# Implications of the Effects of Viscosity, Macromolecular Crowding, and Temperature for the Transient Interaction between Cytochrome *f* and Plastocyanin from the Cyanobacterium *Phormidium laminosum*†

Beatrix G. Schlarb-Ridley,*,‡ Hualing Mi,§ William D. Teale,‡,|| Verena S. Meyer,‡ Christopher J. Howe,‡ and Derek S. Bendall‡

*Department of Biochemistry and Cambridge Centre for Molecular Recognition, University of Cambridge, Building "O", Downing Site, Cambridge CB2 1QW, United Kingdom, and National Laboratory of Plant Molecular Genetics, Shanghai Institute of Plant Physiology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Fenglin Road 300, Shanghai, 200032, China*

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ABSTRACT: The reaction between cytochrome *f* and plastocyanin is a central feature of the photosynthetic electron-transport system of all oxygenic organisms. We have studied the reaction in solution to understand how the very weak binding between the two proteins from *Phormidium laminosum* can nevertheless lead to fast rates of electron transfer. In a previous publication [Schlarb-Ridley, B. G., et al. (2003) *Biochemistry 42*, 4057−4063], we suggested that the reaction is diffusion-controlled because of a strong effect of viscosity of the medium. The effects of viscosity and temperature have now been examined in detail. High molecular mass viscogens (Ficoll 70 and Dextran 70), which might mimic *in vivo* conditions, had little effect up to a relative viscosity of 4. Low molecular mass viscogens (ethane diol, glycerol, and sucrose) strongly decreased the bimolecular rate constant ($k_2$) over a similar viscosity range. The effects correlated well with the viscosities of the solutions of the three reagents but not with their dielectric constants or molalities. A power law dependence of $k_2$ on viscosity suggested that $k_2$ depends on two viscosity-sensitive reactions in series, while the reverse reactions are little affected by viscosity. The results were incompatible with diffusion control of the overall reaction. Determination of the effect of temperature on $k_2$ gave an activation enthalpy, $\Delta H^{\ddagger} = 45$ kJ mol$^{-1}$, which is also incompatible with diffusion control. The results were interpreted in terms of a model in which the stable form of the protein−protein complex requires further thermal activation to be competent for electron transfer.

The reaction between cytochrome *f* and plastocyanin has been intensively investigated because of its central importance in the electron-transport system of oxygenic photosynthetic organisms. It can be conveniently studied in solution with recombinant proteins expressed in *Escherichia coli*; in the case of cytochrome *f*, a truncated form is used that lacks the membrane anchor but contains the whole of the soluble haem-containing portion, which is involved in electron transfer. X-ray crystal structures of the individual proteins are available, and models of the complex between them have been derived by an NMR method for both higher plant proteins (*1*) and those from the cyanobacterium *Phormidium laminosum* (*2*). Despite a basic similarity between the two systems, there are significant differences in the balance between electrostatic and hydrophobic interactions.

The binding between cytochrome *f* and plastocyanin is weak ($K_A \approx 10^3-10^4$ M$^{-1}$), but the rate of electron transfer is rapid (second-order rate constant, $k_2 \approx 10^8$ M$^{-1}$ s$^{-1}$). Hence, the rate of dissociation must be exceptionally fast ($k_{off} \geq 10^4$ s$^{-1}$), which is necessary for rapid turnover of the electron-transport system as a whole. These kinetic characteristics raise the question of how the structural properties of the interface are sufficiently specific for rapid electron transfer at the same time as being loose enough to allow rapid dissociation. This is a problem common to many electron-transfer reactions between soluble proteins (*3*).

Weak binding between protein partners is not confined to electron-transfer systems but is a feature of many other processes, such as cell−cell interactions in the immune system (*4*, *5*), virus capsid assembly (*6*, *7*), some signaling systems (*8*), and enzyme reactions with protein substrates, in which a short-lived or transient interaction is a key feature. In the present context, we arbitrarily define short-lived interactions as those for which $k_{off} \geq 1$ s$^{-1}$. Not all weak interactions ($K_A \leq 10^6$ M$^{-1}$) are short-lived in this sense. For example, lysin from spermatozoa of the abalone mollusc, which plays an important part in fertilization, occurs as a dimer for which $k_{off}$ is $8.8 \times 10^{-3}$ s$^{-1}$, while $K_A \approx 10^6$ M$^{-1}$ (*9*). Long-lived complexes ($k_{off} < 1$ s$^{-1}$) include strong

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* To whom correspondence should be addressed. E-mail: bgs9@mole.bio.cam.ac.uk. Telephone: +44-1223-333684. Fax: +44-1223-333345.

