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Review

Electron transfers amongst cytochrome *f*, plastocyanin and photosystem I: kinetics and mechanisms

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Abstract

The review covers the theory and practice of the determination of kinetic constants for the electron transfer reactions in chloroplast thylakoid membranes between plastocyanin and cytochrome *f* in cytochrome *bf* complexes, and between plastocyanin and the reaction centre of photosystem I. Effects of ionic strength and pH are featured. The contribution of mutant studies is included. It is concluded that nearly all data from in vitro experiments can be interpreted with a reaction scheme in which an encounter complex between donor and acceptor is formed by long-range electrostatic attraction, followed by rearrangement during which metal centres become close enough for rapid intra-complex electron transfer. In vivo experiments so far cast doubt on this particular sequence, but their interpretation is not straightforward. Means of modelling the bimolecular complex between cytochrome *f* and plastocyanin are outlined, and two likely structures are illustrated. The complex formed by plastocyanin and photosystem I in higher plants involves the PsaF subunit, but its structure has not been fully determined.

Keywords: Electrostatic complex; Docking; Rearrangement; pH; Ionic strength; Cytochrome c_6; Acidic residues; Basic residues; Models

Contents

Abbreviations: *A*, generalised electron acceptor; $A_{(1,2)}$, proportion of each of two distinguishable phases in a trace representing e.g. reduction of an electron acceptor; cyt, cytochrome; *D*, generalised electron donor; **D**, diffusion coefficient; *k*, rate coefficient as defined for a particular reaction scheme; Mega-9, nonoyl-*N*-methylglucamide; MD, molecular dynamics (computations); NADP, nicotinamide adenine dinucleotide phosphate; Pc, plastocyanin; PS (I,II), photosystem (I,II); P700, reaction centre of PS I; e-t, electron transfer

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1. Introduction

Electron transfers between the proteins and protein complexes under review are stimulated by light absorbed by the Photosystems (PS) in the chloroplasts of green plants, algae, cyanobacteria and photosynthetic bacteria. With the exception of the bacteria, two types of photosystem are found in the thylakoid membranes, PS I and PS II. Electron transport, from water as donor to terminal acceptors, such as ferredoxin or NADP, is effected by the two PS's aided by a third membrane-located complex, the cytochrome *bf* complex [1]. Plastoquinol functions as an electron carrier between PS II and the *bf* complex, which contains cytochrome *f*, and plastocyanin does the same between the *bf* complex and PS I where it interacts with P700, the reaction centre of PS I.

This review concentrates on the period 1993–1999, but briefly summarises earlier work where relevant. It leans toward the biophysical and quantitative. Appendices cover a couple of esoteric areas, namely the details of kinetics and of ionic strength theories. The exact subject of this article has not been fully reviewed, although sections of reviews, on plastocyanin for example, refer to electron transfer rates and mechanisms [4–6]. Useful reviews exist on electron transfer in proteins with different [7] or similar [8] emphasis to the present review.

2. The given structures

The general arrangement of the thylakoid complexes engrossing cyt *f* and P700 is illustrated in Fig. 1. The structures of the soluble portion of eucaryotic cyt *f* and of plastocyanin are given in Fig. 2. That of a cyanobacterial cyt *f* has recently been established [13] and closely resembles the cyt *f* in Fig. 2. For recent reports of structure determinations of novel plastocyanins, or refinements, see [14–17]. Photosystem I structure is not fully refined, but is generally as shown in Fig. 3 for eucaryotes.

3. Diffusion and formation of an encounter complex

In general terms, electron transfer is the result of several processes of which the first to be considered is diffusion. The membrane-embedded complexes PS I and cytochrome *bf* should be less mobile than plastocyanin, and restrained to two dimensions. Pc has

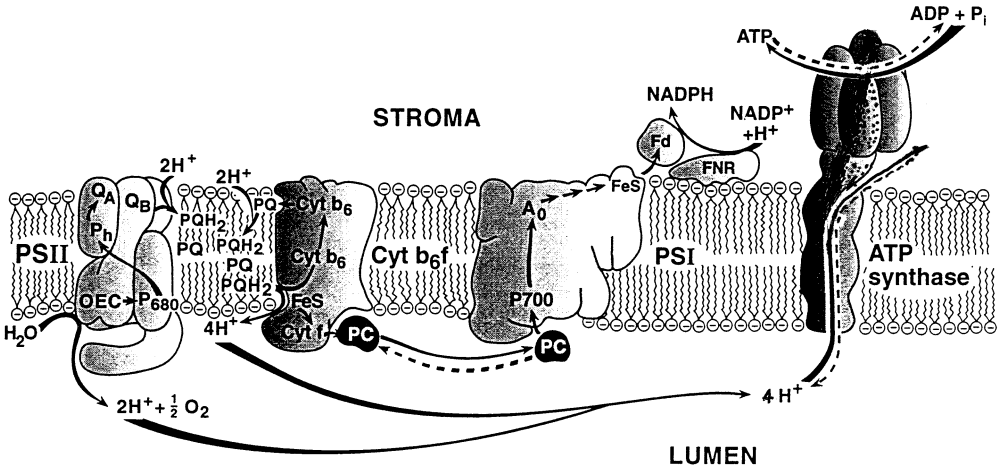

Fig. 1. Arrangement of electron transfer components in the thylakoid membrane of eucaryotes, from [1]. Used with permission of the authors and Kluwer Academic Publishers. The ATP-synthase is now depicted with a proton/ATP stoichiometry of 4 and with indirect proton coupling to the rotary mechanism (see [2,3]).

the possibility to diffuse longer distances in the lumenal space, shuttling between grana and stromal membranes [19,20]. There too, diffusion in vivo is more two- than three-dimensional, due to the intermembrane spacing. Proteins protruding from the thylakoids into the lumen increase the diffusion path of Pc relative to that in free solution, and membrane complexes do the same for cyt *bf* complexes. More tellingly, the masses of *bf* complexes, about 180 kDa for a dimer, and of PS I complexes (ca. 280 kDa) are great relative to Pc with its 10-kDa mass.

If Brownian motion were the only factor, diffusion would lead to an estimated collision rate between Pc and cyt *f* in *bf* complexes of about 10^8–10^9 M^{-1} s^{-1} [21]. Intermolecular forces, particularly long-range electrostatic forces, modify this rate: attractive forces, between charges of opposite sign on amino acid residues, increase k_{on} while the opposite occurs when the charges have the same sign. Then the diffusion-limited rate coefficient is,

$$k_{on} = 4\pi N_A R D \Phi \cdot 10^3 \text{ (units } M^{-1}\ s^{-1}),$$

where N_A is Avogadro's number, $R = R_1 + R_2$, the sum of the radii of the collision partners and $\boldsymbol{D}$ is their mutual diffusion coefficient. Φ is a function of the electric field distribution [22] and is unity in the absence of any field.

At the distance of closest approach, the configuration of the two proteins is called the encounter complex [23]. Its structure, lifetime, and effectiveness for electron transfer depend on a number of factors. The structure will be variable, as one component undergoes rotational and linear motions relative to the other, ('explores configurational space' in the jargon) with many similar interaction energies. The stability of any complex depends on this energy, the 'off rate' under diffusion-controlled conditions being given [22] by:

$$k_{off} = 3D\ \Phi\ \exp(V(R)/kT)/R^2 \text{ in units of } s^{-1}.$$

The ratio k_{on}/k_{off} is the association or binding constant K_a, in M^{-1}, and its reciprocal is the dissociation coefficient $K_d = k_{off}/k_{on}$ in M. As an extreme example, the complex between barnase and its inhibitor barstar has a spectacularly slow dissociation rate [24] the K_d being 10^{-14} M. Electron transfer complexes, not surprisingly, have greater dissociation rates, or else the throughput of electrons would be very slow.

During the lifetime of the encounter complex, electron transfer between donor and acceptor may occur in the familiar activated state, at a rate determined by several factors, conveniently encapsulated in semiclassical theory [25] by equations such as:

$$k_{et} = (4\pi^3/h^2\ \lambda k_B T) \cdot H_{DA}^2 \cdot \exp\left[-(\Delta G^0 + \lambda)^2/4\lambda k_B T\right]$$

In this, we see the product of a frequency factor, an electronic factor and a nuclear factor. H_{DA}^2 is the electronic coupling between donor (D) and acceptor

(A) in the activated state, and depends on details of the electron path, along bonds and through space; in all theories it is extremely sensitive to the distance between D and A. ΔG^0 is the driving force, related to the difference in reduction potential between donor and acceptor by $\Delta G^0 = -F\Delta V^0$, for a one-electron redox reaction. λ is the nuclear reorganisation energy. The inner sphere component of λ for self-exchange electron transfers in the blue-copper proteins such as Pc has been estimated to be 62 kJ/mol or 0.64 eV [26].

