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Laser Flash Photolysis Studies of Electron Transfer to the Cytochrome b_5–Cytochrome c Complex†

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ABSTRACT: Rate constants for electron transfer in the complex between recombinant rat mitochondrial outer membrane cytochrome b_5 or the tryptic fragment of bovine liver cytochrome b_5 and horse mitochondrial cytochrome c were measured by laser flash photolysis of 5-deazariboflavin–EDTA solutions. When an excess of cytochrome b_5 was titrated with increasing amounts of cytochrome c at low ionic strength and electron transfer was initiated by a laser flash, both proteins were rapidly reduced by deazariboflavin semiquinone. The initial photoreduction was followed by a slower second-order reduction of b_5 complexed oxidized cytochrome c by free reduced cytochrome b_5. At an 8:1 ratio of cytochromes b_5 to c, the pseudo-first-order rate constant for reduction of complexed cytochrome c increased 3–5-fold between ionic strengths of 5 and 40 mM, and then dropped precipitously at higher ionic strengths. The ionic strength dependent increase in rate constant is likely to be due to relief of steric hindrance via rearrangement of cytochrome c in the complex. The reaction rate showed no sign of saturation at any ionic strength, indicating a first-order rate constant greater than 10^4 s^{-1} within a transient ternary protein complex; i.e., interprotein electron transfer approaches the largest values previously reported for the stable binary protein complex (approximately 4×10^5 s^{-1}). Our results emphasize the flexibility of electron-transfer protein complexes, which had previously been modeled in a single conformation with specific salt bridges. It appears that a variety of orientations can exist within such protein–protein complexes and that the population of conformations changes with ionic strength. Furthermore, the complex which is most favorable for electron transfer is not necessarily the one which is most stable.

The cytochrome b_5–cytochrome c electrostatic complex is an important model for testing theories of interprotein electron transfer. The large negative charge on cytochrome b_5 provides a binding site complementary to the large positive charge on cytochrome c. The two proteins form a relatively strong complex with a dissociation constant of 0.4 μM at I = 2 mM (Rogers et al., 1988); the value at I = 10 mM is 0.6 μM, but a decrease of more than 2 orders of magnitude occurs by I = 40 mM (Mauk et al., 1991).

A hypothetical computer model of the complex was first proposed by Salemme (1976) and later refined by Mauk et al. (1986) and Wendeloski et al. (1987). In the model, the complementary electrostatic fields of the two proteins were juxtaposed, and the hemes were placed as close as possible to one another and were constrained to be in a parallel orientation, which was presumed to be optimal for electron transfer.

Intracomplex electron transfer for the bovine liver microsomal cytochrome b_5–horse cytochrome c complex measured by pulse radiolysis showed a moderately fast first-order rate constant, k = 1600 s^{-1} (McLendon & Miller, 1986). Measurement by laser flash photolysis using an acridine dye as reductant gave a similar number for the rat liver microsomal cytochrome b_5–horse cytochrome c complex (Qin et al., 1991). However, a stopped-flow analysis of electron transfer from cytochrome b_5 to cytochrome c indicated that intracomplex rate constants might be much faster (Eltis et al., 1991). In fact, the most recent measurements, using a ruthenium derivative of cytochrome b_5 plus cytochrome c, show that the intracomplex electron-transfer rate constant is greater than 10^5 s^{-1} (Willie et al., 1992).

Site-specific mutations of the acidic residues on cytochrome b_5 which are thought to be involved in binding to cytochrome c (E44, E48, and D60), and binding constant measurements, appear to corroborate the hypothetical model (Rogers et al., 1988; Rogers & Sligar, 1991; Qin et al., 1991). Likewise, steady-state kinetic measurements of cytochrome c mutants and a comparison of the kinetics of reduction of native and dimethyl-esterified heme propionate cytochrome b_5 (Eltis et al., 1988; Whitford et al., 1991; Burrows et al., 1991) were in apparent agreement with the model.

Previous investigations of interprotein electron transfer between cytochrome b_5 and cytochrome c have utilized the water-soluble domain of microsomal cytochrome b_5, derived either from trypsin cleavage of the membrane tail of the bovine liver protein (Reid & Mauk, 1982; Mauk et al., 1986; McLendon & Miller, 1986; Wendoloski et al., 1987; Eltis et al., 1991) or from the recombinant rat liver cytochrome b_5 obtained from gene synthesis and bacterial expression of the water-soluble fragment that would have been obtained upon trypsin cleavage of the rat liver microsomal protein (Bodman et al., 1986; Rogers et al., 1988; Rogers & Sligar, 1991; Qin et al., 1991; Willie et al., 1992).