‡ University of Cambridge.

§ Shanghai Institute of Plant Physiology.

|| Present address: Institut für Biologie II, Albert-Ludwigs-Universität Freiburg, Schänzlestrasse 1, 79104 Freiburg, Germany.

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complexes such as enzyme−inhibitor complexes and antibody−antigen complexes, which, from the functional point of view, are effectively permanent once formed, and also nonobligate triggered complexes, which are transient in the sense that they dissociate as part of their biological function when the appropriate trigger (e.g., GTP/GDP exchange or phosphorylation) is provided (*10*, *11*). Weak complexes have been studied much less than those for which $K_A \geq 10^7$ $M^{-1}$ because of the difficulty of obtaining crystal structures of the complex. Nevertheless, the small number of electron-transfer complexes for which structures are available are valuable systems in which to study the structural basis of weak binding because the cofactors and the electron transfer between them provide convenient ways of studying the interactions.

We have previously determined the role of electrostatic interactions in the reaction between cytochrome *f* and plastocyanin from *P. laminosum* (*12*) and have also shown that aromatic residues in the haem-shielding N-terminal peptide of cytochrome *f* have an important role to play (*13*). In this earlier work, we came to the tentative conclusion, based largely on a strong dependence of the rate of reaction on solvent viscosity, that the reaction is diffusion-controlled. Here, we have tested that conclusion more rigorously by examining the effects of viscogens in more detail and those of temperature. The results were capable of a coherent explanation in terms of activation control and a model in which the most stable form of the precursor complex is not capable of rapid electron transfer. In view of the importance of molecular crowding effects for reactions occurring *in vivo*, we also studied the effects of relatively high molecular mass compounds, such as Ficoll 70 and Dextran 70, which may partially mimic the crowded environment of the cell. These compounds also raise the viscosity of the medium, but their influence on the rate of reaction between cytochrome *f* and plastocyanin was found to be very different from that of low molecular mass viscogens.

## MATERIALS AND METHODS

*Molecular Biology*. Molecular biological methods were essentially as described by Sambrook et al. (*14*), and materials were the same as in Schlarb et al. (*15*), except that the *E. coli* strain BL21(DE3) was replaced by BL21(DE3)-$Cd^+$(RIL).

*Protein Methods*. Expression, purification, and characterization of wild-type plastocyanin and cytochrome *f* of *P. laminosum* were carried out as in Hart et al. (*16*). Extinction coefficients used to calculate protein concentrations were 4700 $M^{-1}$ $cm^{-1}$ for plastocyanin and 31 500 $M^{-1}$ $cm^{-1}$ for cytochrome *f*.

*Kinetic Analysis*. Overall rate constants of the reaction ($k_2$) and their temperature dependence and viscosity dependence were determined using an Applied Photophysics stopped-flow spectrophotometer as described in Schlarb-Ridley et al. (*12*), except that, for viscosity dependence, 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)[1] at pH 6.0 was used as the buffer; note that in Figure 3 of ref *12* the concentration of plastocyanin should read micromolar instead of millimolar.

[1] Abbreviations: BLIP, $\beta$-lactamase inhibitor protein; HyHEL, immunoglobulin G1 antibody to hen egg-white lysozyme; MES, 2-(*N*-morpholino)ethanesulfonic acid.

