Transient Kinetics of Electron Transfer from a Variety of *c*-Type Cytochromes to Plastocyanin†

T. E. Meyer, Z. G. Zhao, M. A. Cusanovich, and G. Tollin*

Department of Biochemistry, University of Arizona, Tucson, Arizona 85721

Received November 25, 1992; Revised Manuscript Received February 18, 1993

ABSTRACT: Plastocyanin (PC) and its physiological reaction partner cytochrome (cyt) *f* form a complex which is electrostatically stabilized by interactions between complementary localized charges. We have measured the kinetics of intracomplex electron transfer between several reduced cytochromes and PC using laser flash photolysis. With spinach cyt *f* and spinach PC, we obtain first-order rate constants, $k_{forward}$ = 2780 s^{-1} and $k_{reverse}$ = 1050 s^{-1}, for the reversible reaction and a complex dissociation constant of about 23 μM at an ionic strength (*I*) of 5 mM. The observed rate constant increases by a factor of 2 between *I* = 5 and 40 mM and then decreases monotonically at higher ionic strengths. This indicates that the complex is not completely dissociated until *I* = 150 mM and that the proteins within the electrostatically most stable complex are not optimally oriented for electron transfer. Similar results were obtained with turnip cyt *f* and spinach PC, although in this case intracomplex electron transfer is about 4 times as fast. Horse cyt *c* also forms an electrostatically stabilized complex with PC, and yields a limiting rate constant for intracomplex electron transfer (1750 s^{-1}) and a dissociation constant (10 μM) comparable to those for spinach cyt *f*. The ionic strength dependence shows that the complex is more readily dissociated (complete at *I* = 25 mM) than is that of cyt *f* and that rearrangement is not required for optimal electron transfer. Addition of polylysine results in 10-fold inhibition of the rate of electron transfer. *Pseudomonas* cyt *c*-551 is an acidic cytochrome which does not form a complex with PC. The second-order rate constant for reaction with PC at *I* = 5 mM is about 2 orders of magnitude smaller than for cyt *f* or cyt *c*, and the effect of increasing ionic strength on the cyt *c*-551 reaction is to monotonically increase the rate constant. Addition of polylysine also results in an increase in the rate constant, which is comparable in magnitude to that caused by ionic strength. *Chlorobium* cyt *c*-555 is a basic protein like horse cyt *c*, but the kinetic results indicate that it does not form a complex with PC, although the second-order rate constant is similar to those obtained with the other basic cytochromes. The effect of ionic strength is to monotonically decrease the rate constant in a manner consistent with simple electrostatic theory. For all of the cytochromes, the rate constants extrapolated to infinite ionic strength are comparable in magnitude. However, protein–protein electron-transfer rate constants should correlate with the thermodynamic driving force if the distances and orientations are held constant. The fact that they do not suggests that these factors vary for the different cytochromes in their reactions with PC.

Cytochrome (cyt) *f* and plastocyanin (PC) are physiological reaction partners in plant, algal, and cyanobacterial photosynthetic electron transport. They form an electrostatically stabilized complex due to interaction of localized negative charges on PC and localized positive charges on cyt *f* (He et al., 1991; Modi et al., 1992). Horse mitochondrial cyt *c* also forms a complex with PC (Bagby et al., 1990b). This latter cytochrome is approximately spherical in shape, with one edge of the heme exposed to solvent and surrounded by a region of positive electrostatic charge (Weber & Tollin, 1985). PC is roughly cylindrical, with the copper at one end and most of the negative charge localized on one side. The latter is often referred to as the acidic patch or the eastern site, and is centered over Tyr-83, which is presumably where the basic cytochromes bind (Roberts et al., 1991). The region at which the copper is nearest to the protein surface is known as the hydrophobic or northern patch, and is centered over the copper ligand His-87. The well-known exponential distance dependence for outer-sphere electron transfer (Marcus & Sutin, 1985) would suggest that the hydrophobic region is the preferred site for electron transfer. However, the horse cyt *c*–PC complex was modeled by Roberts et al. (1991), who found that the maximum overlap of electrostatic fields was achieved if the heme-binding Cys-17 of the cytochrome was juxtaposed with Tyr-83 of PC. Experimental confirmation of complexation at the acidic site of PC was obtained by Morand et al. (1989), who found that PC Asp-44 and Glu-59 and/or Glu-61 could be covalently cross-linked to cyt *f* in the complex.

Previous studies of the cyt *f*–PC system have utilized a variety of species of cyt *f* and PC, and have rarely been done with both proteins derived from the same species. Furthermore, experimental methods and conditions of measurement have not been uniform. This is especially true of investigations of the kinetics of electron transfer between cytochromes and PC. These have been performed using a range of techniques, both transient and steady-state, and have yielded rate constants which vary considerably from one another, and are difficult to compare. As a consequence, there is no consensus on how fast intracomplex electron transfer occurs in this system, nor on whether there are one or more sites of electron transfer on PC.

Hope et al. (1992) have indirectly measured electron transfer between cyt *f* and PC in vivo and obtained an estimated rate constant of 2000 s^{-1}. In contrast, He et al. (1991) and Modi et al. (1992) reported estimates of (5.8–6.2) × 10^4 s^{-1}, based on stopped-flow studies at 0.1 M ionic strength, conditions in

† This work was supported in part by grants from the NIH to G.T. (DK 15057) and to M.A.C. (GM21277).

0006-2960/93/0432-4552$04.00/0

which the complex would be largely dissociated at the protein concentrations used for the experiments. In fact, the dissociation constant was reported to be about 100 μM at this ionic strength. Qin and Kostic (1992) calculated a rate constant of 2800 s^{-1} for electron transfer from turnip cyt *f* to french bean PC at low ionic strengths (I = 4 mM) using laser flash photolysis methodology. Although this was not a direct determination, at least the measurement was carried out under conditions in which the complex is stable.

The kinetics of electron transfer from horse cyt *c* to PC have been directly measured by pulse radiolysis by Peerey and Kostic (1989), and using laser flash photolysis by Peerey et al., (1991). A value of 1300 s^{-1} for the intracomplex rate constant at low ionic strength was obtained in these studies. Complex dissociation constants were determined to be in the range of 0.2–20 μM between I = 1 and 40 mM.