4. Kinetic analyses

4.1. Reaction schemes

To describe quantitatively the overall rate of electron transfer, it is necessary to consider the relationships between components in a kinetic scheme, itself of varying complexity (see further Appendix A). For example, Hervás et al. [27] refer to the following equations (Eqs. 1–3) as 'kinetic mechanisms' of types I, II or III, which might be a convenient initial classification, but remembering that type II will behave like type I when internal e-t rate is much greater than association rates (diffusion limited), and type III simplifies to type II if rearrangement rates greatly exceed e-t rate:

$$D + A \leftrightarrow \underset{k_{rev}}{\overset{k_{so}}{\leftrightarrow}} D^+ + A^- \quad (1)$$

$$D + A \leftrightarrow \underset{k^I_{off}}{\overset{k^I_{on}}{\leftrightarrow}} \{D{\cdot}A\} \leftrightarrow \underset{k_{ret}}{\overset{k_{et}}{\leftrightarrow}} \{D^+{\cdot}A^-\} \leftrightarrow \underset{k^{II}_{on}}{\overset{k^{II}_{off}}{\leftrightarrow}} D^+ + A^- \quad (2)$$

$$D + A \leftrightarrow \underset{k^I_{off}}{\overset{k^I_{on}}{\leftrightarrow}} \{D...A\} \leftrightarrow \underset{k_{der}}{\overset{k_{rearr}}{\leftrightarrow}} \{D{\cdot}A\}$$

$$\leftrightarrow \underset{k_{ret}}{\overset{k_{et}}{\leftrightarrow}} \{D^+{\cdot}A^-\} \leftrightarrow \underset{k^{II}_{on}}{\overset{k^{II}_{off}}{\leftrightarrow}} D^+ + A^- \quad (3)$$

While the second-order rate-coefficient in Eq. 1 is relatively uninformative about mechanism, it is often used in a comparative way, for example in estimating the effect of mutations in D or A on the overall rate of e-t; extensive experiments involving mutants are described later.

Since we know that an activated complex must be formed before e-t occurs, Eq. 2 would appear to be more realistic. We see below and in Appendix A that approximations have frequently been employed to analyse data in terms of such a scheme. Note that

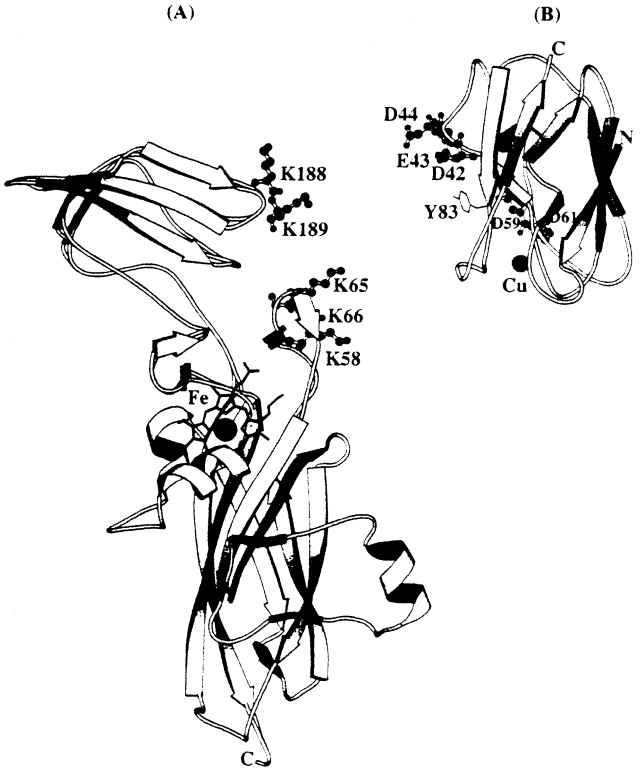

Fig. 2. Schematic diagrams of: (A) the molecular structure of the lumen located portion of cytochrome f; and (B) that of plastocyanin, from [9]. For the original determination of the crystal structure of poplar Pc, see [10]; for that of pea cyt f, see [11,12]. Used with the permission of the authors and the American Chemical Society.

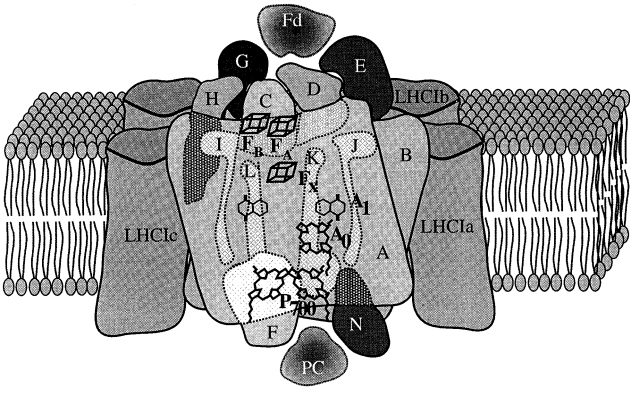

Fig. 3. The arrangement of subunits comprising Photosystem I in eucaryotes [18]. Bold labels signify electron transfer components, others, subunits. Used with the permission of the author and Kluwer Academic Publishers.

the on and off rates during association of D with A may be different from those for dissociation of $\{D^+ \cdot A^-\}$.

Eq. 3 is a description of association of donor and acceptor to form a 'complex' in which for some reason (elaborated later) the probability of electron transfer is quite low. Then, with rate k_{rearr}, rearrangement occurs to a favourable configuration, followed by internal e-t. Much has been made in the literature of 'rearrangement'; both its theoretical and experimental bases are examined below. We will see that very likely Eq. 3 represents the mechanism in all cases; the kinetics may appear to be describable by Eq. 1 or Eq. 2 because of the relative speeds of the electron transfer or rearrangement steps, as already stated.

All the components of the energy of interaction when a complex is formed must be considered [28]: electrostatic forces already mentioned, H-bonds, Van der Waals forces and hydrophobic forces. Consideration of one or, unusually, all of these factors has lead to several molecular models of association complexes between Pc and cyt *f*. These are outlined in more detail later. Particularly relevant in such modelling is the inclusion of water molecules in the complex. Protein molecules are hydrated, but the probability of water molecules being excluded from the area of contact between D and A must be considered. A further question arises – is there a significant change in structure of either of D or A upon complexation?

4.2. Methods of studying electron transfer

Experimental data usually consists of a set of estimates of the change in concentration of the donor and/or acceptor with time after either (a) mixing D with A quickly as in a rapid-mix, stopped-flow experiment (Pc_{ox} with cyt f_{red}), or (b) a single-turnover light flash that generates A_{ox} from A either directly (e.g. $P700^+$ from P700) or indirectly produces a reactant via a fast-reacting radical [29].

If Eq. 1 is appropriate, a second order rate-coefficient k_{so} may be calculated from observed changes in concentration of one of the components. If the donor is in excess concentration, $[A]_t$ has an exponential form, from which is estimated k_{obs}, the pseudo-first-order rate-coefficient. Part of a plot of k_{obs} vs. $[D]$ will be linear; then k_{so} comes from the slope of the plot: $k_{so} = k_{obs}/[D]$. Further information may come from the curvature and maximum value of k_{obs} in the plot. A section below describes such experiments. If Eq. 2 is thought more appropriate, the observed rate(s) k_{obs} is connected with the coefficients in that equation through certain approximations ([8] and Appendix A).

These changes in concentration are estimated from absorbancy changes at suitable wavelengths where there is minimum interference from other spectral changes, or with proper deconvolution to take into account the contributions of all components at each wavelength [30,31]. Since the absorbancy changes are small, of the order of 10^{-4} to 10^{-3} units, signal averaging is normally necessary. Repetitive flashes are delivered at such a frequency that the flash-oxidised component is all re-reduced between flashes; a suitable reductant such as ascorbate is customary for this purpose; its slow reduction of oxidised reactants has to be taken into account in using the data to estimate kinetic coefficients.

As well as using the changes in concentration in the reaction to draw conclusions about the mechanism, additional experiments may reveal other coefficients: use of a mixture of oxidised and reduced D to probe for any difference in on and off rates between complexes $\{D_{red} \cdot A_{red}\}$ and $\{D_{ox} \cdot A_{red}\}$; use of double flashes to see whether an oxidised D needs to leave the complex before another turnover, answering queries about single or multiple binding sites on the acceptor.