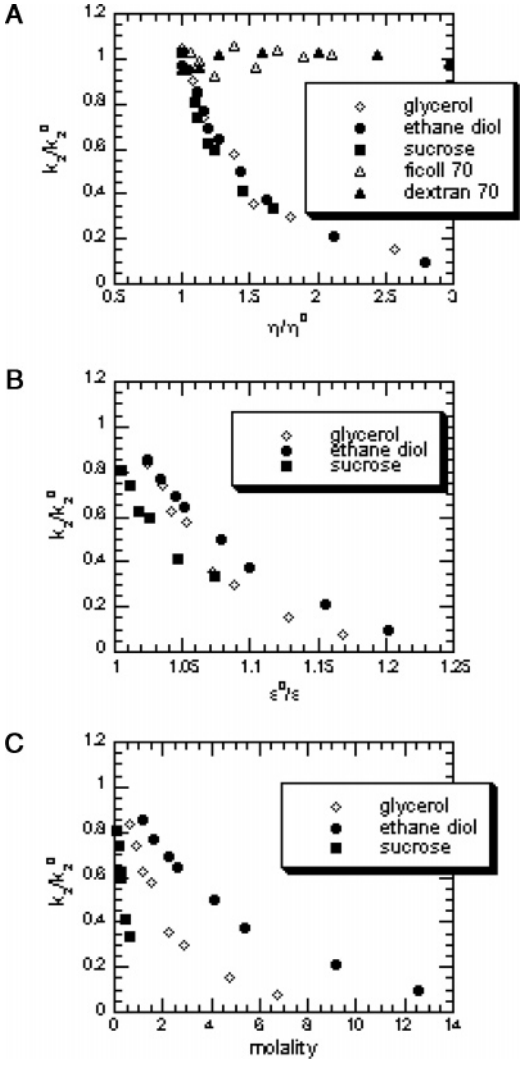


FIGURE 1: Effect of concentration of viscogen on relative $k_2$ ($k_2/k_2^0$). Viscogens used were Ficoll 70, Dextran 70 (high molecular mass), ethane diol, glycerol, and sucrose (low molecular mass). (A) Relative $k_2$ plotted against relative viscosity ($\eta/\eta^0$); viscosities were measured with an Ostwald viscometer. (B) Relative $k_2$ plotted against reciprocal relative dielectric constant ($\epsilon/\epsilon^0$). (C) Relative $k_2$ plotted against molality.

For the temperature dependence of $k_2$, the temperature was controlled by a thermostat connected to a circulating water bath (Grant Ltd. 20G). For the viscosity and crowding dependence of $k_2$, protein stock solutions (6 $\mu$M plastocyanin or 0.4 $\mu$M cytochrome *f* in 20 mM MES at pH 6.0) were mixed with equal volumes of solutions of the appropriate concentration of each viscogen or crowder in water. The relative viscosities of solutions of each viscogen or crowder mixed with equal volumes of 20 mM MES at pH 6.0 were measured using an Ostwald viscometer.

## RESULTS

*Effects of Low Molecular Weight Viscogens.* Figure 1A shows that when the viscosity of the solvent was increased by addition of glycerol, ethane diol, or sucrose the rate of reaction between cytochrome *f* and plastocyanin declined sharply. A plot of the relative second-order rate constant, $k_2/k_2^0$, against the relative viscosity, $\eta/\eta^0$, showed almost identical effects of the three viscogens, strongly suggesting that the inhibitory effect was due to the increasing viscosity

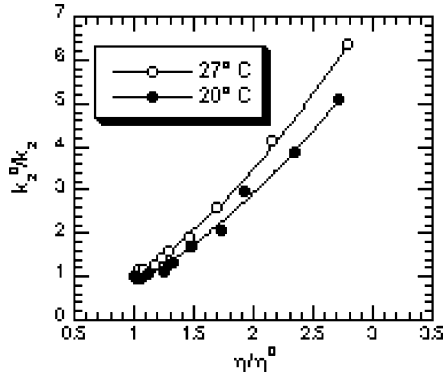


FIGURE 2: Dependence of reciprocal relative rate ($k_2^0/k_2$) on relative viscosity ($\eta/\eta^0$) adjusted with ethane diol. Independent measurements were made at 293 and 300 K. The experimental data were fitted to the equation $k_2^0/k_2 = A(\eta/\eta^0)^x$ by a nonlinear least-squares method (Kaleidagraph from Synergy Software).

rather than some other factor. This was confirmed by observing that when the relative rate constant was plotted against either the reciprocal relative dielectric constant (Figure 1B) or the molality (Figure 1C) the three reagents gave significantly different effects. Thus, the inhibition was not the result of either a change in the dielectric constant of the medium or any colligative effect such as osmotic pressure.