Several studies of chemically modified proteins have also been carried out. Tyr-83 has been modified by Gross and Curtis (1991) and by Christensen et al. (1992), who found that the resulting lowered pK of nitrotyrosine-83 affected the reaction of PC with horse cyt *c* at high ionic strength, consistent with electron transfer occurring at the acidic site on PC. Pan et al. (1990) examined the interaction between PC and horse cyt *c*, the latter modified at specific lysine residues with a photoactivatable ruthenium complex. They found intracomplex rate constants which varied from 500 to 1900 s^{-1} at I = 5 mM, which actually became larger for some but not all derivatives at higher ionic strengths. They concluded that the electrostatically stabilized complex was not optimized for electron transfer but required some rearrangement, which is consistent with the conclusions of Peerey and Kostic (1989) and Peerey et al. (1991) based on studies of covalently cross-linked complexes. Using horse cyt *c* with zinc substituted for iron, Zhou and Kostic (1992a) concluded that the inhibitory effect of solvent viscosity on electron transfer to PC also implies that intracomplex rearrangement is required for the most efficient electron transfer. Zhou and Kostic (1992b), using cross-linked zinc horse cyt *c* and PC in electron-transfer reactions with free PC, found deviations from simple electrostatic behavior in their ionic strength studies which were similar to those observed by Pan et al. (1990). However, they concluded that these resulted from the importance of dipolar as well as ionic interactions in complex formation, rather than being a consequence of changes in protein–protein orientation within a complex.

Takabe and Ishikawa (1989) cross-linked cyt *f* to PC and found that the covalent complex would not react with photosystem I reaction centers. Takabe et al. (1984) modified carboxyl groups on PC which inhibited the reaction with cyt *f*, but not with photosystem I. These results suggest different reaction sites on PC for cyt *f* and for reaction centers. Potentially interesting site-specific mutants of PC have been constructed by Nordling et al. (1991), He et al. (1991), and Modi et al. (1992ab), but these have not yet been fully characterized at low ionic strength where they would be expected to form stable complexes with cyt *f* or with horse cyt *c*.

In an attempt to clarify the variety of results reported, and to obtain additional measurements of intracomplex electron-transfer rates, we have studied the cytochrome–spinach PC system by laser flash photolysis using two species of cyt *f*, horse cyt *c*, *Pseudomonas* cyt *c*-551, and *Chlorobium* cyt *c*-555. Using this methodology, we have been able to determine a value for the intracomplex electron-transfer rate constant at low ionic strength for the spinach cyt *f*–spinach PC system, as well as a lower limit for this rate constant for the turnip cyt *f*–spinach PC pair. In both systems, we have measured protein–protein electron-transfer rate constants as a function of ionic strength, which we interpret in terms of the role of electrostatic forces in optimizing protein–protein orientations. We conclude that reorientation is less important for the horse cyt *c*–PC system, i.e., that the electrostatically most stable complex is optimal for electron transfer, and that cyt *c*-551 and cyt *c*-555 do not form stable complexes with PC.

MATERIALS AND METHODS

The laser flash photolysis apparatus and methods of data analysis were as previously described (Tollin et al., 1986; Tollin & Hazzard, 1991; Watkins, 1986). The sample buffer contained 0.5 mM EDTA, 1 mM phosphate, pH 7, and 50–100 μM flavin. Excitation was at 400 nm for 5-deazariboflavin and at 445 nm for lumiflavin and FMN. Flavin semiquinone was generated in less than 1 μs after a laser flash, and its concentration was less than 1 μM. The error in rate constant determinations is estimated to be ≤10–20%. Protein stock solutions were typically prepared in 5 mM phosphate, pH 7. Spinach and turnip cytochromes *f* were purchased from Sigma in a lyophilized state, suspended in 5 mM phosphate, pH 7, plus 1% octyl glucoside detergent, and centrifuged to remove insoluble material. The stock solution was titrated with potassium ferricyanide to stoichiometrically oxidize the heme without adding an excess. Attempts to oxidize the cyt *f* with excess ferricyanide, followed by pressure dialysis, led to precipitation and denaturation of the protein. Spinach PC was prepared according to the method of Yocum (1982). *Pseudomonas aeruginosa* strain ATCC 10145 cyt *c*-551 was prepared according to the method of Ambler and Wynn (1973) and *Chlorobium thiosulfatophilum* strain Tassajara cyt *c*-555 according to Meyer et al. (1968). Polylysine (MW 1000–4000) was obtained from Sigma.

RESULTS AND DISCUSSION

It has generally been considered that spinach cyt *f* is not desirable for in vitro kinetic studies because of its low solubility, whereas cyt *f* obtained from turnip or rape is thought to have a higher level of solubility. However, we found that there was little difference between the solubility of lyophilized spinach and turnip cytochromes *f* obtained from Sigma. In attempts to prepare solutions whose concentrations were greater than about 200 μM, we found that some protein was soluble in buffer alone, more dissolved in detergent (1% octyl glucoside), and roughly half of the protein remained insoluble. Despite this problem, we have compared these two photosynthetic cytochromes with one another, and with other cytochromes which are more easily studied, in their electron-transfer reactions with spinach PC. We attempted to determine whether they reacted at the same site, whether the physiological partners showed any greater specificity than nonphysiological cytochromes, and whether one could force cytochromes to react at different sites on PC.