5. General features of results

5.1. The reaction between cyt f and Pc

Table 1, for reactions between cyt *f* from various sources and Pc, recalls earlier estimates of k_{so} and k_{rev}, and shows more recent and ingenious experiments in which K_a and k_{et} were estimated as well as k_{so} [34]: as a complex was formed between cyt *f* and Pc, the Soret band of cyt *f* was slightly enhanced, enabling estimation of K_a. This phenomenon proved elusive later [36], but K_a could be estimated from changes in the proton NMR spin relaxation time T2 of V39γH in Pc, yielding values not too

Table 1
Kinetic coefficients for the reaction between plastocyanin and cytochrome *f*

Plant material *D*, *A*	$10^{-7} \times k_{so}$ (M^{-1} s^{-1})	k_{rev}	K_a (M^{-1}) (K_d (μM))	10^{-7} k_{on} (M^{-1} s^{-1})	10^{-3} k_{et} (s^{-1})	pH	*I* (mM)	Ref.
Brassica komatsuma 0.3 μM cyt f^{II} 1–20 μM Pc^{II}	4.5	1.9	–	–	–	7.0	200	[32]
Radish 0.22 μM cyt f^{II} 2.2 μM Pc^{II}	6.0	–	–	–	–	7.0	100	[33]
0.22 μM cyt f^{II} (rape seed) 2.2 μM Pc^{II} (pea)	4.06	–	10^4 [b] (100)	4.35	62[c]	6.0	100	[34]
0.25 μM cyt f^{II} (Turnip[a]) 2.5 μM Pc^{II} (French bean)	20	–	–			7.0	100	[29]
Spinach equi-M, 15 μM	–	–	(23)	6.7	2.8	7.0	5	[35]
6 μM cyt f^{II} (Turnip) 6 μM Pc^{II} (Spinach)	18	–	7100[d] (140)	–	26	6.0, 7.5	100	[36]

Mostly the stopped-flow technique was used, or flash-generated oxidant, to initiate the reaction. *D* is donor, *A*, acceptor.
[a]Turnip cyt f^{III} had only slow autoreduction compared with spinach.
[b]Complex formation observed through enhancement of the Soret band of cyt *f*.
[c]Based on a steady-state approximation (see text).
[d]From proton NMR T_2 (see text); k_{so} from ΔA_{422}.

dissimilar from the earlier ones. The 'steady-state' approximation in [34] which led to the estimate for k_{et} was criticised [35] and in the later results [36] replaced by a 'minimum estimate' for k_{et}, namely k_{so}/K_a. It is notable that the traces representing oxidation of cyt *f* do not appear to be biphasic like those for $P700^+$ reduction (next section). This is despite the association constants being comparable (Tables 1 and 2). However, there are differences in the experiments. In stopped-flow, rapid-mix experiments any complex between Pc^I and cyt f^{II} is not preformed, whereas it is, in flash experiments.

5.1.1. Product binding

Little information on this exists. An assumption was made in [39] that K_A for the reaction complexes $\{Pc^{II}\cdot cyt\ f^{III}\}$ or $\{Pc^{I}\cdot cyt\ f^{II}\}$ was the same as that for $\{Pc^{II}\cdot cyt\ f^{II}\}$. This was seen as reasonable in that the metal centres might be too far apart for changes therein to influence the binding process. Unpublished data showed the same constant for Cd-substituted Pc when binding with either oxidised or reduced cyt *f*, and the value was the same as for Cu-Pc.

Experiments involving cyt *f* in cyt *bf* complex [37] have been omitted as inaccurate because the oxidation of cyt *f* was rate-limited by the formation of Pc^{II} from its reaction with $P700^+$; though a kinetic analysis of this system is possible, it is not as reliable as from a stopped-flow experiment, provided the mixing time in the latter is suitably small. As well, with cyt *bf* complex, the FeS centre reduction of oxidised cyt *f* may be a complication, though the presence of a suitable inhibitor, such as stigmatellin, would tend to simplify the reaction scheme. Experiments using cyt *bf* complex together with other reactants from the same plant are arguably more relevant than those with isolated cyt *f* and heterogeneous reaction partners, but remain to be done.

Results of earlier studies using Pc mutant Tyr83-Leu have been corrected [38] so that the retardation factor for its reduction by cyt *f* is now agreed as 1.4, not 14 as in [34,39]. This means that k_{on} is not as much altered as previously thought by the change at Tyr-83, which is adjacent to an acidic patch on Pc.

5.2. The reaction between Pc and P700

Table 2 lists some results for the Pc/PS I reaction [37,40,41], with a complete description of all the parameters in Eq. 2 above becoming available [42] from the types of experiment already outlined in Section 4.2. In the presence of a high enough concentration of Pc, such that there exists a sufficient fraction of $\{P700\cdot Pc^I\}$ before a flash, the re-reduction signal of $P700^+$ appears biphasic. There is a fast, ca. 10 μs fraction of which the rate (usually called k_1), but not the proportion (A_1) is independent of $[Pc^I]$. The rest of the reduction is slower and its rate (k_2) and proportion (A_2) are both dependent on $[Pc^I]$. An analysis of the rate equations of Eq. 2 can lead to explicit solutions for k_{on}, k_{off} and k_{et} in terms of the parameters got from the biphasic signals, but only after several simplifying assumptions ([40], and Appendix A). Refining these solutions with the aid of

other experiments, Drepper et al. came to the following conclusions (see [42] for details):

1. The values of k^{I}_{on} and k^{I}_{off} did not depend on the oxidation state of P700.
2. When P700 was oxidised, there was a decrease in the binding affinity for Pc^{II} compared with Pc^{I} (K_D was increased); this has the effect of accelerating the release of Pc^{II} after its formation in the complex, in preparation for another turnover.
3. Pc^{II} must leave $\{P700^{+}\cdot Pc^{II}\}$ before that $P700^{+}$ can be reduced.
4. In the complex $\{P700^{+}\cdot Pc^{I}\}$ the driving force is as little as 65 mV, corresponding to a $K_e(=k_{et}/k_{ret})$ of 16, whereas for the free donor and acceptor, it is about 110 mV.
5. There was no kinetic indication of a rearrangement step.

Refinements may be possible, in that usually a small fraction (0.1–0.2) of P700 when oxidised by a flash is reduced slowly or hardly at all in the time of the observation. This residual may look like an equilibrium (since the reverse reaction rate k_{ret} cannot be neglected), but could be an inactive fraction [30,37,40].

Similar data for $P700^{+}$ reduction by Pc have been analysed in terms of Eq. 3 above, to yield kinetic coefficients including those for rearrangement from an inactive to a competent complex [43,44]. Accepting the conclusions cautiously, they are that the rearrangement is rate-limiting, in that k_{rearr} is considerably less than k_{et}, and that the equilibrium constant for the rearrangement is about 4. A Tyr83-His mutation in Pc [43] caused an estimated slower e-t rate to $P700^{+}$ (but less than expected of the change in reduction potential of the mutant), and a considerably diminished k_{rearr}. Relatively small effects were found [43] of Pc mutations Tyr83Phe or Tyr83Leu, suggesting that electron transfer to $P700^{+}$ does not occur through Tyr-83.

In further studies with a comprehensive set of mutants of Pc (the hydrophobic (Northern) area in [45], the acidic patches in [46], the same analysis was presented, to show apparent effects on rearrangement and e-t rates. The general conclusions that both acidic patches are involved in the association between Pc and PS I, and that electron transfer occurs via the copper ligand His-87, agree with other reports (see Section 6.3). The whole of this work on site-directed mutagenesis of Pc is reviewed in [6].

6. Ionic strength effects

6.1. Local charge determines electrostatic interaction

Ionic strength was varied quite early in these studies [32,47] because the interacting proteins were known to have an overall charge, and, as mentioned earlier, enhancement or reduction of collision rates could lead to corresponding changes in observed rate coefficients, depending on the details of the reaction. It was soon realised that local, positive charge on cyt *f* was more relevant than its overall, net negative charge [29,33,47]. Fig. 4 shows an example of such

Table 2
The reaction of Pc^{I} with $P700^{+}$ in PS I particles (ca. 200 chl/P700)

Material	$10^{-7}\times k_{so}$ (M^{-1} s^{-1})	K_d (μM)	10^{-3} k_{et} (s^{-1})	pH	I (mM)	Ref.
Spinach ca. 4 μM P700 150–370 μM Pc^{I}	3.7	170	38	7.5	25	[40]
Spinach 0.29 μM P700 40–50 μM Pc^{I}	4.9	60	58	7.2	50	[41]
Pea 0.25 μM P700 4 μM Pc^{I}	5.0	–	–	7.8	40	[37]

Spinach Pc and PS I particles	$10^{-8}\times k_{on}$		$10^{-3}\times k_{off}$			K_d (μM)			10^{-3} k_{et} (s^{-1})	10^{-3} k_{ret} (s^{-1})	
	I	II	I	II	II^{+}	I	II	II^{+}			
chl *a*/chl *b* = 10	3.5	1.7	2.4	6.7	11	8	40	100	58	4.5	[42]

Flash–oxidation of P700 initiated the reaction in most cases. The re-reduction of $P700^{+}$ was usually observed through ΔA_{702}, or ΔA_{830} which is preferable. I and II refer to complex formation between PC^{I} or PC^{II} and P700; II^{+} refers to complex formation between PC^{II} and $P700^{+}$.