A second type of membrane-anchored cytochrome b_5 has been discovered in the outer membranes of rat liver mitochondria (Sottocasa et al., 1967; Fukushima & Sato, 1972; Nishimoto et al., 1977; Ito, 1980; Lederer et al., 1983). This outer mitochondrial membrane cytochrome b_5 has been shown to participate in the rotenone-insensitive NADH–cytochrome c reductase system (Sottocasa et al., 1967), suggesting to us that it might be a physiological partner of cytochrome c in rat

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liver mitochondria. The proteolytically cleaved outer membrane cytochrome b_5 was first purified to homogeneity by Ito (1980), who also carried out immunological reactions with rabbit antibodies prepared against both microsomal and outer membrane cytochromes b_5, which demonstrated that they are distinct proteins. Lederer et al. (1983) reported the complete amino acid sequence of the trypsin-cleaved portion of the outer membrane cytochrome b_5 and showed that it differs from that of the microsomal protein at 38 of 91 positions.

In order to investigate the spectroscopic, redox, and kinetic differences between microsomal and outer membrane cytochromes b_5, we have recently synthesized the gene coding for the water-soluble domain of this protein by back-translating the known amino acid sequence (Lederer et al., 1983) and expressed it in *Escherichia coli* (Rivera et al., 1992). NMR, EPR, and UV–visible spectroscopic investigations have shown that the rhombic perturbation of the ferric heme center of outer membrane cytochrome b_5 is essentially identical to those of the microsomal beef, rabbit, chicken, and rat cytochromes b_5. With this similarity in mind, we undertook an investigation of electron transfer between this protein and cytochrome *c*. As a control, we have carried out the same set of experiments on the trypsin-cleaved bovine microsomal cytochrome b_5/cytochrome *c* system. For the two forms of cytochrome b_5, we have found analogous electron-transfer processes that differ only slightly in rate constant. The results further show that electron-transfer rates in transient ternary complexes of cytochrome b_5 and cytochrome *c* can approach those in stable binary complexes under the appropriate conditions.

MATERIALS AND METHODS

The laser flash photolysis apparatus and methods of data collection and analysis were as previously described (Tollin et al., 1986; Eltis et al., 1988; Tollin & Hazzard, 1991). A laser flash at 400 nm excites 5-deazariboflavin to the triplet state, which in turn oxidizes EDTA, resulting in formation of 5-deazariboflavin semiquinone in less than 1 μs. All kinetic experiments were performed under pseudo-first-order conditions in which the concentration of 5-deazariboflavin semiquinone generated by the laser flash (<1 μM) was smaller than the cytochrome b_5 or cytochrome *c* concentrations (>1 μM). Thus, when both cytochromes were present simultaneously in a preformed complex at low ionic strength, only one of the components was likely to undergo reduction during the laser flash. Protein stock solutions were prepared in 5 mM phosphate, pH 7. The flash buffer contained 0.5 mM EDTA, 1 mM phosphate, pH 7, and 100 μM 5-deazariboflavin. The ionic strength was varied by addition of aliquots of 4 M NaCl. Most experiments were performed with the rat mitochondrial outer membrane cytochrome b_5, except where stated otherwise.

The DNA sequence that encodes the tryptic fragment of rat liver outer mitochondrial membrane cytochrome b_5 was obtained by back-translating the amino acid sequence reported by Lederer et al. (1983). The appropriate DNA sequence was synthesized using two pairs of self-primed single-stranded oligonucleotides (Rivera et al., 1992), by a method similar to the one reported by Funk et al. (1990). Codons frequently used by *E. coli*, start and stop signals, and restriction sites aimed to facilitate future site-directed mutagenesis were included in the design of the gene. The synthetic gene was then cloned into the pET11-a plasmid (Studier & Moffatt, 1986). Transformation of *E. coli* strain BL21(DE3) with this recombinant plasmid (MRL2) resulted in colonies that upon induction with IPTG (isopropyl β-D-thiogalactopyranoside) synthesize outer mitochondrial membrane cytochrome