In order to obtain information about the intrinsic reactivity of cyt *f*, we determined second-order rate constants for the reaction between these proteins and lumiflavin semiquinone at I = 96 mM, for which we have a large data base (Meyer et al., 1983). With spinach cyt *f*, we obtained a rate constant of 1.5×10^8 M^{-1} s^{-1} (data not shown), which is approximately 1.5 times larger than that for cytochromes of comparable redox potential. This suggests a larger degree of exposure of the heme to solvent than, for example, in cyt *c*. For turnip cyt *f*, a value of 2.8×10^8 M^{-1} s^{-1} was obtained (data not

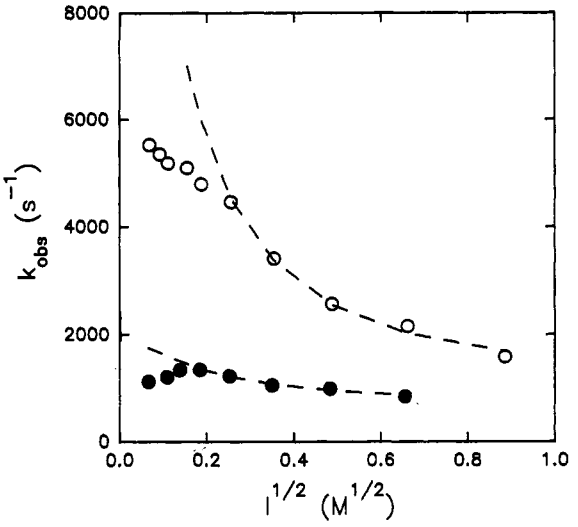

FIGURE 1: Effect of ionic strength on the reduction of cytochromes *f* by FMN semiquinone. (Open circles) 19 μM turnip cyt *f*; (closed circles) 13 μM spinach cyt *f*. The reaction mixture contained 70 μM FMN, 0.5 mM EDTA, 0.1% octyl glucoside, and 0.8 mM phosphate buffer, pH 7.0. Ionic strength was varied by adding aliquots of NaCl. The dashed lines are theoretical fits using the Watkins equation (see text), with an interaction radius of 5 Å, as used previously (Meyer et al., 1984).

shown), a result which is consistent with that reported by Qin and Kostic (1992). As we will show below, the redox potential of the turnip cytochrome appears to be somewhat larger than that of spinach cyt *f*. This could account for only a small portion of the observed increase in reactivity, and thus we suggest that a difference in heme exposure is the most significant contributor. As we will show below, the turnip cytochrome is more reactive toward PC as well.

The effect of ionic strength on the kinetics of the reaction of cyt *f* with the negatively charged FMN semiquinone provides a measure of the magnitude of electrostatic charge at the site of electron transfer on the cytochrome (Meyer et al., 1984). The results obtained with the spinach and turnip proteins are shown in Figure 1, in experiments done at constant flavin and cytochrome concentrations by adding small aliquots of a concentrated NaCl solution. With both cytochromes, anomalous results are apparent at low ionic strengths (below I = 32 mM for spinach cyt *f* and below I = 66 mM for turnip cyt *f*), whereas above these values of ionic strength, the data are well fit by the electrostatic theory of Watkins (1986), which we have found to give excellent results in other systems (Meyer et al., 1984: Tollin et al., 1984). It should be noted that the magnitude of the deviation of the k_{obs} values from the theoretical curves (dashed lines) is well outside the range of our experimental error. From the theoretical fits, we conclude that both cytochromes display a localized positive charge at the site of electron transfer, which is smaller for the spinach protein compared to that from turnip. The corresponding electrostatic interaction energies, V_{ii}, are −1.1 and −2.7 kcal/mol, and the rate constants extrapolated to infinite ionic strength are 5.7×10^7 and 6.9×10^7 M^{-1} s^{-1}, respectively. The V_{ii} value for the spinach cytochrome is approximately one-third that obtained for horse cyt *c*, and indicates a smaller active-site charge (approximately +2), whereas the turnip protein appears to have a charge which is comparable to that of the horse cytochrome. The k_∞ values for both cytochromes *f* are about 25% of the rate constants obtained for lumiflavin semiquinone, which is consistent with results for other cytochromes, and attributed to steric effects of the FMN side chain (Meyer et al., 1984). We should note that our results

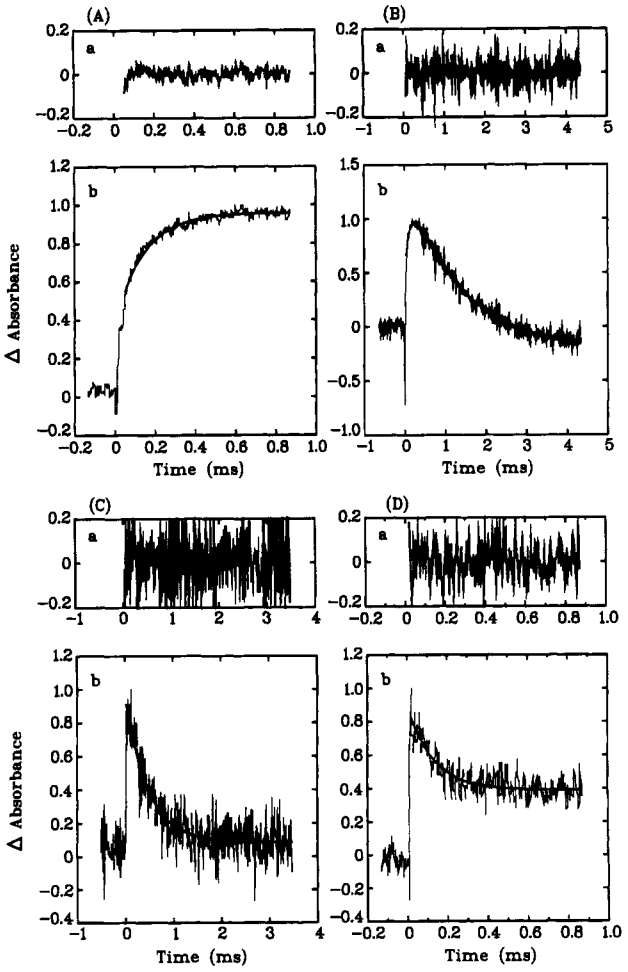

FIGURE 2: Kinetic transients obtained upon laser flash photolysis of protein solutions at I = 5 mM. (A) Reduction of 17.2 μM horse cytochrome *c* by laser flash photolysis of 100 μM 5-deazariboflavin solution in 0.5 mM EDTA and 0.8 mM phosphate buffer, pH 7, monitored at 550 nm; (B) reduction and reoxidation of horse cytochrome *c* by equimolar spinach plastocyanin; other conditions as in (A); (C) reduction and reoxidation of 14.7 μM spinach cytochrome *f* by equimolar spinach plastocyanin, monitored at 554 nm; other conditions as in (A) except for addition of 0.1% octyl glucoside; (D) reduction and reoxidation of 15.3 μM turnip cytochrome *f* by equimolar spinach plastocyanin, monitored at 554 nm; other conditions as in (C). Solid lines in lower panels (b) are exponential fits to the data, using single exponentials in (A), (C), and (D) and double exponentials in (B). Traces in upper panels (a) correspond to the residuals for the fits. Absorbance scales in arbitrary units.

with the turnip cytochrome in the higher ionic strength range are similar to those reported by Qin and Kostic (1992).