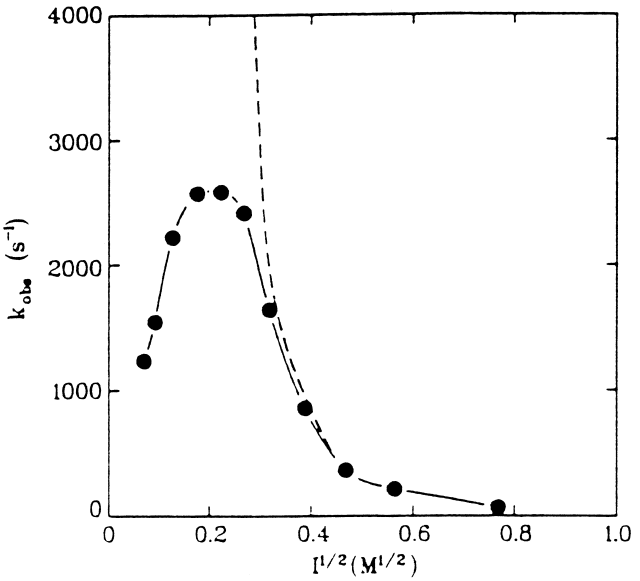

Fig. 4. A plot of k_{obs} against the square root of ionic strength (NaCl added) for a reaction between 14.7 μM soluble spinach cytochrome *f* and equimolar spinach Pc [35]. The dashed line is a fit to data for $I > 150$ mM using the Watkins equation (see Appendix B). Used with permission of the authors and the American Chemical Society.

an experiment. Similar shaped plots have been obtained for the Pc/P700 reaction [48,49]. It is seen that the observed rate varies in a complex way, increasing, then decreasing, as I is increased. If the overall, negative charges on each of Pc and cyt *f* were the determining factors, $z_1 \cdot z_2$ would be positive, and an upward sloping curve would be expected (see Appendix B > for a discussion of the expected relationships between k_{so} and I). The main part of the curve, for $I > 0.1$ M, is only compatible with the product $z_1 \cdot z_2$ being negative, the electrostatic enhancement of the collision rate tailing off as the field is reduced by ion shielding at high I. Hence it was concluded that local charges are the determining factor, positive on cyt *f*, negative on Pc. The later visualisation of the structure of cyt *f* [11,12] (Pc crystal structure was known at the time [10]) was nicely in agreement with the idea of local interacting charges, because several lysines are to be found near the solvent-exposed ligand to the haem in cyt *f*, and the acid patches on Pc were well-known.

6.2. Meaning of the bell-shaped relationship

What of the rates at low I? Now arises the idea of 'rearrangement' [25,35,50,51]. Rates lower than those expected from almost any theory for k vs. I, that is, deviations from the dashed line in Fig. 4, may be explained by supposing that as I is lowered, the electrostatic energy of interaction becomes higher and higher, eventually 'freezing' the complex into a configuration where, due to an unfavourable separation of haem Fe from Cu in Pc, electron transfer is very slow. Meyer et al. [35] estimated k_{et} in complexes of Pc with cyt *f* at $I = 4$ mM as 2800 s^{-1} (cf. 30 000–60 000 for $I = 100$ mM, Table 1). Furthermore, as seen below, some (not all) computer models of the complexes between cyt *f* and Pc agree with the conclusion that 'electrostatic complexes' are in the wrong configuration to support fast intermolecular electron transfer.

An example of experiments where the ionic strength relationship was not bell-shaped has been reported [52] for *Anabaena*, implying no necessity for rearrangement after complex formation, for effective e-t. Rather, this probably meant that k_{rearr} was much greater than k_{et}; in fact the e-t rates were surprisingly low, about 500–700 s^{-1}.

6.3. Studies with mutants

6.3.1. Pc mutants in the Pc/cyt f reaction

The concept of an electrostatic complex resulting from interaction between the acidic patch(es) on Pc with opposite charges on cyt *f* and PS I has been exhaustively probed with the use of point mutations. This follow extensive experiments with chemically modified Pc and cyt *f* [33,47,53,54], and with cross-linked cyt *f*:Pc [55], which, while less specific with respect to the altered residues, all confirmed the importance of the local charges on Pc and cyt *f*.

Pc has been mutated so that the acidic patches were converted to neutral or positively charged residues [36,56]. The mutants and wild-type were then compared in experiments yielding the second-order rate-coefficient in the stopped-flow reaction cyt f^{II}/Pc^{II}. The data as a function of ionic strength were analysed according to the Watkins formulation (Appendix B) to yield k_{so}^{∞} and V_{ii} the interaction energy in units of $k_B T$. Table 3 lists these mutants and key findings and Fig. 5 shows some of the rate-coefficients. In this table, K_a is the binding constant in M^{-1} (see Section 5.1) and k_{so}^{∞} represents the reaction

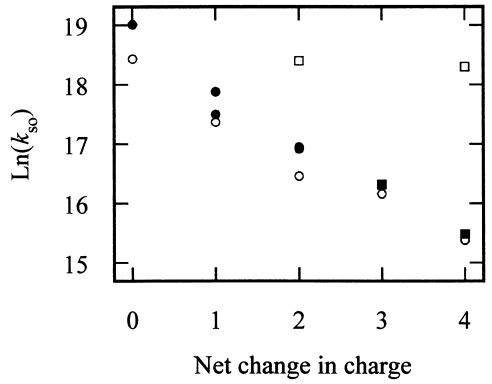

Fig. 5. Data from studies with mutant Pc in reaction with cyt *f*, plotted or replotted from [36,56]. Ln (k_{so}) at pH 7.5 or 8.0 and $I = 100$ mM has been plotted against the number of charges changed on Pc. Open symbols from [56], closed symbols from [36]. Squares are data from mutants of the small acidic patch.

rate in the absence of electrostatic effects, at infinite ionic strength. Since k_{so}^{∞} was relatively unchanged in the mutants, as was the estimated k_{et} [36], and because of the decreases in K_a, it was concluded that the main effect of the mutations was upon k_{on} as expected of altered electrostatic interactions.

Unfortunately, there is unexplained disagreement between the results from mutants of the minor acidic patch at Glu-59, Glu-60; mutations here were indistinguishable in effect from those in the large acidic patch in one study [36], but were more like the wild-type in the other [56]. However, there is agreement that the southern acidic patch on Pc is a key area of interaction between Pc and cyt *f* or PS I in solution.

6.3.2. Pc mutants in the Pc/PS I reaction

When the Pc mutants of Lee et al. [56] were used in the reaction with PS I, the small acidic patch mutants were progressively different from the wild-type, but the decrease in estimated second-order rate per changed charge was less than for mutations in the large acidic area (Fig. 8 in [56]). Incidentally, no specific ion effects of Mg^{2+} were found. Reichert et al. [57] agree that the Southern acidic patch is the more important, with the minor patch providing fine tuning.

7. The effects of varied pH

Studies of pH effects have been sparse, and interpretation has been inconclusive, or partly wrong. Fig. 6 recalls some of these experiments. What is expected? As pH is lowered, acidic residues in Pc (particularly) should become protonated, lowering electrostatically mediated collision rates. As pH is raised, basic residues will become deprotonated, with a similar outcome with regard to collisions. Unfortunately, other effects of pH are present, at least known for eucaryotes: with a pK near 5, the His-87 ligand to Cu^{I} in Pc is broken [58,59], the resultant

Table 3
Mutants of Pc from spinach used in the Pc/cyt *f* reaction

Pc species	K_a	$10^{-6}\ k_{so}^{\infty}$	V_{ii}	Ref.
Wild-type	–	2.7	−12.5	[56]
	7100	4.1	−13.1	[36]
Gln88Glu	15040	3.7	−42.0	[36]
Asp42Asn	–	2.2	−9.3	[56]
	2650	4.2	−14.9	[36]
Glu43Asn	2860	3.3	−11.7	[36]
Glu43Lys	–	2.4	−5.7	[56]
	660	3.3	−8.5	[36]
Asp42Asn/Glu43Lys	–	2.1	−5.4	[56]
Glu43Lys/Asp44Lys	–	1.5	−4.5	[56]
Glu43Gln/Asp44Asn	700	1.8	−6.5	[36]
Glu59Lys	–	2.9	−11.6	[56]
Glu59Lys/Glu60Lys	–	2.7	−11.7	[56]
Glu59Lys/Glu60Asn	312	3.9	−7.0	[36]
Glu59Lys/Glu60Gln/Glu43Asn	–	3.3	−2.1	[36]

Data on k vs I were analysed to yield the parameters listed in columns 3 and 4.