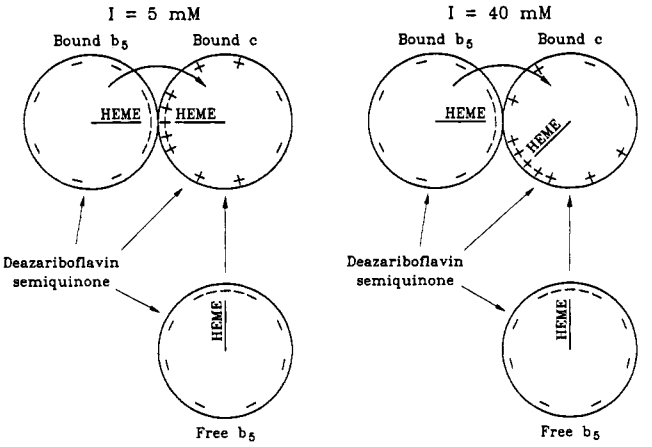

FIGURE 1: Schematic representation of the cytochrome b_5–cytochrome *c* system. Deazariboflavin semiquinone is rapidly generated by a flash of laser light at 400 nm, and subsequently reacts with free cytochrome b_5 and with the bound cytochromes b_5 and *c* within a stabilized protein complex as shown by the arrows. All three rate constants for this process are within an order of magnitude of one another as detailed in the text. Any cytochrome b_5 which is reduced in this initial process is reoxidized by the bound cytochrome *c*, also shown by arrows. Intracomplex electron transfer from bound cytochrome b_5 to bound cytochrome *c* was studied by Willie et al. (1992), and interaction of free cytochrome b_5 with bound cytochrome *c* is the subject of this report.

b_5 very efficiently. The protein was purified by standard methods of column chromatography (Rivera et al., 1992). The tryptic fragment of bovine liver microsomal cytochrome b_5 was prepared from fresh liver as described previously (Reid & Mauk, 1982; Reid et al., 1984). Horse cytochrome *c* was purchased from Sigma and used without further purification. The cytochrome *c* solution did contain some reduced protein, which was oxidized by a few crystals of ferricyanide in the presence of 0.5 M NaCl in buffer, which was removed by several cycles of concentration and dilution in an Amicon Diaflo YM5 apparatus, with a final buffer change to 5 mM phosphate. *Rhodobacter capsulatus* cytochrome c_2 was prepared as previously described (Bartsch, 1978).

RESULTS AND DISCUSSION

Laser flash photolysis of flavin/EDTA solutions provides a means of rapidly generating a reductant in situ, which can be used to initiate electron transfer in multicenter proteins or multiprotein complexes, and subsequently to follow intramolecular or intracomplex electron transfer without the complication of chemical modification of the proteins (Tollin et al., 1986; Tollin & Hazzard, 1991; Cusanovich, 1991a). The scheme shown in Figure 1 illustrates the electron-transfer reactions which occur in our experimental system, and should be consulted throughout the following discussion. We had previously studied the bovine liver cytochrome b_5–cytochrome *c* electrostatic complex by this method, but could not observe any intracomplex electron transfer from b_5 to *c* in a 1:1 mixture (Eltis et al., 1988). This was attributed to the greater reactivity of free cytochrome *c* with lumiflavin semiquinone due to the large difference in the redox potentials of cytochrome b_5 (around 0 mV; Reid et al., 1982; Walker et al., 1988) and cytochrome *c* (about 260 mV). We have confirmed this in the present work with the rat outer membrane cytochrome b_5, and have found an approximately 4-fold difference in reactivity of flavins with free cytochrome *c* over free cytochrome b_5. Deazariboflavin semiquinone was chosen as reductant for the cytochromes in the present study because it is more reactive as a consequence of having a much lower redox potential ($E_{m,7}$ = –650 mV) than lumiflavin semiquinone ($E_{m,7}$ = –231 mV).

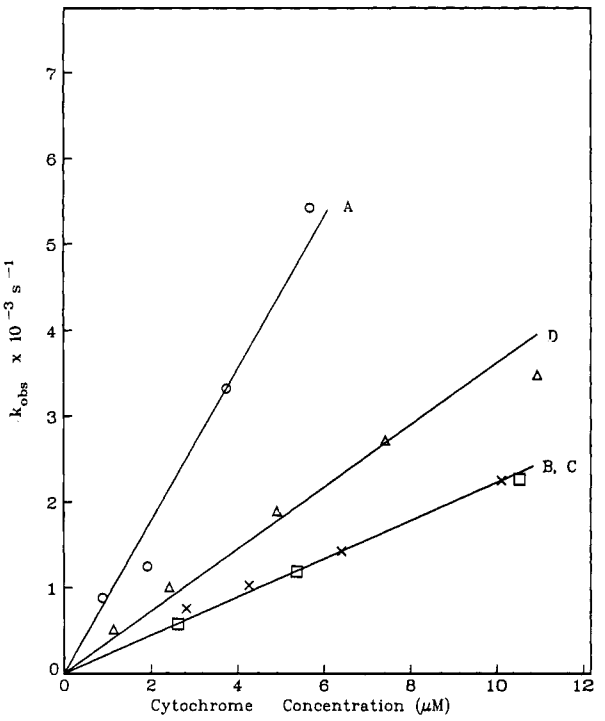

FIGURE 2: Second-order plots for reaction of deazariboflavin semiquinone with individual proteins. (A) Horse cytochrome *c* (○); (B) rat mitochondrial outer membrane cytochrome b_5 (×); (C) bovine liver cytochrome b_5 (□); (D) *Rb. capsulatus* cytochrome c_2 (△). The reaction solution contained 1 mM phosphate, pH 7, 0.5 mM EDTA, about 70 μM deazariboflavin, and the indicated amount of protein.