An increase in solvent viscosity would be expected to slow the diffusional association of the two proteins, that is, to decrease $k_{on}$. Stokes' law suggests that $k_{on}$ should be inversely proportional to viscosity. Similarly, Kramers' theory for reaction rates in terms of diffusion over a potential barrier also gives an inverse dependence on viscosity. Such behavior has been observed for reactions of certain proteins (*17*, *18*), but more frequently, a plot of inverse relative rate against relative viscosity has given a slope either significantly less than 1 (*19*) or greater than 1 (*20*, *21*). In the present case, such a plot (Figure 2) gives not only a slope greater than 1 but also a distinct upward curvature. Fitting the results to the equation

$$k_2^0/k_2 = A(\eta/\eta^0)^x \qquad (1)$$

gives a value for the exponent $x = 1.8 \pm 0.1$ at both 293 and 300 K. The significance of $x > 1$ will be discussed below.

*Effects of Macromolecular Crowding Reagents.* Ficoll 70 and Dextran 70 were used as high molecular mass crowding reagents, which would help to mimic more realistically the solution conditions likely to be encountered in the living cell. The maximum concentrations used, 7% (w/v) in the case of Ficoll and 5% (w/v) in the case of Dextran, were in the range corresponding to *in vivo* conditions, and these solutions had substantial relative viscosities (2.1 and 2.9, respectively). Surprisingly, a range of concentrations up to these maximum values had little or no effect on the rate of reaction between cytochrome *f* and plastocyanin, even though solutions in which the viscosity had been increased to similar values by the addition of the low molecular weight viscogens caused substantial inhibition (Figure 1A). However, crowding reagents can be expected to influence rates of reactions in several different ways, with some enhancing the rate and some causing inhibition, and these may compensate each other (see below).

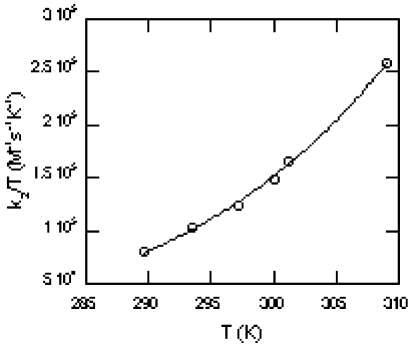


FIGURE 3: Effect of temperature on $k_2$. The experimental data were fitted to the Eyring equation $k_2 = \kappa k_B T/h \exp[\Delta S^{\ddagger}/R] \exp[-\Delta H^{\ddagger}/RT]$ by a nonlinear least-squares method (Kaleidagraph from Synergy Software).

*Effect of Temperature.* The marked decrease in $k_2$ when the viscosity of the medium was increased by addition of glycerol, ethane diol, or sucrose (Figure 1A) indicated that diffusion plays a part in the overall kinetics of the reaction. To test whether the reaction was diffusion-controlled, the effect of temperature on $k_2$ was determined. The effect of temperature on the viscosity of water suggests that for a diffusion-controlled reaction the Arrhenius activation energy would be about 20 kJ $mol^{-1}$ (*22*). An Arrhenius plot of $\ln(k_2)$ against $1/T$ (not shown) gave a value for the activation energy of 47.2 ± 1.2 kJ $mol^{-1}$, substantially higher than expected for a simple diffusion-controlled reaction. A plot of $k_2/T$ against $T$ (Figure 3) according to the Eyring equation

$$k_2 = \kappa \frac{k_B T}{h} \exp\frac{\Delta S^{\ddagger}}{R} \exp\frac{-\Delta H^{\ddagger}}{RT} \qquad (2)$$

where the transmission coefficient, $\kappa$, $\leq 1$, $k_B$ is the Boltzmann constant, and $h$ is Planck's constant, gave $\Delta H^{\ddagger} = 45.3 \pm 1.1$ kJ $mol^{-1}$. This is in good agreement with the Arrhenius activation energy, which, in principle, can be equated with $\Delta H^{\ddagger} + RT$. With $\kappa$ set to 1, the maximum value of the entropy of activation was $\Delta S^{\ddagger} = 52.7$ J $mol^{-1}$ $K^{-1}$, giving $T\Delta S^{\ddagger} = 15.8 \pm 1.1$ kJ $mol^{-1}$ at 300 K and $\Delta G^{\ddagger}$ (minimum) $= 29.5 \pm 3.7$ kJ $mol^{-1}$. The interpretation of this result will be discussed below.