The anomalous behavior of these cytochromes at low ionic strength in their reaction with FMN has not been observed with any other cytochromes in our previous studies (Meyer et al., 1984). Such results can be rationalized in two ways: (i) FMN semiquinone forms a complex with the cytochromes, thus changing the rate-limiting step in the reaction mechanism; (ii) cyt *f* forms aggregates at low ionic strength which partially block access to the heme. We will consider these possibilities further below.

In order to increase the rate at which electrons are transferred to the cytochrome so as to be able to follow rapid reoxidation by plastocyanin, we utilized 5-deazariboflavin semiquinone (dRfH) as a reductant (Meyer et al., 1983). All of the cytochromes studied here were rapidly reduced by dRfH generated in situ by laser flash photolysis, as shown for horse cyt *c* in Figure 2A. The second-order rate constants determined at I = 5 mM were all of comparable magnitude [(4–5)

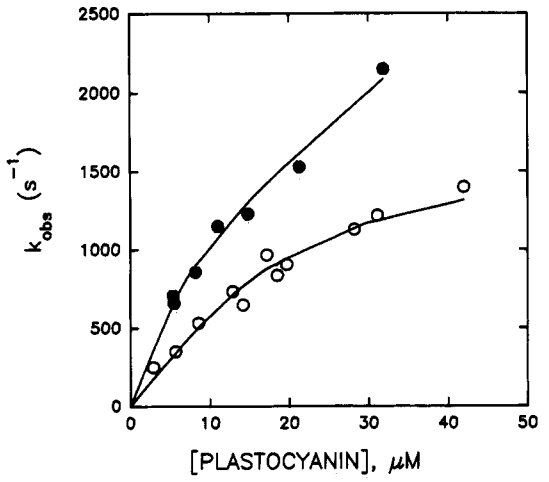

FIGURE 3: Second-order plots for the reoxidation of 14.7 μM spinach cytochrome *f* (closed circles) and 17.2 μM horse cytochrome *c* (open circles) by varying amounts of spinach plastocyanin. The buffer conditions were the same as in Figure 2. The solid lines are theoretical fits assuming a rapid binding equilibrium followed by electron transfer as described in the text.

× 10^8 M^{-1} s^{-1}; data not shown], suggesting that diffusion rates were dominant. PC is also rapidly reduced by dRfH with a rate constant of 2.4 × 10^8 M^{-1} s^{-1} (data not shown). In a mixture of the two proteins, cyt *f* and PC, both should react with substoichiometric amounts of free flavin semiquinone, although correspondingly more electrons should go to cyt *f* because of the 2-fold larger rate constant. Inasmuch as PC has a redox potential (370–381 mV) slightly higher than that of cyt *f* (355–370 mV) (Morand et al., 1987; Bagby et al., 1990a), there should also be a reversible secondary electron transfer from cyt *f* to PC. For the other cytochromes studied here, the potential differences are much larger, resulting in essentially irreversible transfer from cytochrome to PC. In order to ensure that most of the electrons initially go to reduce the cytochrome component, we started with an excess of cytochrome and gradually increased the concentration of PC over the course of the experiment. As shown in Figure 2B,C,D, the initial reduction of cytochrome by dRfH (monitored at the peak of the α band) was followed in all cases (although to varying extents) by reoxidation of cytochrome by PC. That PC was indeed being reduced was confirmed by monitoring the reaction at a cytochrome isosbestic point or at 600 nm, where PC has an absorption maximum and cytochrome shows little or no absorbance change (data not shown).

A second-order plot for reoxidation of spinach cyt *f* by PC at I = 5 mM is shown in Figure 3. Due to the difficulty of obtaining data points at low PC concentrations, caused by the similarity of the redox potentials of the two proteins, which results in a small extent of cytochrome reoxidation, we have included in this plot data obtained with greater than stoichiometric PC to cyt *f* ratios, assuming that the only effect of the extra PC was to increase the amount of complex. In support of this assumption, all of the points appear to fall on the same curve. The concentration dependence obtained is clearly nonlinear, suggesting that there is a change in the rate-limiting step at the higher PC concentrations, probably due to formation of an electrostatically stabilized complex at low ionic strengths as previously reported. The limiting first-order rate constant should reflect the sum of the rate constants for forward and reverse electron transfer between cyt *f* and PC, as a consequence of their comparable redox potentials (Morand et al., 1989; Bagby et al., 1990a); i.e., the equilibrium constant for the overall reaction is near 1. At a 1:1 ratio of cyt *f* to PC, it can be seen in Figure 2C that cyt *f* is nearly completely oxidized by PC (compare with Figure 2B), indicating that the equilibrium favors PC reduction under our experimental conditions. The largest difference in redox potentials reported in the literature for this pair is about 25 mV, and the equilibrium constant corresponding to this difference is 2.65. Assuming a minimal two-step mechanism involving a rapid binding equilibrium followed by reversible electron transfer, and using the above value for the equilibrium constant, we calculate the following values of the kinetic constants by nonlinear least-squares fitting of the data (Simondsen et al., 1982; Simondsen & Tollin, 1983): k_{12} = 6.8 × 10^7 M^{-1} s^{-1}; k_{21} = 1540 s^{-1}; k_{23} ($k_{forward}$) = 2780 s^{-1}; and k_{32} ($k_{reverse}$) = 1050 s^{-1}. The calculated dissociation constant, K_d ($=k_{21}/k_{12}$), for the complex is thus approximately 23 μM. The second-order rate constant is similar to that reported for the *Brassica* cyt *f*–spinach PC reaction (2 × 10^8 M^{-1} s^{-1} at I = 50 mM and 6 × 10^7 M^{-1} s^{-1} at I = 100 mM) by Takabe et al. (1984), and for the spinach cyt *f*–spinach PC reaction, 5 × 10^7 M^{-1} s^{-1} at I = 110 mM (Ho & Krogmann, 1980), but measured by stopped-flow spectrophotometry. A similar value was also obtained by laser flash photolysis for the turnip cyt *f*–french bean PC reaction (2 × 10^8 M^{-1} s^{-1} at I = 100 mM; Qin & Kostic, 1992). These numbers are in reasonable agreement with one another considering that they were obtained for different species and measured under different conditions. The reactions are clearly very fast and approach diffusion-controlled rates.

