, it must be determined whether or not such modifications alter the dependence of complex stability on pH or the relative abundance of protein–protein complexes within a family of near isoenergetic complexes. If the dependence of the stability constant for complex formation on pH is not evaluated, then comparison of apparent variations in complex stability following surface modification can be misleading. This concern is increased when the possible effects of such substitutions on the relative abundance of individual members of a family of putative complex structures such as those predicted by Brownian dynamics simulations is considered. Finally, the precision of modelling studies is restricted to some extent by limitations of the crystallographically determined structures of the two cytochromes in that many of the electrostatically charged surface amino acid side chains are sufficiently disordered that their positions are not defined by diffraction analysis.

To overcome these challenges, a comprehensive, multidisciplinary approach is required in which a wide range of physical, spectroscopic, and theoretical approaches is employed. At least three fundamental advantages arise from such an approach. First, potential blindspots of individual approaches are avoided. For example, although potentiometric titrations of protein–protein complex formation are highly precise and accurate, they are only able to detect complex formation that is pH-dependent. Spectroscopic techniques should permit detection of interactions that are not pH-dependent and that result in spectroscopic changes. Second, the application of new, emerging theoretical and experimental techniques to characterization of a model system such as the cytochrome *c*–cytochrome b_5 complex provides both insight into the interaction of these two proteins and into the limitations and capabilities of the new techniques prior to their application in the study of more sophisticated and less well characterized systems. Finally, the use of Brownian dynamics simulations as a guide to mutagenesis and as an aid in data analysis provides an unusually effective synergism between theory and experiment. In our experience, Brownian dynamics has been useful both in identification of the electrostatically favored sites of protein–protein contact and in interpretation of functional studies involving variants.

Future studies of the cytochrome *c*–cytochrome b_5 complex will employ many enhancements of available techniques as well as implementation of new techniques that are currently emerging as applicable to the study of proteins. For example, NMR experiments to evaluate the interaction of these proteins in solution will be enhanced through the combined use of site-directed mutagenesis, chemical modification, and isotopic enrichment (e.g., Moore *et al.*, 1995). Calorimetric methods may be applicable for some pairs of interacting proteins (e.g., Jelesarov and Bosshard, 1994), though it is not currently apparent whether this technique will be applicable to the interaction of cytochrome *c* and cytochrome b_5 (Mauk *et al.*, 1982). Another issue that has been virtually ignored in previous work is the contribution of hydrophobic interactions to the stability of protein–protein complex formation. Clearly, variant cytochromes can be designed that should alter the dielectric of the protein–protein interface or that would modify the steric properties of uncharged residues present at this interface. While the electrostatic consequences of such modifications may be small or difficult to assess, such variants could be of considerable value in stabilizing the protein–protein complex and of use in NMR studies. In fact, enhancement of the stability of cytochrome complexes through protein engineering could be an important means of gaining insight into the structural basis for more stable complexes formed by hormones and their membrane-bound receptors to overcome structural ambiguities and uncertainties normally encountered with these more sophisticated systems.

Recent advances in electrospray mass spectrometry suggest that perhaps this technique may be applicable to the study of protein–protein complexes involving small proteins. While it is uncertain whether or not the behavior of these complexes in the gas phase is representative of their behavior in solution, the possibility of directly evaluating the charge properties of such complexes and possibly the number of such complexes directly by this method merits evaluation. The relatively brief time between ionization and detection (hundreds of microseconds) in such experiments may be sufficiently short that significant changes in the structures of the complexes do not occur. As indicated above, such studies at the least will provide useful information concerning the capabilities of the technique if not the properties of the system under study.

In future modelling studies, the utility of simulations of electron transfer rates between metalloproteins will increase as more detailed modelling of the intrinsic electron transfer step is incorporated. For instance, the most advanced reactivity model used so far, the simple exponential distance decay model, is undoubtedly inadequate. The knowledge of the variable reactivity of the protein surfaces is being made available by bond pathways analysis (Beratan *et al.*, 1992; Siddarth, 1994; Siddarth and Marcus, 1990), and these theories could be included in simulations by incorporation of special reactivity functions. In addition, it may be feasible to employ contemporary electrostatic algorithms to model the results of potentiometric measurements of protein–protein interaction and in this way develop mechanistic models for the involvement of specific surface residues in formation of protein–protein complex. The results of such modelling studies would then be of use for the design of further site-directed variants that could be used in subsequent experimental and modelling studies.

ACKNOWLEDGMENTS

Research in the authors' laboratories related to the subject of this review has been supported by NIH Grants GM-33804 (to A. G. M.) and GM-34248 (to S. H. N.), by the BBSRC/EPSRC through support of the UEA Centre for Metalloprotein Spectroscopy and Biology via their Biomolecular Sciences Panel, by the Donors of the Petroleum Research Fund Administered by the American Chemical Society (to S. H. N.), and by NATO Travel Grant 0145/87 (to A. G. M. and G. R. M.). The authors also acknowledge the important contributions of their students, postdoctoral fellows, and collaborators who have participated in work concerning this topic over the past several years and whose names appear in the references cited.

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