## DISCUSSION

*Influence of Molecular Size on the Viscosity Effects of Cosolvents.* The study of electron transfer and other biological reactions in dilute solution is necessary if we are to understand the chemical and kinetic factors influencing their behavior, but the biological significance of the results obtained must be considered against the very different conditions prevailing inside the cell. One way to bridge the gap is to study the effects of artificial macromolecular crowding on the reaction in solution. To this end, we examined the effects of two reagents, Ficoll 70 and Dextran 70, on $k_2$ for the reaction between cytochrome *f* and plastocyanin. Both reagents have an average molecular weight of about 70 kDa, and neither would be expected to interact specifically with either protein. It should be borne in mind, however, that the natural environment for the reaction studied is the thylakoid lumen, and according to current estimates, the total concentration of soluble protein in the lumen contents is of the order of 20 mg $mL^{-1}$, whereas

higher concentrations (50−400 mg mL$^{-1}$) normally prevail in cell cytoplasm (*23*); furthermore, the lumen is a tightly confined space so that effects of confinement rather than crowding may be more important.

When the viscosity of the solution was raised by addition of ethane diol, glycerol, or sucrose, a marked inhibition of $k_2$ was observed (Figure 1A), as would be expected for a reaction in which diffusion plays an important part. It was remarkable then that increasing the solution viscosity to a similar extent by addition of Ficoll 70 or Dextran 70 had little or no effect on $k_2$. A similar lack of effect on reaction rates has been observed in some other systems (*20*, *24*). The simplest explanation for these observations is compensation between the two opposing effects of macromolecular crowding. On one hand, the volume exclusion effect enhances macromolecular association by increasing the effective $K_A$ and $k_{on}$. The excluded volume of 7% (w/v) Ficoll is about 60% from osmotic pressure measurements (*24*) so that $K_A$ and $k_{on}$ should be enhanced by a factor of $1/0.4 = 2.5$. On the other hand, this should be offset by an increase in viscosity. Under our conditions, the relative viscosity of 7% Ficoll was 2.1, which would almost compensate for the excluded volume effect if there was an inverse dependence on viscosity. Nevertheless, it would be surprising if such compensation would be maintained over a range of crowder concentrations; the true behavior in a solution of Ficoll or Dextran is almost certainly more complex than this simple model implies. Kozer and Schreiber (*20*) found that the effect of crowding reagents on the association between $\beta$-lactamase and its inhibitor protein depended markedly on the chemical nature of the reagent. In particular, they found that Ficoll 70 had little effect on $k_{on}$, even at a relative viscosity of 5, whereas Haemaccel, which is a partial hydrolysis product of gelatin with an average mass of 35 000 Da, gave the same response to viscosity as did ethane diol with a mass of 62 Da. They suggested that Ficoll solutions are nonuniform and that association between Ficoll monomers left relatively large volumes of free solution in which the reacting protein molecules could diffuse unhindered, even though the bulk solution has a high viscosity. Moreover, rotational diffusion, which plays an important part in protein−protein association, is little affected by macromolecular crowding (*25*). A further possibility, which would probably diminish the effective rate of binding, is self-association of plastocyanin or cytochrome *f* molecules. The Macromolecular Structure Database shows that plastocyanin from *P. laminosum* is hexameric in the crystal structure and that cytochrome *f* is dimeric, although the asymmetric units are monomolecular. Thus, self-association is a possibility for both proteins.

The results obtained with high molecular mass crowders strongly suggest that crowding is unlikely to be a significant factor for relating the solution kinetics of the system to its behavior *in vivo*. Other factors not examined here may, nevertheless, significantly modify the behavior of the system. In particular, there is the fact that the reaction normally occurs in the narrow, confined space of the thylakoid lumen, as mentioned above, and also the possibility of two-dimensional diffusion over the membrane surface rather than three-dimensional liquid-phase diffusion (*26*, *27*).