Qin and Kostic (1992) reported a rate constant of 2800 s^{-1} for turnip cyt *f* and french bean PC at I = 4 mM, for an experiment in which there was very little reoxidation of the cytochrome and using an equation derived on the basis of a steady-state approximation, which is not applicable in single-turnover experiments. Hope et al. (1992) reported a rate constant of 2000 s^{-1} from in situ measurements. Values of (5.8–6.2) × 10^4 s^{-1} for the reaction of rape cyt *f* with spinach or pea PC at I = 100 mM have also been reported on the basis of extrapolations from stopped-flow measurements, also incorrectly utilizing a steady-state approximation (He et al., 1991; Modi et al., 1992). It is important to note that the present experiment constitutes the first measurement of the intracomplex rate constant in vitro for a *physiological* reaction pair at low ionic strength where the complex is stable. The values previously reported at high ionic strength are too high by an order of magnitude and are based on faulty analysis, whereas the in vivo result is similar to ours.

We examined the effect of ionic strength on the reaction of spinach cyt *f* with PC as shown in Figure 4. These experiments were done at a constant cyt *f* to PC ratio (1:1) by adding small aliquots of a concentrated NaCl solution. The observed rate constant for cyt *f* oxidation by PC doubles between I = 5 and 40 mM, and then decreases in a manner consistent with a simple plus–minus electrostatic interaction at higher ionic strength. The data points at I = 150 mM and above were fit using the electrostatic analysis of Watkins (1986). The electrostatic interaction energy, V_{ii}, obtained in this way is –15.6 kcal/mol, and the second-order rate constant at infinite ionic strength, k_∞, is 2.3 × 10^6 M^{-1} s^{-1}. Our value for V_{ii} is only slightly smaller than that obtained by Qin and Kostic (1992) for turnip cyt *f* and french bean PC (–20 kcal/mol), using the same method of analysis. This difference is probably not significant, considering the limited number of data points and the uncertainty with regard to the influence of residual complex at the lower ionic strengths.

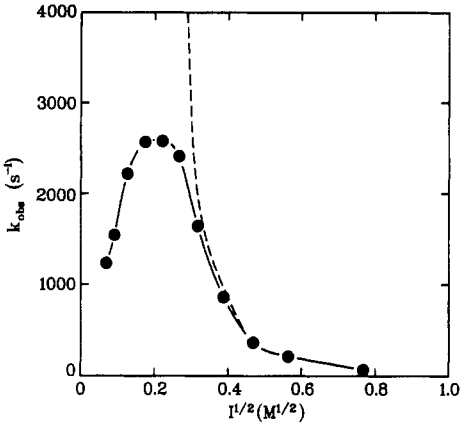

FIGURE 4: Effect of ionic strength on the reoxidation of 14.7 μM spinach cytochrome *f* by equimolar spinach plastocyanin. The buffer conditions were the same as in Figure 2. The ionic strength was varied using NaCl. The solid line is a smooth curve connecting data points, whereas the dashed line is a thoretical curve based on fitting data points at I = 150 mM and above using the Watkins equation as described in the text (the interaction radius was assumed to be 7.25 Å).

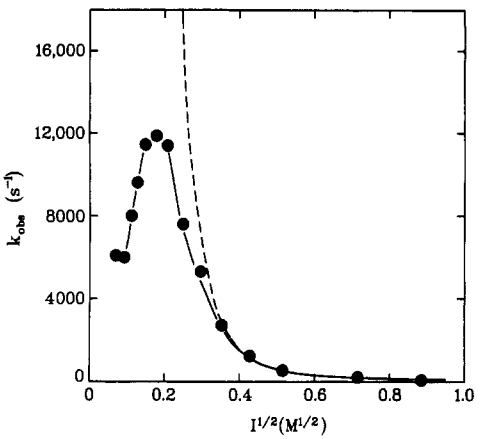

FIGURE 5: Effect of ionic strength on the reoxidation of 15.3 μM turnip cytochrome *f* by equimolar spinach plastocyanin. The buffer conditions were the same as in Figure 2. Solid and dashed lines as in Figure 4.

The initial increase in rate constant with ionic strength is analogous to the results obtained with FMN noted above (Figure 1), and thus is probably caused by the same phenomena. Again, the deviation from the simple electrostatic model (dashed line) is well outside of the experimental error. In the case of PC, we know that a complex is formed, as evidenced by the nonlinear second-order plot shown in Figure 3. Thus, it seems logical to assume that complexation is involved, although we cannot rule out a contribution from cyt *f* aggregation. However, as we will show below, anomalous ionic strength effects are also observed for the horse cyt *c*–PC reaction, where cytochrome aggregation is not a factor. Furthermore, again as shown below, cytochromes which do not form complexes with PC do not exhibit this type of ionic strength dependence. One interpretation is that the complex at the lowest ionic strength is not optimized for electron transfer and the two proteins must rearrange in order to achieve a maximal rate constant, which occurs at intermediate ionic strengths. Such rearrangement is inhibited by the stronger electrostatic forces existing at I = 5 mM. Above I = 40 mM, the complex begins to dissociate, and the ionic strength behavior approaches that expected for a simple second-order plus–minus electrostatic interaction at about I = 150 mM. As noted in the introduction, this explanation has also been invoked by other workers (Peerey & Kostic, 1989; Peerey et al., 1991), although Zhou and Kostic (1992b) have used a dipole model of protein–protein interactions to account for similar behavior. An alternative explanation is that the reaction of free reduced cytochrome with the cytochrome–PC complex makes a major contribution to the overall reaction, and is much slower than either the second-order reaction of free proteins or the limiting first-order reaction within the complex. However, the contribution of this ternary reaction should decrease with increasing ionic strength, and the contribution of the first-order intracomplex reaction should also decline to the same extent. Furthermore, the rate constant for the second-order reaction between the free uncomplexed proteins should drop dramatically with ionic strength, as shown by the results at high salt concentrations. Thus, we are forced to conclude that the rate constant for the first-order intracomplex reaction increases with ionic strength and that protein–protein rearrangement is the best explanation for the bell-shaped ionic strength dependence.