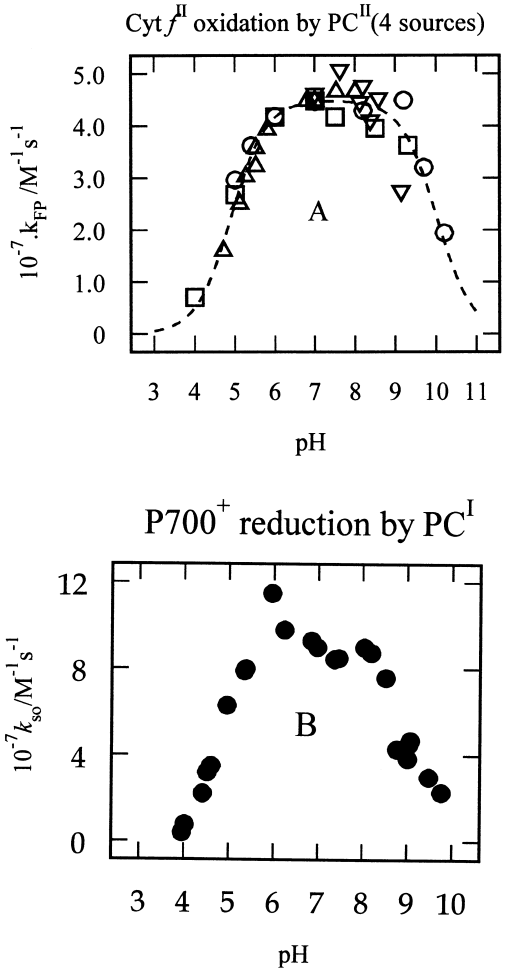

Fig. 6. A collection of representative data on the effect of varied pH on the rates of reactions between Pc nd cyt *f* or PS I. (A), from [62], shows the second-order coefficient for the oxidation of cyt *f* by Pc, from four studies; data were normalised around 4.5×10^{7} M^{-1} s^{-1}. (B), also from [62], is for the reduction of $P700^{+}$ by Pc. Used with the permission of the publishers of Australian Journal of Plant Physiology.

structure having a raised redox potential and therefore more difficult to oxidise; the reduction of Pc^{II} is, however, unchanged over this pH range. An effect of pH > 9 on a haem ligand in cyt *f* has been reported [60], the redox potential of Fe^{III} becoming more positive. Finally, at pH below about 5.5, PS I particles have been reported to coagulate [61], but this was not apparent until pH < 4.5 in our experiments [62] in which Mega-9 detergent was present.

Rather wilfully, we [62] chose to interpret effects of lowered pH on Pc/cyt *f* and on Pc/PS I reactions as due to protonation of the local negative charges on Pc, causing diminished k_{on}, and of raised pH as the results of the deprotonation of positive residues in cyt *f* and PS I. Sigfridsson [48] observed small effects of pH between pH 4.5 and 8.7 on the reaction between Pc and PS I from spinach, and only minor changes in rate k_2 in mutants Thr79His and Lys81His compared with wild-type. The discussion inclined towards the protonation/deprotonation explanation, but was aware of the pH sensitivity of the His Pc ligand.

Some progress was made in disentangling the pH effects on Pc/PS I [49] when it was found that with pea-derived Pc and PS I, the slopes of ln k_{so} versus $I^{1/2}$ were similar at pH 7.5 and 5.05, suggesting unchanged $z_1 \cdot z_2$. This means that the p*K* for protonation of the acidic patch(es) on pea Pc is lower than 5.05. By contrast, at pH 8.6 there was an indication of less positive charge on PS I. pH could also affect the structure of the activated complex and hence k_{et}.

With *Synechocystis* PCC 6803 [63], where acid residues do not dominate (see Section 9), there were similar pH effects (pH 4–8) on $P700^{+}$ reduction by Pc to those seen in peas [62].

The observed rate-coefficient in the reaction between oxidised P700 from spinach with Pc or cyt c_6 from *Anabaena* [52] showed a decrease going from pH 8 to 5.5, with an apparent p*K* of 7.0–7.2, quite different from when reactants are both from higher plants (above). A tentative explanation is given later in the comparison between Pc and cyt c_6.

More study of pH is needed.

8. The structure of the encounter complexes

8.1. Pc with cyt f

Several approaches to envisaging the structure of the bimolecular complexes formed between Pc and cyt *f* have been made in recent years. The complex between Pc and PS I has been thought less accessible because the crystal structure of PS I is not sufficiently defined, although several medium resolution structures have been published [64,65]. When the concept of electrostatic interaction between Pc and cyt *f* was clear and acceptable, and the molecular structures of both solved, modelling of possible complexes commenced.

8.1.1. Visual pre-docking, electrostatic energy minimised

Visual docking, with prevention of overlap at Van der Waals radii, was done by Pearson et al. [66] and Soriano et al. [67], with various criteria for successful docking, such as minimum distance between key opposing residues. The electrostatic fields around Pc and cyt *f* have been computed [66–68] using the programs *Grasp* [69] or *DelPhi* [70]. One probable structure, named DC1 in [67], was manifestly a close association between the acid patches of Pc and the basic residues on the surface of cyt *f*, in the region of the N-terminus and the haem (see Fig. 2). Such configurations where a minimum in the total electrostatic energy appears to have been reached will be referred to as 'electrostatic complexes'. In structure DC1 the shortest path for electrons between Cu in Pc and the haem Fe in cyt *f* was about 2.4 nm. If this structure were to be the 'final' one before electron transfer occurred at activation, the rate would be low, possibly of the order of 1 s^{-1}. Other manually docked structures named DC2 and DC3 [67] were arrived at, with Cu–Fe distances of 1.4 to 1.7 nm. These had no close contact between oppositely charged residues.

8.1.2. Brownian motion, electrostatic forces

In [71] Brownian motion, guided by the known electrostatic forces, was simulated to attempt to guide molecules to probable docking situations. The (rigid) molecules were initially placed 8.9 nm apart with Pc mobile, cyt *f* fixed. The frequency of occurrence of 'end' structures such as those with 'triplet contacts' was noted. Simulation of the effect of ionic strength was achieved through its relation with the electric fields surrounding the molecules. A 'solvent' presence was included through the assumption of the value for the dielectric constant of the space between the molecules. An examination of the structures of the dominant (more probable) complexes showed that they were rather similar (Fig. 5 in [71]), the conclusion being that simply electrostatic forces were sufficient to guide Pc into a single preferred conformation with cyt *f*, one that might be suitable for electron transfer. Thus the average Cu–Fe distance was about 1.7 nm for $I = 50$ mM. A further outcome of this study included estimations of second-order rate coefficients at various ionic strengths; for example, ca. 7×10^8 M^{-1} s^{-1} at $I = 50$ mM, a little on the high side of observations (Table 2 above).

8.1.3. Molecular dynamics, total energy minimisation

More realistic simulations should include other interatomic forces, those in hydrogen bonds, and hydrophobic and Van der Waals forces, in addition to electrostatic ones. Molecular dynamics (MD) methods (e.g. see [72,73]) include all these factors in the 'force fields' used. In such simulations, the motion of each atom is considered, which in protein molecules may be unworkable, because of the very large number of tiny time intervals necessary before the total energy reaches a valid minimum, suggesting a probable, stable structure. Ullmann et al. [74] have modeled the motions of Pc and cyt *f* without restricting intramolecular motion (i.e. the interacting molecules were not enforcedly rigid except during pre-docking) and included thousands of water molecules between Pc and cyt *f*. Six pre-docking configurations were found by a Monte Carlo procedure. These configurations were chosen from those with the lowest electrostatic energy of interaction. Full molecular dynamics conditions were then used on these six likely conformations, and water introduced explicitly. MD run time was 260 ps, followed by energy minimisation. Finally, calculations were made of the total energy of interaction of these 'final' structures, called A–F. Estimations were obtained of the relative (electronic coupling)^2 with respect to electron tunnelling from Cu to Fe, in the six configurations, using the *Pathways* method [75].

Which, if any, is a likely electron transferring complex? Are any of the complexes interconvertible (their energies being similar)? Table 4 lists some of the relevant properties of A–F. We see that configurations A, B, C and F are not suitable for fast e-t because of the separation, > 3 nm, of Cu and Fe. Complex E with the highest total energy $-\Delta G_t$ and with $R_{Cu-Fe} = 2.0$ nm is promising but the best electronic tunnelling path in complex D (1.4 nm) has a (relative coupling)^2 which is orders of magnitude greater than E. Fig. 7 shows these two structures, the authors proposing that E rearranges into D by an easy twist and a short translation through space. The difference in total energy between E and D, depending on certain assumptions, is 22–60 kJ/mol if