Thus, the second-order rate constant for reduction of free cytochrome *c* by 5-deazariboflavin semiquinone is 9.0×10^8 M^{-1} s^{-1} (Figure 2A), and that for free cytochrome b_5 is 2.4×10^8 M^{-1} s^{-1} (Figure 2B). The observed rate constants for cytochrome b_5 reduction by 5-deazariboflavin semiquinone increased by a factor of 2–3-fold with an increase in ionic strength from 5 to 240 mM, whereas those for cytochrome *c* decreased slightly (data not shown). This may be due to an increase in the redox potential of cytochrome b_5 as a result of masking the large negative surface charge, and to a decrease in the redox potential of cytochrome *c* due to masking the large positive surface charge (Margalit & Schejter, 1973; Goldkorn & Schejter, 1976; Reid et al., 1982). For the same reason, the rate constants for reduction of the two proteins by 5-deazariboflavin semiquinone within an electrostatically stabilized complex should be closer to one another than for the free proteins (ignoring steric hindrance of one protein by the other as discussed below).

Upon flash photolysis of a 1:1 cytochrome b_5–cytochrome *c* mixture at 5 mM ionic strength using 5-deazariboflavin, we found no observable absorbance change at 557 nm, which is the wavelength maximum for reduced cytochrome b_5 and an isosbestic point for cytochrome *c*. However, there was a large absorbance increase at 550 nm, which is the wavelength maximum for reduced cytochrome *c*. This is identical to the results reported previously by Eltis et al. (1988). These results can be interpreted in either of two ways: (1) cytochrome b_5 in the complex is not reduced by 5-deazariboflavin semiquinone due to the large difference in individual rate constants as reported above, or due to steric constraints; (2) intracomplex electron transfer is faster than the rate of cytochrome b_5 reduction under these conditions. It had previously been reported from two separate laboratories that intracomplex electron transfer was within the range of detection by our methods. McLendon and Miller (1985) used pulse radiolysis to generate hydrated electrons to initiate electron transfer and reported an intracomplex rate constant of 1600 s^{-1} for the bovine microsomal cytochrome b_5–cytochrome *c* complex. Qin et al. (1991) used laser flash photolysis of an acridine dye to initiate electron transfer in the rat microsomal cytochrome b_5–cytochrome *c* complex and obtained a rate constant of 1700 s^{-1}. In contrast to these reports, however, the most recent results, obtained with a tris(bipyridyl)ruthenium–cysteine-65 derivative of cytochrome b_5 in a 1:1 complex with cytochrome *c*, indicate that the rate constant for intracomplex electron transfer is 4×10^5 s^{-1} (Willie et al., 1992), which is too fast to be resolved in our experiment because of a rate limitation by the flavin to protein electron input reaction from 5-deazariboflavin semiquinone.

In order to determine whether cytochrome b_5 could be reduced in the complex under our experimental conditions, we completely photoreduced cytochrome *c* in a 1:1 mixture of the two proteins by steady-state preillumination and then examined the reduction of bound oxidized cytochrome b_5 initiated by flash photolysis. The bound cytochrome b_5 was in fact reduced with a second-order rate constant approximately twice that of the free protein, which is consistent with the results of the effect of ionic strength on the individual rate constants noted above, as well as with similar observations with lumiflavin semiquinone in an earlier study (Eltis et al., 1988). This supports the second interpretation noted above, i.e., that there is no steric hindrance to reduction of cytochrome b_5 by 5-deazariboflavin semiquinone due to complexation with cytochrome *c* and that intracomplex electron transfer is rapid. We thus conclude that there was partial reduction of cytochrome b_5 in the experiment with a 1:1 complex of oxidized proteins, followed by rapid intracomplex transfer of electrons to cytochrome *c*.