To determine whether the results we obtained with spinach cyt *f* are unique or whether they are generally applicable to other cyt *f* species, we examined turnip cyt *f* as well. The magnitude of the reoxidation of turnip cyt *f* by PC is much smaller than that which occurs between spinach cyt *f* and PC (compare Figure 2C and Figure 2D); thus, it is much more difficult with the turnip protein to obtain data over a wide concentration range. This is probably due to a higher redox potential for turnip cyt *f*, which is closer to that of PC. Our best estimate for the second-order rate constant is based on only two data points and is 6×10^8 M^{-1} s^{-1}. This is somewhat larger than the 1.7×10^8 M^{-1} s^{-1} obtained for the spinach cyt *f*–PC reaction and is consistent with the results obtained with lumiflavin and FMN semiquinones. We were unable to obtain a value for the limiting first-order rate constant (k_{23}) for the same reason.

The effect of ionic strength on the turnip cyt *f*–PC reaction was similar to that for spinach cyt *f* with PC (Figure 5), in that the observed rate constant increased by approximately 2-fold with ionic strength up to I = 32 mM before decreasing at higher ionic strength. The electrostatic interaction energy, V_{ii}, obtained using the data points at I = 125 mM and above, is –16.2 kcal/mol and $k_\infty = 4.1 \times 10^6$ M^{-1} s^{-1}. These values are also similar to those for spinach cyt *f*. It is important to note that the comparable V_{ii} values are in contrast with the results obtained with FMN, which suggested a larger active-site charge on the turnip cytochrome. We interpret this as indicating that, within the much larger interaction domain experienced by PC during the electron-transfer reaction, the two cytochromes have similar charges. Comparing Figure 4 and 5, obtained under similar conditions, it can be seen that turnip cyt *f* is about 4 times as reactive as spinach cyt *f* with PC. This is consistent with the larger heme exposure suggested by the lumiflavin and FMN experiments.

Horse cyt *c* has been reported to form a complex with PC, which has been used as a model for the cyt *f*–PC system because the horse cytochrome has a known three-dimensional structure and its kinetics of electron transfer with a variety of small molecules and other proteins have been characterized (Meyer et al., 1984; Kostic, 1991; Tollin & Hazzard, 1991). The reaction of reduced horse cyt *c* with PC is shown in Figure 2B. It can be seen that this cytochrome is completely reoxidized by PC in an essentially irreversible reaction. This is due to the much larger spread in redox potentials (260 vs 381 mV). Furthermore, one can obtain much higher quality data with this system. The second-order plot for the horse cyt

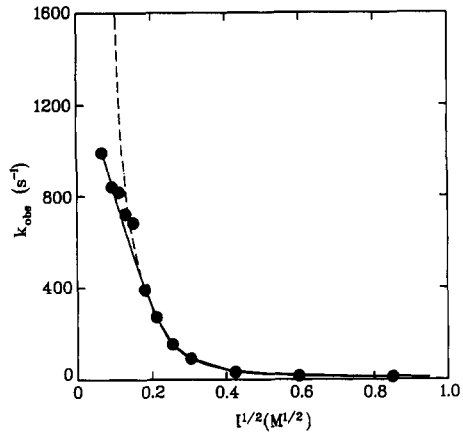

FIGURE 6: Effect of ionic strength on the reoxidation of 17.2 μM horse cytochrome c by equimolar spinach plastocyanin. The buffer conditions were the same as in Figure 2. Solid and dashed lines as in Figure 4.

c–PC reaction at I = 5 mM is clearly nonlinear (Figure 3), consistent with complex formation followed by intracomplex electron transfer [see also Peerey and Kostic (1989) and Peerey et al. (1991)]. Using a simplified version of the method of data analysis noted above, which assumes irreversible electron transfer from cytochrome to PC, the second-order rate constant $k_{12} = 8.9 \times 10^7$ M^{-1} s^{-1}, the dissociation constant K_d = 10 μM, and the limiting first-order rate constant for electron transfer k_{23} ($k_{forward}$) = 1750 s^{-1}. The intracomplex rate constant for the horse cyt c complex is smaller than for spinach cyt f at this ionic strength, in spite of its lower redox potential which should have resulted in a larger value due to the greater driving force (see below). The dissociation constant for the horse cyt c–spinach PC complex has been independently measured by an NMR method and reported as 67 μM at low ionic strength (Bagby et al., 1990). Peerey and Kostic (1989) and Peerey et al. (1991) reported a limiting rate constant of 1300 s^{-1} and K_d values of 0.2–20 μM at I = 1–40 mM for horse cyt c and french bean PC. These latter results are in reasonable accord with ours.

The effect of ionic strength on the reaction of horse cyt c with spinach PC, shown in Figure 6, is quite different from that for cyt f. Instead of increasing at low ionic strength, the rate constants decrease up to about I = 20 mM, although more slowly than predicted by the simple electrostatic model (dashed line), and then decrease in accord with the model above that value. This result is also indicative of complex formation at low ionic strength. However, there are three important differences from the cyt f system. First, the horse cyt c complex dissociates at a lower ionic strength than either of the two cyt f complexes. Second, the electrostatic interaction energy is smaller for the horse cyt c–PC reaction (V_{ii} = –8.5 kcal/mol) and k_∞ is also somewhat smaller (5×10^5 M^{-1} s^{-1}), again despite the increased thermodynamic driving force. Third, the most stable complex at I = 5 mM is the most efficient for electron transfer (i.e., has the largest rate constant), and rearrangement is not necessary to optimize the reaction. It should also be noted that the smaller electrostatic interaction energy for horse cyt c, which is about half that for the cytochromes f, indicates that the latter proteins are interacting with a much larger surface of PC.