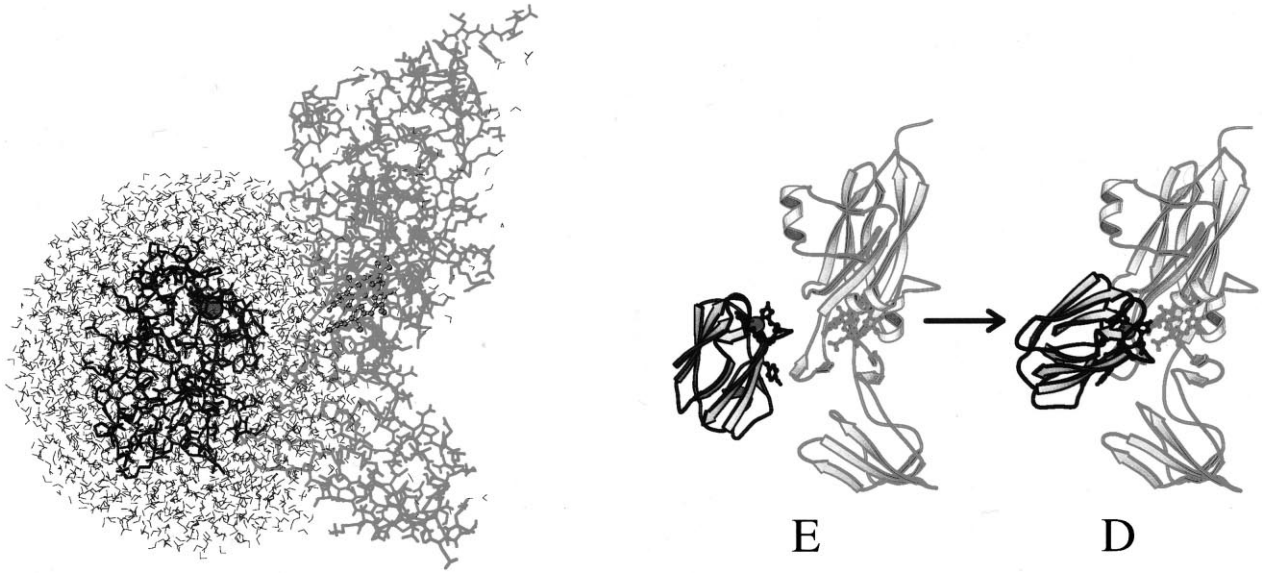

Fig. 7. Left: computed structure D of a complex of Pc with cyt *f*, which includes water of hydration around the proteins. Right: complex E is shown translating to complex D. See Section 8.1 for a discussion of the means used to compute the structures and of their suitability in electron transfer. Used from [74] with permission of the authors and the American Chemical Society.

the protein dielectric constant is 4, 100–140 if 2 (said to be similar for $I = 100$ and 300 mM); these energies are considerable compared with an RT of 2.3 kJ/mol at 295 K, raising the question of the probability of that particular rearrangement.

Three other matters are to be noted from this study. It was concluded that the likely electron path in D was from Cu–Cys-84–Pro-86 to haem propionate in cyt *f*; for complex E, a lesser path involving a cation-π interaction between the aromatic ring of cyt *f* Tyr-83 and Lys-65, etc, in cyt *f* was mooted, but the cation-π interaction was later discarded (G.M. Ullmann, personal communication). Secondly, upon forming a complex such as D, a small reorientation of a β-sheet in cyt *f* was noted, contrasting with no detectable changes at all from the solution structure of Pc. Structural and spectral changes to Pc, attributed to changes in the active site Cu–cysteine geometry occurred upon complexing with lysine peptides [76], while complexing cyt *f* with aspartic acid peptides [77] led to unattributed changes to spectra and reduction potential, the latter towards easier oxidation in the adduct, compared with in solution.

8.1.4. A test-tube complex

Ubbink et al. [78] created physically tangible Pc/cyt *f* complex by mixing millimolar concentrations of

Table 4
Some relevant properties of six model complexes between plastocyanin and cyt *f*, in the theoretical study of Ullmann et al. [74]

Complex	ΔG_E (kJ/mol)	ΔG_T (kJ/mol)	Best-path coupling[a]	R_{Cu-Fe} (nm)
A	−1368	−1488	310	3.4
B	−1863	−1957	8.1	3.1
C	−1717	−1833	7.6	3.7
D	−1718	−1875	2.4×10^{12}	1.4
E	−1872	−1979	2.8×10^{7}	2.0
F	−1613	−1712	44	3.5

The energies refer to E for electrostatic and T for total.
[a]Given as $10^{20}\times$(relative coupling)^2, for the choice of 'hydrated+isotropic metal ligand covalency+aromatic ring coupling parameter of 1.0': see [74].

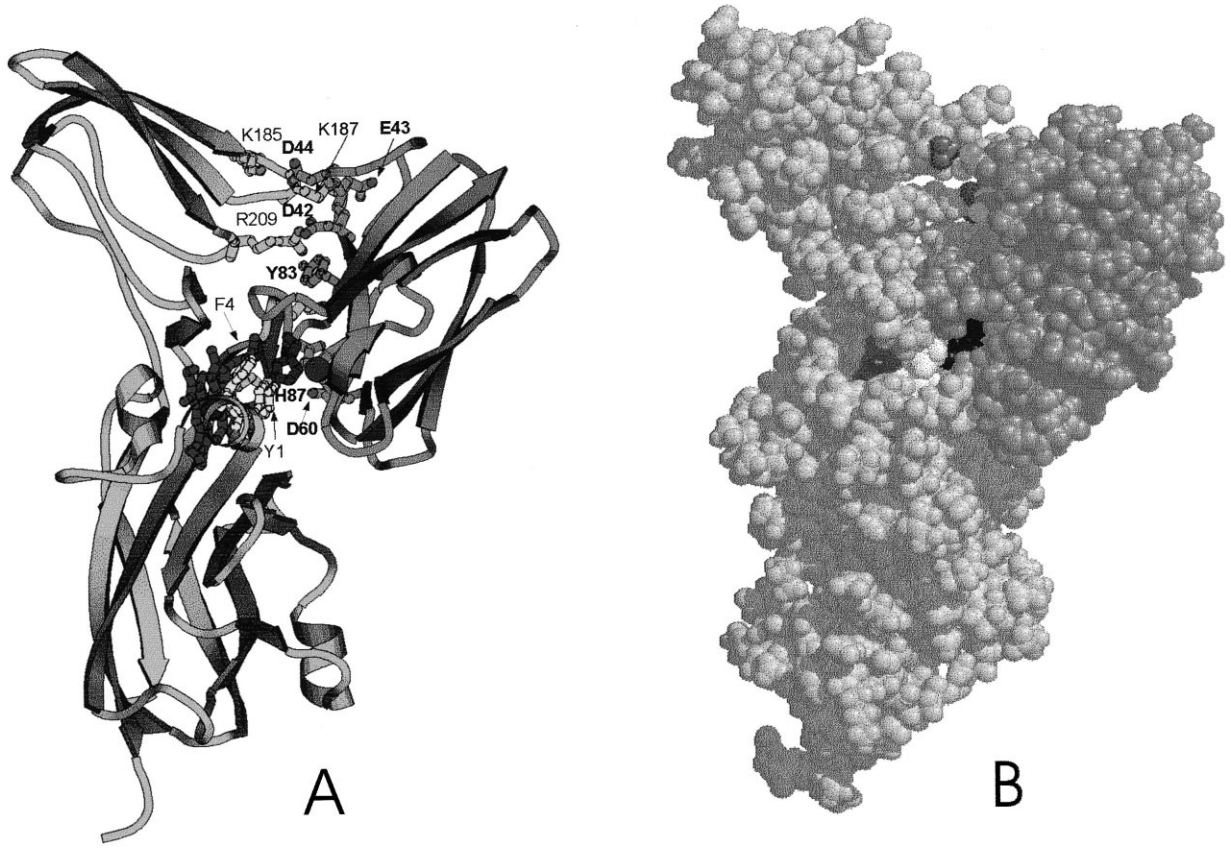

Fig. 8. Molecular diagram of a complex between Pc and cyt *f* developed by solving the structure of biochemically formed adduct between equi-millimolar partners. The labels are similar to those in Fig. 2. Used from [78] with permission of the authors and publishers of Structure.

^{15}N-enriched spinach Pc with soluble turnip cyt *f*, at ca. 40 mM ionic strength. Then the ensuing structure was solved by a combination of NMR techniques, rigid-body molecular dynamics and restrained energy minimisation. The result was striking: as seen in Fig. 8, the negatively charged residues Asp-42–44 on Pc are close to basic Arg-209, Lys-187 and Lys-185 of cyt *f*, reminiscent of an 'electrostatic complex', while His-87 of Pc is adjacent to Tyr-1 of cyt *f*, thus fulfilling the requirement of facile e-t as well. Why such a structure did not emerge from modelling studies, such as that of Ullmann et al. [74] is not clear; it is stated as being close, but not identical, to their configuration D above, but with an average Cu–Fe distance of 1.09 nm.

When Pc and cyt *f* form a close association, does water separate the residues or do ion pairs form between oppositely charged residues? Ullmann et al. ([74] and see [79]) proposed that hydration energies would be greater than those of ion pairs, such that water would not in fact be squeezed out at any such points of contact. However, in model D, Van der Waals contact exists between cyt *f* haem propionate and Pro-86 in Pc, which appears to mean that water can be excluded at those points.