*Response to Low Molecular Mass Viscogens.* The results shown in Figure 2 show a more complex influence of viscosity than the simple inverse dependence predicted either by the Stokes−Einstein equation, which applies to particle diffusion in a liquid phase, or by Kramers' theory, which applies more generally to any process or reaction in which the rate of passage over a potential energy barrier is influenced by solvent friction (*28*, *29*). Although such behavior has been observed, for example, in the binding of antithrombin to thrombin (*17*) or of a monoclonal antibody to cytochrome *c* (*18*), more complex responses are not infrequent. Kozer and Schreiber (*20*) observed that a plot of 1/relative $k_{on}$ against relative viscosity for the binding of the inhibitor protein BLIP to $\beta$-lactamase gave a slope of 2, and in the case of the binding of HyHEL-5 or HyHEL-10 to hen egg-white lysozyme, Xavier and Willson observed a slope of 2.5 (*21*). The latter authors suggested that the large response could be explained by the osmotic effect of the cosolvent. Formally, a linear response with a slope greater than 1, if it is a genuine effect of viscosity, suggests that the effective microviscosity experienced by the protein solutes is greater than the macroviscosity measured, for example, with an Ostwald viscometer, but this distinction does not correspond to physical reality. More frequently, there has either been a linear response with a slope less than 1, or the plot has shown a downward curvature, indicating a diminished response as the viscosity is increased. Two types of explanation have been offered for these effects. On one hand, there is the inclusion of a protein friction term ($\sigma$) in addition to the solvent friction or viscosity, so that $\eta$ is replaced by $(\eta + \sigma)$ (*30*−*33*); on the other hand, viscosity is represented as a power term, so that $(1/k) \propto \eta^x$. The exponent $x$ has been found to have a value less than 1, suggesting a partial shielding from the frictional effects of solvent by the protein matrix (*19*, *31*, *32*, *34*−*36*).

Such explanations are inadequate, however, in the case of the reaction between cytochrome *f* and plastocyanin for which Figure 2 implies a value of the exponent $x$ in eq 1 of 1.8. This suggests that $k_2$ depends on two processes occurring in series. An upward curvature of the plot as in Figure 2 has not previously been observed in any protein system, as far as we are aware, and can only be explained by a power law behavior with $x > 1$. Such a situation would not be consistent with the reaction being diffusion-controlled (when $k_2$ is dependent on $k_{on}$ alone), but could, in principle, be accommodated if it were activation-controlled. In the latter case, the simplest possible scheme for electron transfer (Scheme 1) would give the following equation for $k_2$

Scheme 1

$$Pc_{ox} + Cyt\ f_{red} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} [Pc_{ox}Cyt\ f_{red}] \xrightarrow{k_{et}} [Pc_{red}Cyt\ f_{ox}] \longrightarrow Pc_{red} + Cyt\ f_{ox}$$

$$k_2 = \frac{k_{on}k_{et}}{k_{off}} \tag{3}$$

Equation 3 would demand that both $k_{on}$ and $k_{et}$ were dependent on viscosity, whereas $k_{off}$ was not. It is generally expected, however, that a true $k_{et}$, independent of any part of the binding process, would not be influenced by solvent viscosity, so that a simple one-step binding process is unlikely to offer an explanation of the observed viscosity dependence.

Binding has frequently been described as a two-step process (*1*, *16*, *37*, *38*) such as shown in Scheme 2. The

Scheme 2

$$Pc_{ox} + Cyt\ f_{red} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} [Pc_{ox}Cyt\ f_{red}]_1 \underset{k_{-r}}{\overset{k_r}{\rightleftharpoons}}$$

$$[Pc_{ox}Cyt\ f_{red}]_2 \xrightarrow{k_{et}} [Pc_{red}Cyt\ f_{ox}] \longrightarrow Pc_{red} + Cyt\ f_{ox}$$

general equation for this scheme is

$$k_2 = \frac{k_a k_r}{k_{-a}(k_{et} + k_{-r}) + k_r k_{et}} k_{et} \quad (4)$$

(*39*, *40*), which, in its reciprocal form, may be rearranged to show how the overall reaction time can be represented as the sum of the transit times of the three individual steps

$$\frac{1}{k_2} = \frac{1}{k_a} + \frac{1}{K_A k_r} + \frac{1}{K_A K_R k_{et}} \quad (5)$$

In eq 5, $K_A = k_a/k_{-a}$ and $K_r = k_r/k_{-r}$. The equation may be approximated and simplified depending on which step in Scheme 2 is rate-limiting. If the initial binding is rate-limiting, that is to say $k_{et} > k_{-r}$ and $k_r > k_{-a}$, the reaction is diffusion-controlled and $k_2 = k_a$. Again, this does not offer an explanation of the viscosity dependence. A second possibility is that rearrangement is rate-limiting. This will be the case when $k_r < k_{-a}$ and $k_{et} > k_{-r}$, which leads to the equation

$$k_2 = \frac{k_a k_r}{k_{-a}} \quad (6)$$

The third regime may be described as activation-controlled and occurs when $k_{et} < k_{-r}$ and $k_r \leq k_{-a}$. Under these conditions