Cyt f and horse cyt c are likely to bind to similar sites on PC, i.e., in the vicinity of the acidic region centered over Tyr-83 as modeled by Roberts et al. (1991). This site is relatively distant from the copper, approximately 12 Å as opposed to the 6-Å distance at the hydrophobic site centered over the

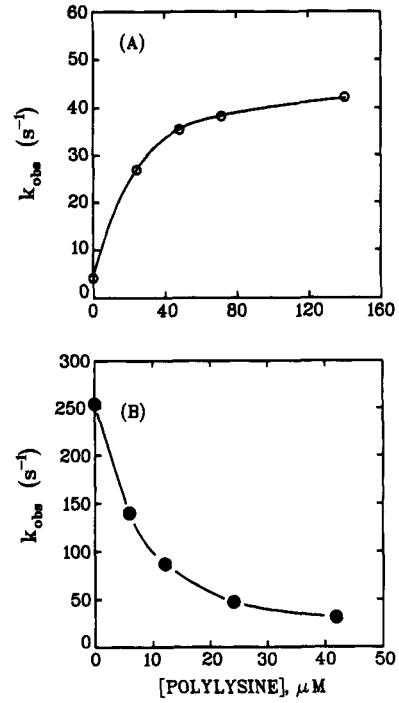

FIGURE 7: Effect of polylysine concentration on the reoxidation of (A) 24 μM cyt c-551 by equimolar PC and (B) 42.5 μM horse cytochrome c by 5.7 μM spinach plastocyanin. The buffer conditions were the same as in Figure 2. The solid lines are smooth curves connecting data points.

copper ligand His-87. One would predict an approximately 400-fold faster reaction at the His-87 site compared with the Tyr-83 site, due to the exponential distance dependence for outer-sphere electron transfer (Marcus & Sutin, 1985). Because both second-order and first-order electron-transfer rate constants for horse cyt c and cyt f do not differ by that much, our results are consistent with reaction at the Tyr-83 site of PC for both proteins. In order to see whether we could force reaction to occur at what should be the more favorable site in terms of distance, we examined the effect of polylysine on the interaction of horse cyt c with PC. We expected the polylysine to bind to the acidic region of PC and neutralize it, thus preventing complex formation with cyt c and perhaps forcing it to react at the His-87 site. The results are shown in Figure 7; an approximately 10-fold inhibition of electron transfer was obtained. From these data, the dissociation constant for polylysine was estimated to be about 3 μM. Our results are thus consistent with inhibition due to alteration of both electrostatic effects and steric effects, but do not provide evidence for a change in reaction site.

We reasoned that if proteins containing strongly basic active sites preferentially bind and react at the Tyr-83 site of PC, then proteins which have relatively weak charges or acidic active sites might react at the His-87 site. *Pseudomonas* cyt c-551 is such a protein; it has very little charge near the exposed edge of the heme and displays a small negative charge in its reactions with other charged molecules (Meyer et al., 1984; Tollin et al., 1984). Furthermore, the redox potential is about the same as for horse cyt c, i.e., 270 mV. Upon laser flash photolysis at I = 5 mM, we found that the reaction with PC is relatively slow; the second-order plot shown in Figure 8 can be accurately fit with a straight line ($k_2 = 1.7 \times 10^5$ M^{-1} s^{-1}), with no indication of complex formation at the highest PC concentrations used. This value is 3 orders of magnitude smaller than the rate constants obtained for cyt f and horse cyt c with PC at the same ionic strength. The effect of ionic strength also differs from these other cytochromes in that

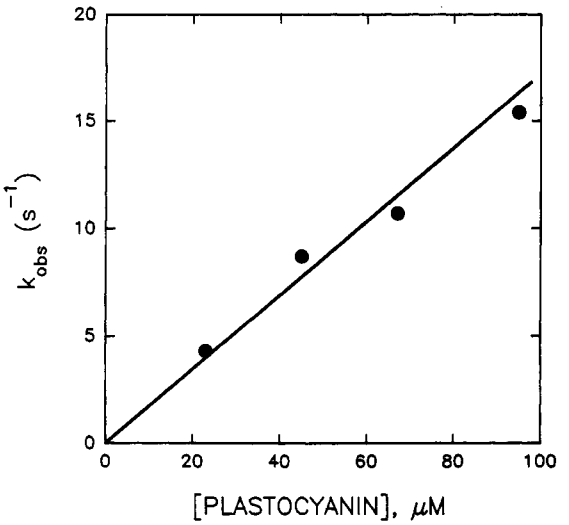

FIGURE 8: Second-order plot for reoxidation of *Pseudomonas* cytochrome *c*-551 by spinach plastocyanin. The buffer conditions were the same as in Figure 2.

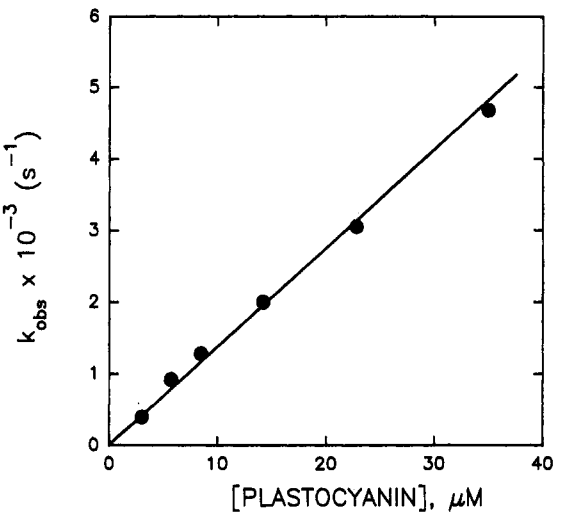

FIGURE 10: Second-order plot for reoxidation of 35.5 μM *Chlorobium* cytochrome *c*-555 by spinach plastocyanin. The buffer conditions were the same as in Figure 2.

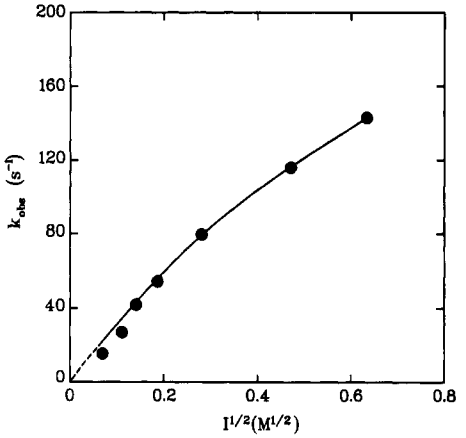

FIGURE 9: Effect of ionic strength on the reoxidation of 24 μM *Pseudomonas* cytochrome *c*-551 by 95 μM spinach plastocyanin. The buffer conditions were the same as those in Figure 2 and NaCl was used to vary ionic strength. The solid line is a theoretical fit to the data as in Figure 3.