8.2. Pc and PS I: docking and rearrangement

Low values for the second-order rate for Pc reacting with highly purified PS I [80,81], omitted from Table 2, raised the suspicion that a subunit, missing from that PS I preparation, was essential for efficient electron transfer [82]. For higher plants, ionic strength and other studies are clear in that PS I must have localised positive charge. The PsaF subunit in PS I has been found to contain at the N-terminal a series of basic amino acids (Lys-12, Lys-16, Lys-19, Lys-23) that recognise the acidic area (the

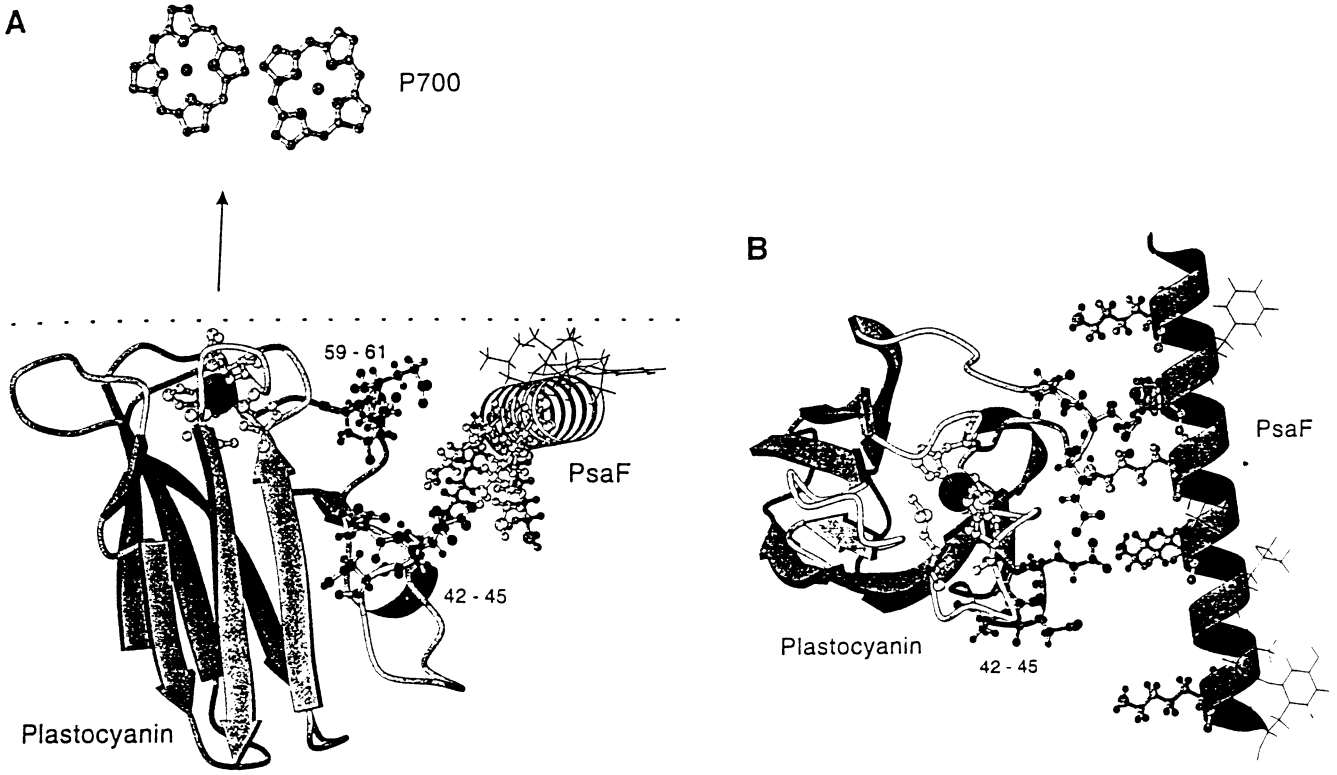

Fig. 9. Model of the interaction between Pc and the PsaF binding domain in PS I of eucaryotes. (B) The major acidic area of Pc in proximity to positively charged residues on an amphipathic helix of PsaF. (A) shows another view, with the flat hydrophobic (Northern) patch of Pc in close proximity to the P700 dimer. Used from [83] with permission of the authors and the European Molecular Biology Organisation.

'Southern' patch) on Pc [83,84]. Kinetic studies of wild-type and mutants [85] have elegantly confirmed the requirement of PsaF for high rates of electron transfer between Pc and PS I in higher plants and algae (here *Chlamydomonas*). A requirement for the PS I *N* subunit (lumen-located) has been reported for 'efficient' electron transfer from plastocyanin [86]; transformants lacking PSI N had a k_{so} 55% of that in wild-type. This requirement may have little to do with docking; the criterion for a residue or subunit to be essential in that respect should be a large diminution of the rate-coefficient; for example, a change in one charged residue could result in a change in rate of more than 2× as in Fig. 5.

The 'electrostatic complex' envisaged for Pc/PsaF is from the point of view of fast internal e-t a predocking one; rearrangement, as suggested from the ionic strength experiments (above), possibly by one-dimensional diffusion along one of the PsaF helices, could bring the Pc Cu ligand His-87 to within a healthy 1.45 nm edge-to-edge distance from P700 [83]. Fig. 9 illustrates this idea. The ensuing hydrophobic interaction had already been proposed following mutant studies [41] in which leucine at Ala-90 or Gly-10 of Pc was said to prevent complex formation, the fast 12–15 μs component of $P700^+$ reduction being absent. This conclusion was later modified [57] in that a stable complex between Ala90Leu and PS I was obtained but that inter-protein electron transfer was altered.

The low rates when algal Pc is offered to cyanobacterial PS I are increased by the insertion of a PsaF-like ending into the native cyanobacterial PS [87]. The comparison with cyanobacteria, and the contrast of cyt c_6 with Pc, follow below.

9. Comparison of Pc with cyt c_6, and of procaryotes with eucaryotes

When there is insufficient Cu, some algae and cyanobacteria express cytochrome c_6 instead of Pc (see

[88]). It acts analogously to Pc, ferrying electrons from cyt *f* in the *bf* complexes to oxidised P700 in PS I. The solution structure of this cytochrome from the green alga *Monoraphidium* [89] and from the cyanobacterium *Synechococcus* [90] has been determined. Sequences and structural aspects, together with conjectures about evolution, have been reviewed [91]. In *Synechococcus elongatus*, cyt c_6 is the sole carrier expressed. Its oxidation by the reaction centre does not need the PsaF gene product in photosystem I [92], despite the inference of a negative area which interacts with the cytochrome.

Here attention is concentrated on electron transfer properties, studied extensively by the Spanish group [27,52,61,63,91,93–95]. The same shape of the ionic strength relationship with k_{obs} was found for the reaction between PS I particles and either Pc or cyt c_6 from *Monoraphidium* [61]. This is consistent with negative charge on both of these donors being recognised by positive residues on PS I. This was the case whether Na^+ or Mg^{2+} was used to alter *I*, though specific ion effects were apparent. The plots resembled that in Fig. 4 above. The estimated rate coefficients using cyt c_6 were generally about half of those for Pc at pH 7, but were more similar at pH 5.5.

When similar observations were made with the cyanobacterium *Anabaena*, Table 2 in [52], the ionic strength data implied that $z_1 \cdot z_2$ was negative, both when the oxidising component was PS I from spinach and from *Anabaena*. However, the effect of added ions on the spinach PS I/Pc (or c_6) reaction was quite modest. Since spinach PS I has a positive patch, it was concluded that the cyanobacterium PS I did, also. However, the net charge on *Anabaena* Pc or cyt c_6 at neutral pH is also positive (unpublished data quoted in [52]), which suggested that a different local, negative patch on either of these donors interacts with the PS I particles. This was an error, the present interpretation being as follows: both Pc and c_6 from *Anabaena* have in fact *basic* patches, lysines, in the areas corresponding to the minor acidic patches on the 'Eastern' side of higher-plant Pc (see Fig. 2 in [91]). The recognition site on *Anabaena* PS I has a negative patch. The weak interaction of *Anabaena* Pc with spinach PS I was the result of Pc finding one or more negative residues probably not on the PsaF subunit. The interaction was unfavourable, with a k_{obs} of only 20 s^{-1}, compared with about 200 s^{-1} when both reactants were sourced from *Anabaena*.

Synechocystis forms an interesting contrast with *Anabaena* [63]. There was no detectable ca. 10 μs phase in the reduction of oxidised P700. The plot of k_{obs} vs. donor protein concentration at pH 7 was linear over a large range for both Pc and cyt c_6 (and see [92] for PS I/cyt c_6 in *Synechcoccus*). The rates increased with *I*, indicating that $z_1 \cdot z_2$ was positive-screening of local charges by added ions encouraged a faster 'on' rate. The absence of a fast phase and lack of observable maximum in the relation between k_{obs} and [*D*] mean that K_d for the formation of the initial complex is much greater than the highest concentration of *D* employed, 300 μM, so the concentration of complex in the experiments would have been very small. The authors [63] concluded that e-t involved "a simple bimolecular collisional mechanism" (Eq. 2 above). As we have reiterated, this is apparent only, in that Eq. 3 might describe the observations under different conditions; for example, concentrations in vivo might be more like K_d.