$$k_2 = \frac{k_a k_r}{k_{-a} k_{-r}} k_{et} \quad (7)$$

Equations 6 and 7 open up the possibility of $k_2$ being dependent on $\eta^2$. Both $k_a$ and $k_r$ would be expected to be sensitive to solvent friction. The extensive studies of Kostic and colleagues on electron transfer between the flash-induced triplet state of Zn-substituted cytochrome *c* and plastocyanin or cytochrome $b_5$ have shown that electron transfer within the preformed complex is dependent on a viscosity-sensitive rearrangement step ($k_r$) (*31*, *32*, *35*). A similar viscosity dependence of the reaction between ruthenium-substituted cytochrome *c* and plastocyanin has also been observed (*41*). In both types of experiment, a first-order apparent $k_{et}$ was being measured.

In the case of the bimolecular reaction between cytochrome *f* and plastocyanin reported here, the $\eta^2$ dependence would not have been observed unless $k_{-a}$ was independent of viscosity or only weakly dependent. If eq 7 is more appropriate, $k_{-r}$ would have to be similarly insensitive to viscosity. The latter requirement would be met if $k_{-r}$ is fast, owing to a very small activation energy, as illustrated in Figure 4, so that $K_r < 1$. Under these conditions, $k_{-a}$ is equivalent to $k_{off}$. There is conflicting experimental evidence regarding the viscosity dependence of $k_{off}$. Grove and Kostic (*42*) have interpreted their kinetic data on electron transfer between zinc-substituted cytochrome $c_6$ and cytochrome *f* from *Chlamydomonas reinhardtii* as indicating that the binding constant is independent of viscosity, and hence that $k_{off}$ is inversely proportional to viscosity because this is true for $k_{on}$. On the other hand, there is evidence from diverse systems for either a weak viscosity dependence of $k_{off}$ or none at all (*20*, *43*−*45*). Although one might expect dissociation of a protein complex to be influenced by solvent friction, the effect can be masked at low relative viscosities by a larger protein friction term (*46*). The most plausible kinetic model to explain our results is thus one in which the stable form of the complex formed by diffusional interaction of the two proteins requires an additional activation to an unstable form before rapid electron transfer can occur, as illustrated in Figure 4. Thus, in Scheme 2, $K_r < 1$ and either eqs 6 or 7 may be appropriate.

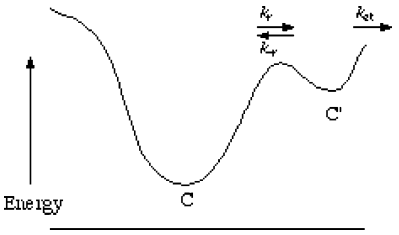


FIGURE 4: Energy diagram for the proposed reaction coordinate. C represents the most stable form of the precursor complex, which requires further activation ($k_r$) to a highly unstable form of the complex, C′, for electron transfer to occur.

The kinetic model developed above implies that the form of the complex between plastocyanin and cytochrome *f* from *P. laminosum* deduced from chemical-shift changes (*2*) is not capable of rapid electron transfer. Although the NMR experiments yielded 24 slightly different structures, they all had in common that His92, the copper ligand of plastocyanin, makes contact with Phe3 of cytochrome *f* and not with Tyr1, as in the higher plant complex (*1*). Phe3 provides a much less efficient pathway for electron transfer than Tyr1, and thus, a picture of the activation process emerges in which a small rotation of one protein relative to the other or possibly a conformational change of one protein brings His92 into contact with Tyr1.

Finally, the above discussion suggests a possible interpretation for those cases of protein association in which a plot of the reciprocal of $k_{on}$ against the relative viscosity gives a slope greater than 1. The crucial point is that $k_{on}$ should depend on the rate constants of two reactions in series. One of them would correspond to $k_a$ in Scheme 2 and as a diffusional process would be expected to show the theoretical dependence on $\eta^{-1}$. The second would be a rearrangement, $k_r$, which, although sensitive to solvent friction might be partially shielded from it so that $k_r$ would depend on $\eta^{-x}$, where $x \leq 1$. This condition would be satisfied by two-step binding, corresponding to that involved in Scheme 2, and for which Selzer and Schreiber give $k_{on} = k_a k_r/k_{-a}$ (*37*). The second necessary condition would be that $k_{-a}$ should either be independent of viscosity or dependent on $\eta^{-x}$ with $x \ll 1$. As mentioned above, this is possible if the effect of solvent viscosity is masked by a larger protein friction term. The net result would be that $k_{on}$ would depend on $\eta^{-x}$, where $1 < x < 2$. In practice, it would be difficult to distinguish between a linear relationship with a slope of 2 and a power dependence with, say, $x = 1.4$. This model could

also be adapted to three-step binding as described by Schreiber (*38*).