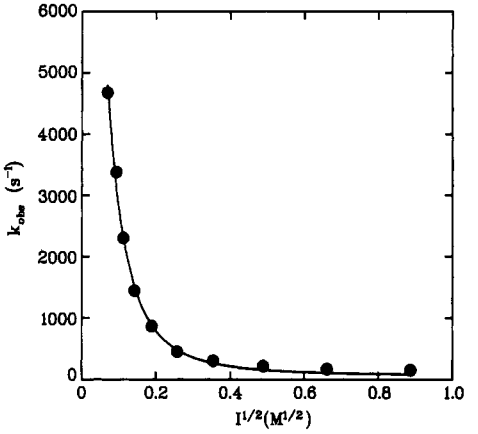

FIGURE 11: Effect of ionic strength on the reoxidation of 35.5 μM *Chlorobium* cytochrome *c*-555 by equimolar concentrations of spinach plastocyanin. The buffer conditions were the same as those in Figure 2. The solid line is a theoretical fit to the data using the Watkins equation as in Figure 4.

there is a simple minus–minus electrostatic interaction between cyt *c*-551 and PC (Figure 9), as would be expected for this negatively charged cytochrome if it were interacting with the negative site on PC. No indication of complex formation is apparent in these data as well, and all of the points can be fit by the Watkins (1986) equation, resulting in V_{ii} = +2.9 kcal/mol and k_∞ = 2.0 × 10^6 M^{-1} s^{-1}. The small electrostatic interaction energy is consistent with our previous observations, as noted above. It is also important to point out that the rate constant at infinite ionic strength is remarkably similar to those for cyt *f* and for horse cyt *c* (whereas it should have been faster due to driving force alone), which suggests that cyt *c*-551 reacts at a site distant from the copper. The effect of polylysine is similar to that of increasing ionic strength; i.e., electron transfer is enhanced by an order of magnitude as shown in Figure 7, apparently due to relief of electrostatic repulsion which is dominant over steric inhibition.

Another basic cytochrome, structurally distinct from horse cyt *c*, is *Chlorobium* cyt *c*-555. In this protein, the charge is more or less uniformly distributed over the surface, and it shows a weak positive charge in its electron-transfer reactions with other charged molecules (Meyer et al., 1984; Tollin et al., 1984). It also has a relatively low redox potential, 145 mV, thus providing an even stronger thermodynamic driving force for electron transfer. *Chlorobium* cyt *c*-555 was found to rapidly react with plastocyanin as shown in Figure 10. As with cyt *c*-551, there is no evidence for complex formation, and k_2 = 1.4 × 10^8 M^{-1} s^{-1} at I = 5 mM. The effect of ionic strength on the kinetics of electron transfer from cyt *c*-555 to PC also gives no indication of complex formation, as shown in Figure 11. It is important to point out that this is what one would have expected the horse cyt *c* and cyt *f* curves to look like if there was no complex formation at low ionic strength. All of the data points can be fit with the Watkins (1986) equation to give V_{ii} = −5.8 kcal/mol and k_∞ = 3.9 × 10^6 M^{-1} s^{-1}. The apparent lack of complex formation is consistent with the weaker electrostatic interaction as compared with horse cyt *c* and cyt *f*, which is probably a consequence of the uniform charge distribution. There is no correlation between k_∞ and redox potential differences, consistent with the conclusion noted above that other factors such as distance and orientation dominate the reaction rate in the cytochrome–PC system. Inasmuch as the rate constant at infinite ionic strength is similar to those for reaction between the other cytochromes and PC, our results are consistent with interaction at a distant site (presumably near Tyr-83) rather than at the proximal His-87 site.

CONCLUSIONS

The complex nature of the factors which influence protein–protein electron-transfer reactions is clearly illustrated by the results reported here. Thus, the magnitude of the observed rate constant depends upon a balance between a number of features of protein collisional complexes, including driving force, electrostatic interaction forces, and geometrical parameters such as redox center orientation and distance. The fact that rate constants for physiological reaction partners are not always optimal at the lowest ionic strengths is perhaps not surprising, inasmuch as biological systems have not evolved under such conditions (e.g., physiological ionic strength is about 150 mM). This is certainly not a unique feature of the cytochrome–PC system, and biphasic ionic strength dependencies similar to those shown in Figures 4 and 5 have been reported for a large number of redox protein electron-transfer reactions, including PC–photosystem I and cytochrome c_6–photosystem I (Hervas et al., 1992a), ferredoxin–ferredoxin: $NADP^+$ reductase (Walker et al., 1991), photosystem I–ferredoxin (Hervas et al., 1992b), photosystem I–flavodoxin (Medina et al., 1993), cytochrome *c*–cytochrome *c* peroxidase (Tollin & Hazzard, 1991), cytochrome *c*–cytochrome *c* oxidase (Tollin & Hazzard, 1991), and cytochrome b_5–cytochrome *c* (Willie et al., 1992; Meyer et al., 1993). Within these systems, intracomplex rate constants vary by more than 3 orders of magnitude, without any real patterns emerging. The cyt *f*–PC system lies approximately in the middle of this range.

The apparent lack of dependence of electron-transfer rate constants on the thermodynamic driving force is perhaps somewhat more surprising, although one must keep in mind that the comparisons between various cytochromes are being made under conditions in which all other factors are not maintained equal; i.e., it is likely that the geometries of the collisional complexes between PC and the various cytochromes differ as a consequence of surface topographical differences etc. Thus, although extrapolations to infinite ionic strength may eliminate electrostatic variables, it cannot control other types of variability. It seems clear that the effects of distance and orientation on protein–protein electron-transfer rates are not likely to be clarified until it becomes possible to systematically vary parameters in a controlled manner. This will require extensive studies utilizing the methods of site-directed mutagenesis, three-dimensional structural determination, and transient kinetic characterization. Studies along these lines with several protein systems are presently underway in our laboratory.

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