Another way to ensure that cytochrome b_5 is initially reduced in a mixture of both proteins is to have an excess of cytochrome b_5 over cytochrome *c*. In an 8:1 mixture of cytochrome b_5 to cytochrome *c* at 5 mM ionic strength, we observed a rapid biphasic initial increase in the absorbance at 557 nm followed by a slower decrease as shown in Figure 3A. At 550 nm, there was a biphasic absorbance increase following the initial transient (Figure 3B). At both wavlengths, the initial very rapid increase in absorbance is due to 5-deazariboflavin semiquinone formation. We attribute the second fast phase in both cases to reduction of the individual proteins, both free and in the complex, by 5-deazariboflavin semiquinone, and the slow phase to intermolecular electron transfer from free cytochrome b_5 to bound cytochrome *c* in a transient ternary protein complex. The alternative explanation, that free cytochrome b_5 reacts with bound cytochrome b_5 followed by more rapid intracomplex electron transfer to bound cytochrome *c*, is less likely due to electrostatic repulsion between free and bound cytochrome b_5. Consistent with the above interpretation, the cytochrome b_5 reoxidation reaction is second order ($k = 2.6 \times 10^8$ M^{-1} s^{-1}) as shown in Figure 4A. As the ratio of cytochrome b_5 to cytochrome *c* approaches 1, the signal intensity at 557 nm approaches 0 due to most of the electrons going directly into the complex. There is no indication of a change in the rate-limiting step in the reaction of b_5 and *c*, and thus electron transfer within the transient ternary protein complex must have a rate constant greater than 6000 s^{-1}, which is the largest pseudo-first-order rate constant we measured at $I = 5$ mM. This is to be contrasted with electron transfer in the stable binary complex, which should be close to 10^5 s^{-1} under these conditions (Willie et al., 1992).

We examined the effect of ionic strength on the reaction between free cytochrome b_5 and the stable binary complex by adding increasing amounts of NaCl to a solution containing

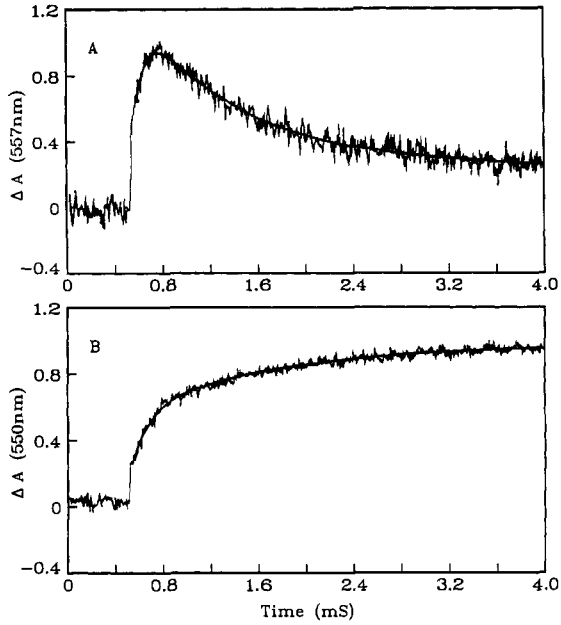

FIGURE 3: Typical transients obtained upon laser flash photolysis of an excess of rat outer membrane cytochrome b_5 (45 μM) over cytochrome c (3.7 μM) using 5-deazariboflavin/EDTA/phosphate at an ionic strength of 5 mM: (A) 557 nm; (B) 550 nm. The solid line in each case is the theoretical fit assuming two exponential reactions following the initial rapid transient. Parameters were (A) $k_A = 1.16 \times 10^4$ s^{-1} and $k_B = 1.08 \times 10^3$ s^{-1}, (B) $k_A = 9.1 \times 10^3$ s^{-1} and $k_B = 8.4 \times 10^2$ s^{-1}.

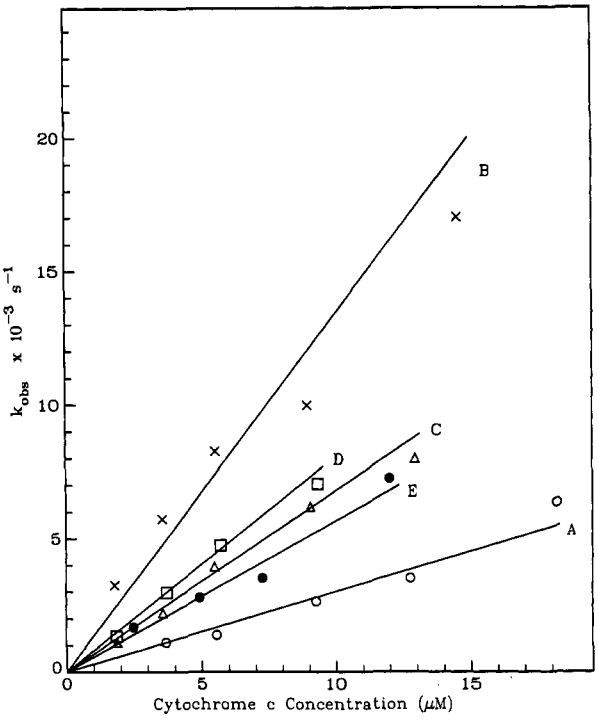

FIGURE 4: Second-order plots of the reaction of free rat outer membrane cytochrome b_5 with the electrostatically stabilized b_5–c binary complex at three ionic strengths: (A) 5 mM (○); (B) 35 mM (×); (C) 87 mM (△). (D) Bovine liver cytochrome b_5–c complex at 5 mM (□). (E) Rat outer membrane cytochrome b_5–c_2 complex at 5 mM (●).