*Temperature Dependence.* One conclusion from the above discussion of the effects of low molecular mass viscogens is that the overall reaction cannot be described as diffusion controlled. Activation parameters derived from the effect of temperature lead to the same conclusion. In particular, $\Delta H^{\ddagger} = 45.3$ kJ mol$^{-1}$ is more than double what would be expected for a simple diffusion-controlled reaction. The activation enthalpy is partially offset by a positive activation entropy, which suggests that the complex must pass through a looser configuration in the transition state before electron transfer can occur.

The growth temperature of *P. laminosum* in its natural environment is 45 °C, substantially higher than the temperature of the measurements reported above. In a previous publication (*12*), we concluded that at the physiological temperature cytochrome *f* and plastocyanin would interact at least as rapidly as does the higher plant system if we assumed an enthalpy of activation, $\Delta H^{\ddagger}$, of 40 kJ mol$^{-1}$. The measured value of 45 kJ mol$^{-1}$ confirms this conclusion.

The activation energy for $k_2$ is the sum of the activation energies of the individual rate constants in eqs 6 or 7, giving the reverse rate constants negative values. Let us suppose that eq 7 applies, that is to say that $k_{et} < k_{-r}$. The difference in activation free energies for $k_a$ and $k_{-a}$ is given by $\Delta\Delta G^{\ddagger} = -RT \ln K_a \approx -14$ kJ mol$^{-1}$, because $K_a \approx 300$ (*2*). If we assume $K_r = 0.1$, as a modest example, we obtain $\Delta\Delta G^{\ddagger} = 6$ kJ mol$^{-1}$ for the second step of Scheme 2. Because $\Delta G^{\ddagger}$ for the overall reaction was found to be 30 kJ mol$^{-1}$, we can calculate that, for $k_{et}$, $\Delta G^{\ddagger} = 30 + 14 - 6 = 38$ kJ mol$^{-1}$. For this reaction, $\Delta G^{\circ} \approx 0$, and hence, from Marcus theory, $\Delta G^{\ddagger} = \lambda/4$, with $\lambda$ being the reorganization energy, so that $\lambda = 152$ kJ mol$^{-1}$ or 1.6 eV. Although there is no independent measurement of $\lambda$ for this reaction, the value of 1.6 eV lies within the range of values for similar reactions.

If, on the other hand, we assume that $k_{et} > k_{-r}$ so that eq 6 applies, we can calculate that $\Delta G^{\ddagger}$ for $k_r$ is 44 kJ mol$^{-1}$. The Eyring equation (eq 2), with $\kappa = 1$, then gives $k_r = 1.2 \times 10^5$ s$^{-1}$. For eq 6 to be plausible, $k_{et} \geq 1.2 \times 10^6$ s$^{-1}$ would be required. Application of the equation of Moser and Dutton (*47*, *48*)

$$\log k = 13 - 0.6(R - 3.6) - 3.1(\Delta G^0 + \lambda)^2/\lambda \quad (8)$$

gives the edge−edge distance, $R = 6.9$ Å, for $k = 1.2\ 10^6$ s$^{-1}$ and $\lambda = 1.6$ eV, as calculated above. This is a plausible distance if the ring of the histidine ligand to the Cu atom is included as part of the Cu cofactor.

The above calculations provide only approximate estimations of parameters, but, nevertheless, they serve to confirm the conclusion from the study of the effects of low molecular mass viscogens that the reaction can plausibly be described with the assumption that the most stable form of the interprotein complex is not capable of rapid electron transfer. A thermally activated rearrangement is required, such that $K_r < 1$. The information available, however, does not enable a distinction to be made between limitation by electron-transfer itself, when $k_{et} < k_{-r}$ and eq 7 applies, and limitation by the rearrangement step, when $k_{et} > k_{-r} > k_r$ and eq 6 applies.

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