an excess of cytochrome b_5 over cytochrome c at an initial ionic strength of 5 mM as shown in Figure 5A. The rate constant increases by approximately 3–5-fold at I = 5–40 mM and then drops rapidly at higher ionic strengths. We ascribe the increase in rate constant to a relief of steric hindrance through rearrangement of cytochrome c relative to cytochrome b_5 in the complex, making the complexed

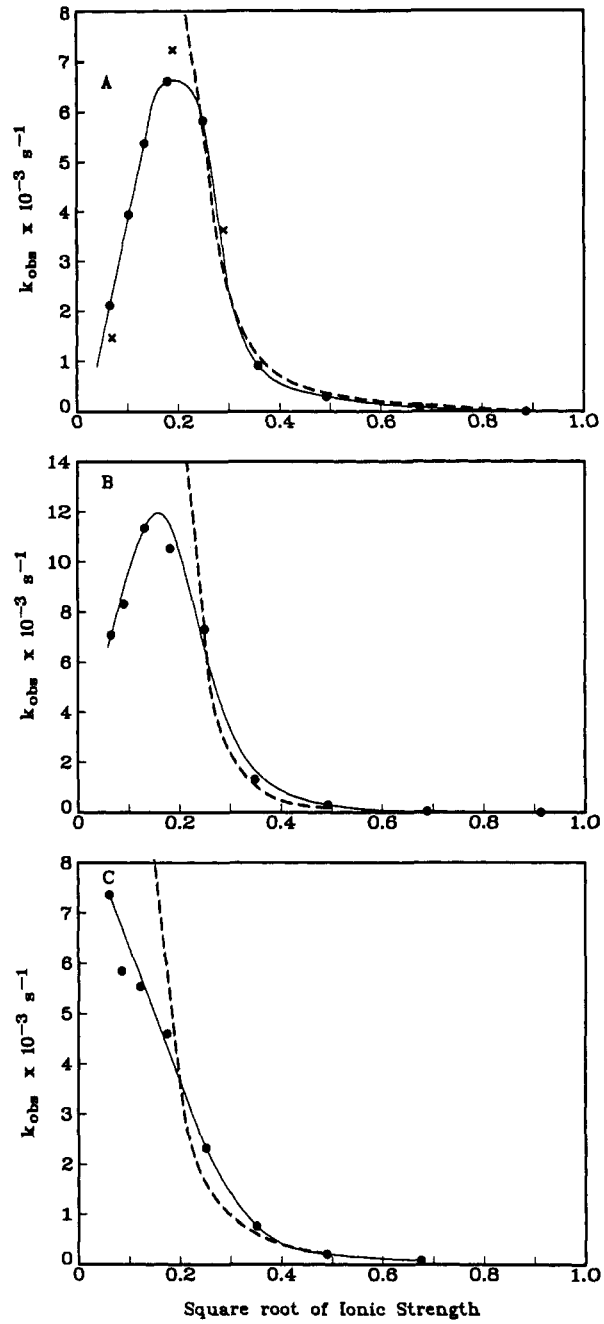

FIGURE 5: Effect of ionic strength on reaction of cytochrome b_5 with the b_5–c complex. (A) A solution of 45 μM rat outer membrane cytochrome b_5 and 5.5 μM cytochrome c in 1 mM phosphate/0.5 mM EDTA, pH 7, was titrated with 4 M NaCl (●). Three points from separate measurements of second-order rate constants have been included (×). The data points were empirically connected with a solid line. (B) The experiment in (A) repeated with 40 μM bovine liver cytochrome b_5 and 9.3 μM cytochrome c. (C) Experiment (A) repeated with 35 μM rat outer membrane cytochrome b_5 and 12 μM *Rb. capsulatus* cytochrome c_2 substituted for cytochrome c. The electrostatic interactions of the free proteins at or above 40 mM ionic strength were fit using the method of Watkins (1986), and the charge product was calculated from the equation $Z_1Z_2 = V_{ii}\alpha^{-1}R_{12}^{-1}\rho^2D_e$, where V_{ii} is the energy of interaction and is obtained from the curve-fitting process, α = 128.47, R_{12} (the distance of closest approach) was assumed to be 3.5 Å, ρ (the radius of the interaction site) was assumed to be 7.25 Å, and D_e (the dielectric constant at the interface) was assumed to be 10 [see Tollin et al. (1984) for further details]. Theoretical curves for the high ionic strength data are shown by means of a dashed line.

cytochrome c more accessible to free cytochrome b_5 as illustrated in Figure 1. An increase in the concentration of free cytochrome c due to partial dissociation of the complex, which might occur in this ionic strength range, may also

account for a small increase in the rate constant. However, our results indicate that there is no appreciable dissociation of complex until above 40 mM ionic strength. For example, the magnitude of the absorbance change due to interprotein electron transfer begins to increase at ionic strengths higher than 40 mM, as a consequence of complex dissociation and thus less rapid intracomplex electron transfer. The decrease in rate constant at high ionic strength is to be expected for a plus–minus electrostatic interaction of the free proteins. The high ionic strength portions of the curves in Figure 5 were fit using the model of electrostatic interactions developed by Watkins [1986; cf. also Tollin et al. (1984)], and a charge product of −17 was obtained. Assuming equal interacting charges on the two reactants, then there are approximately 4 ($17^{1/2}$) side chains participating at the interface of each protein, with a charge of +4 on cytochrome *c* and −4 on cytochrome b_5. This matches the theoretical model of the complex quite well (Salemme, 1976). It must be noted, however, that the value of the charge product obtained from the electrostatic model depends on the parameters used. Thus, the agreement could be fortuitous, although these parameters have been used successfully for other protein–protein systems (Tollin et al., 1984). The second-order rate constants independently measured for the free cytochrome b_5–bound cytochrome *c* reaction (Figure 4) at $I = 5$ mM (2.6×10^8 M^{-1} s^{-1}), 35 mM (1.3×10^9 M^{-1} s^{-1}), and 87 mM (6.7×10^8 M^{-1} s^{-1}) parallel the k_{obs} values, and pseudo-first-order rate constants corresponding to these measurements are also plotted in Figure 5A. Again, no saturation effects were observed in these experiments at the highest cytochrome *c* concentration used. Thus, electron transfer within the transient ternary protein complex at $I = 35$ mM must be greater than 17 000 s^{-1}, which is the largest pseudo-first-order rate constant we measured (Figure 4B). On the other hand, electron transfer in the stable binary protein complex is about 40 000 s^{-1} at the same ionic strength (Willie et al., 1992). Thus, the rate constants for electron transfer in the stable binary and the transient ternary protein complexes appear to be converging just before complete dissociation of the electrostatically stabilized binary complex.

Because there were conflicting reports in the literature concerning the rate constant for intracomplex electron transfer in the bovine liver and rat liver microsomal cytochrome b_5–cytochrome *c* complexes (McLendon & Miller, 1985; Qin et al., 1991; Willie et al., 1992), and because we were using rat mitochondrial outer membrane cytochrome b_5 for most of our studies, we repeated our experiments with the tryptic fragment of bovine liver cytochrome b_5. We obtained results entirely analogous to those with the rat outer membrane cytochrome b_5. Reduction of the two proteins by 5-deazariboflavin semiquinone had virtually identical kinetics (Figure 2B,C). Electron transfer from bovine liver cytochrome b_5 to horse cytochrome *c* was also second-order, but was about 3 times faster than that for rat outer membrane cytochrome b_5; i.e., $k_2 = 8 \times 10^8$ M^{-1} s^{-1} at $I = 5$ mM (Figure 4D) vs 2.6×10^8 M^{-1} s^{-1} (Figure 4A). When a 4:1 excess of bovine liver cytochrome b_5 over horse cytochrome *c* was titrated with NaCl, the b_5 to *c* rate constant increased approximately 2-fold between 5 and 17 mM ionic strength and then dropped precipitously at higher concentrations of salt (Figure 5B). The calculated charge product was −20.5, corresponding to about 4.5 interacting charges on each protein. On the basis of this result, it appears that the bovine liver cytochrome b_5–cytochrome *c* complex has somewhat different characteristics than the rat outer membrane cytochrome b_5–cytochrome *c* complex (maximum k_{obs} at $I = 25$ mM for the former vs 40 mM for the latter). The rate constant for electron transfer within the transient ternary protein complex must be greater than 11 000 s^{-1}, which is the largest pseudo-first-order rate constant observed. Our results are thus consistent with the report of Willie et al. (1992) that electron transfer within the stable binary protein complex is very fast (10^5 s^{-1}).

We also tested the effect of substituting cytochrome *c* with the homolog *Rhodobacter capsulatus* cytochrome c_2 to see whether it would behave in a consistent manner. Cytochrome c_2 is approximately 40% similar in amino acid sequence to the mitochondrial cytochromes *c* and has a net negative charge (Ambler et al., 1979). Nevertheless, there is positive charge at the site of electron transfer with the negatively charged FMN and flavodoxin semiquinones (Meyer et al., 1984; Tollin et al., 1984), which would be expected to interact with cytochrome b_5 to form a complex, albeit weaker than that between cytochrome b_5 and horse cytochrome *c*. Cytochrome c_2 is less reactive with 5-deazariboflavin semiquinone ($k = 3.6 \times 10^8$ M^{-1} s^{-1}; Figure 2D) than is horse cytochrome *c* ($k = 9 \times 10^8$ M^{-1} s^{-1}), and is more comparable to cytochrome b_5 ($k = 2.4 \times 10^8$ M^{-1} s^{-1}). Thus, more electrons should initially enter cytochrome b_5 in a complex with cytochrome c_2. Nevertheless, in a 1:1 mixture of cytochrome c_2 and rat outer membrane cytochrome b_5 at $I = 5$ mM, we could not observe any cytochrome b_5 reduction or intracomplex electron transfer, results which are similar to those we obtained with horse cytochrome *c*. When there was a 3:1 excess of cytochrome b_5 over cytochrome c_2, initial reduction of cytochrome b_5 was followed by a slower second-order reduction of cytochrome c_2, with $k_2 = 5.7 \times 10^8$ M^{-1} s^{-1} (Figure 4E). This is almost twice the value for the rat outer membrane cytochrome b_5–horse cytochrome *c* reaction at the same ionic strength, which suggests a somewhat different structure for the complex that provides greater accessibility to the cytochrome c_2 heme. The effect of ionic strength on this reaction is shown in Figure 5C. Instead of an increase in rate constant between $I = 5$ and 40 mM for the cytochrome b_5–cytochrome c_2 transient ternary protein complex, there was an apparently simple monophasic decrease, as would be expected for a plus–minus electrostatic interaction between the free proteins. The charge product obtained from the Watkins analysis was −11.2, corresponding to about 3.3 interacting charges on each protein. That the electrostatic interaction is not as simple as appeared from the monophasic ionic strength behavior became apparent during the fitting procedure; i.e., the first three points were much too low to be adequately fit, undoubtedly due to the presence of a stable binary complex within this ionic strength range. We interpret our results as being due to formation of a weaker complex between cytochrome b_5 and cytochrome c_2, with different orientations and less steric hindrance, perhaps comparable to the cytochrome b_5–*c* complex at 40 mM ionic strength. Apparently, for this system, the most stable complex at the lowest ionic strength provides the greatest access to cytochrome c_2. This may be due to the more restricted surface area over which the positive charge is distributed in cytochrome c_2 relative to horse cytochrome *c*, which would not allow as much freedom for rearrangement in the binary complex before dissociation.

The picture which emerges from these studies is that the most stable complexes at low ionic strength are not necessarily optimized for a series of coupled electron transfers in a pathway involving more than two reactants. Furthermore, a single static structure with specific salt bridges between proteins may not be the best general model for electron-transfer complexes, which probably exist in a variety of conformations, the population of which changes with ionic strength. This

had been noted during refinement of the cytochrome b_5–cytochrome c model (Mauk et al., 1986), by NMR measurements of the complex (Burch et al., 1990), and by measurements of proton linkage to complex formation (Mauk et al., 1991). It was also apparent from the crystal structure of the yeast cytochrome c peroxidase (CCP)–cytochrome c complex (Poulos et al., 1987) and from modeling of the CCP–cytochrome c electrostatic complex (Northrup et al., 1988). Experimental verification was provided by measurements of rate constants for reduction of CCP and of cytochrome oxidase by cytochrome c, which also showed initial increases with ionic strength (Hazzard et al., 1988, 1991). Intracomplex protein–protein electron transfer has been reviewed (Cusanovich, 1991b; Kostic, 1991; Tollin & Hazzard, 1991), and additional examples of rearrangements necessary for optimization of the process have been presented. Our results provide further support for the idea that some flexibility or rearrangement of cytochrome c is required within the electrostatically stabilized binary protein complex as the ionic strength is increased in order to maximize interaction with free cytochrome b_5. For the stable binary protein complex, the rate constant was observed to decrease monophasically with ionic strength between I = 2 mM and 1 M (Willie et al., 1992), probably due to an increase in the number of orientations sampled that are less efficient in electron transfer, at least between I = 2 and 40 mM, which is the apparent range of stability of the complex. Otherwise, one would expect no change in rate constant with ionic strength until dissociation of the complex occurred. The same rearrangement, which decreases the rate constant within the stable binary protein complex, makes cytochrome c in the transient ternary protein complex more accessible to an additional cytochrome b_5 molecule, and the two rate constants approach one another at 40 mM ionic strength. This may provide a more realistic model of the way cytochrome c functions in the mitochondrial electron-transfer chain. If the ionic strength is too low, cytochrome c may be frozen into a complex or family of complexes with one of its redox partners and thus unable to efficiently interact with a third redox partner, unless conditions are optimized for the overall reaction.

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