The contrasts between spinach, *Monoraphidium* and *Anabaena*, and all accessible or calculable rate-coefficients have been reviewed [27,93], and used to categorise into 'mechanisms'. Some of the calculated rates have to be treated with caution, since the common assumption of irreversibility of the electron transfer step within a rearranged complex is unwise. Thus, the relation between the fraction of any fast phase and e.g. K_{rearr} will be inaccurate. As expressed elsewhere in the review, there is doubt that the kinetic coefficients relating to rearrangement have been reliably calculated from existing data.

Experiments with mutants of Pc from *Synechocystis* PCC 6803 on $P700^+$ reduction [95] form the first such study using a cyanobacterium. Pc from this organism is overall almost neutral. Ionic strength experiments showed the wild-type Pc and PS I as having the same sign of charge (the rate increased with *I*) but the increase was very modest between $I^{1/2} = 0.2$ and 0.6, indicating a small $z_1 \cdot z_2$. In mutant Asp44Lys, the rate decreased with ionic strength, indicating that a net change of +2 in the charge on Pc altered its interaction with PS I to an attractive one. With mutant Asp44Arg/Asp47Arg, the ionic strength

plot was bell-shaped, with a decrease in rate after $I >$ ca. 0.1 M. Thus, the recognition site on PS I is negatively charged; the site may be in the PsaA/PsaB heterodimer as suggested in [65]. With this mutant, rearrangement is inferred towards an effective e-t configuration. The same remarks apply as made previously, about the distinction between kinetic behaviour and mechanism.

With *Pseudoanabaena* PCC 6903 [94], the kinetics of the PS I/Pc reaction were said to follow Eq. 2, in spite of the fact that rearrangement could be implied from the bell-shaped ionic strength relation. For the PS I/c_6 reaction, Eq. 3 was indicated because k_{obs}, extrapolated to infinite [cyt c_6] was two to three times smaller than 'the experimental value of k_{et}' (from the fast component, which is an approximation only). Since all except k_{obs} are coefficients derived through possibly faulty relationships, this conclusion is suspect, although the reality may be Eq. 3.

10. The contrast between in vitro and in vivo situations

Studies using *Chlamydomonas* cells genetically engineered with altered cyt *f* have raised serious doubt that an electrostatic pre-docking complex is necessary for fast e-t between cyt *f* and Pc in intact cells. Thus, the observed pseudo-first order rate-coefficient for flash-induced cyt *f* oxidation was diminished only mildly in cells where the 'docking lysines' of cyt *f* had been replaced by neutral or negatively charged residues [9,96]. The extreme range of the half-times appeared not to be more than a factor of two (my reanalysis taking into account both the rise and fall kinetics, which does not alter the conclusion), between wild-type on the one hand, and cyt *f* with a net change in charge of −6. The expected change in rate of cyt *f* oxidation was stated as 100×, from solution experiments. There was an apparent decrease in the rate of re-reduction of oxidised cyt *f* of the same factor, 2, in corresponding cell types.

The use of nebulised and permeabilised *C. reinhardtii* cells [97] showed a factor of 10× diminution of the oxidation rate of a four-fold mutant cyt *f* compared with wild-type; the re-reduction rates were unchanged in the corresponding cells, in disagreement with the experiments described above [96]. With intact cells, there was a lesser range of rates of photo-oxidation, about 2×. It is difficult to compare these experiments without knowing the prevailing ionic strength and pH in all cases. For the ionic strength of the thylakoid lumen, a value of 0.4 M is quoted in [9], but we [98] estimated about 0.2 M from ionic shift experiments; the correct way to estimate I is debatable when electric double layers overlap as they may do in this phase.

Several possible explanations have been offered for the unexpected absence of changes in rate upon altering the lysine complement of cyt *f* [9]. The limitation of diffusion of Pc in the restricted dimensions of the lumen of thylakoids has already been noted. It is thus possible that rearrangement from electrostatic complex to electron-transferring complex is much slower than in solution and becomes rate-limiting. In terms of Eq. 3 in Section 4.1, k_{rearr} is much smaller than k_{et}. If permeabilised cells [97] were to have an expanded lumenal space and hence less diffusion limitation, it might account for the more noticeable effect of removal of lysines of cyt *f*.

Another possibility to explain the absence of rate-limitation through electrostatic docking was suggested [9] to be partial occlusion of lysines by the Rieske subunit in cyt *bf* in vivo. A third possibility, that Pc tends to bind or adsorb to the thylakoid membranes, might be expected to affect both k_{on} and k_{rearr}, so rate-limitation is not clear. More study of intact cells is warranted.

11. Concluding remarks

It is concluded beyond reasonable doubt that in vitro, and in higher plants and algae, Pc acidic patches are attracted to positive residues both on cyt *f* and PS I by long-range electrostatic forces to form an 'electrostatic complex' which must undergo rearrangement before a suitable configuration exists for activation, leading to inter-protein electron transfer. There is doubt that the same sequence happens in vivo, but further experiments are necessary. Rearrangement rate should depend on ionic strength amongst other things.

In the rearranged complex, e-t occurs from cyt *f* to Pc from Fe through Tyr-1 or Phe-4 to His-87 to Cu. Several valid models exist of the molecular shape of the effective complex between Pc and cyt *f*; one of

these [78], is based on the structural determination of a biochemically produced complex rather than an entirely computed structure [74], and might be supposed the most valid.

Electron transfer from Pc^I to $P700^+$ in eucaryotes takes place in a complex between Pc and the PsaF subunit of PS I, after rearrangement from an initial electrostatic complex to form additional, hydrophobic associations of the Northern area of Pc with P700.

A more general theory relating kinetic data to ionic strength is needed. Kinetic analyses of redox data need refining to exclude simplifying assumptions about the reversibility of the electron transfer step and absence of changes in association parameters when a complex is formed.

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Appendix A. Kinetic schemes, the relation between observables and kinetic coefficients of the schemes

Is it possible to relate observed quantities, such as k_1, k_2 and the fractions of fast and slow components of a P700 reduction curve to the kinetic coefficients in Eq. 2 or Eq. 3? The answer is that it is not generally possible without simplifying assumptions. What then is the validity of the approximate solutions obtained? Put another way, what is the error involved in a particular approximation?

(a) The steady-state approximation. An earlier approach was to assume a steady-state for $\{D{\cdot}A\}$, such that its formation and conversion to $\{D^+{\cdot}A^-\}$ had equal rates. Alternatively, it might be assumed that rates relating to intermediates are small compared with those for reactants and products. It is simple to show with a simulation that the first assumption of a true steady-state cannot be generally true either in stopped flow or flash experiments, where the intermediate complexes will wax and wane during measurement. The extent of the error has not been determined.

(b) Explicit solutions for the relation between the observables and k_{on}, K_d, etc. were obtained by Nordling et al. [40] and further discussed by Drepper et al. [42]. It was assumed that: (1) the reductant Pc was much in excess of P700; (2) the electron transfer step was irreversible; and (3) the dissociation constant K_d was the same for complexes of Pc with either of P700 and $P700^+$. Then, relationships similar to the following are true:

$$k_{on} = (k_1k_2/[\mathrm{Pc}])/\{k_1 + k_2 - [k_1^{-1} + A_1\ (k_2^{-1} - k_1^{-1})/ (A_1 + A_2)]^{-1}\} \quad \text{(A1)}$$

$$k_{off} = [k_1^{-1} + A_1(k_2^{-1} - k_1^{-1})/(A_1 + A_2)]^{-1} - k_{on}[\mathrm{Pc}] \quad \text{(A2)}$$

$$k_{et} = k_1k_2/k_{on}[\mathrm{Pc}] \quad \text{(A3)}$$

A simulation of Eq. 2 in Section 4.1 was done, using likely rate-coefficients (from [42], and with $k_e/k_{ret} = 5$, [Pc] = 40 μM). By fitting exponentials to the output for $[P700^+]_t$, and allowing for a residual due to slow reduction by ascorbate, values for k_1, k_2, A_1 and A_2 were extracted. When these were inserted into (Eqs. A1–A3), it could be seen that k_{on} and k_{off} are underestimated, k_{off} by a large factor; k_{et} is overestimated by 20%. These figures are illustrative only, and depend on the starting parameters.

In [42], the authors worked around the approximations using independent data to reach more reliable estimates. The procedure, which is probably the only recourse at present, was to match data with simulations obtained by solving the appropriate differential equations with successively optimised parameters.

Can the rates of rearrangement be estimated (Eq. 3)? Can Eq. 3 be distinguished from Eq. 2? Three criteria have been cited [43–46] as indications that Eq. 2 is too simple, and that Eq. 3 is a better description of the data. Criterion 4 can justly be added:

1. The proportion of the fast component in the biphasic re-reduction curve for $P700^+$ ($A_1/(A_1+A_2)$) saturates at less than 1.0, as [Pc] is increased.

2. Three kinetic components (one of which is slow reduction by ascorbate) persist even at high concentrations of Pc.
3. The value of k_2, at a saturating concentration of Pc, falls short of the expected value k_{et}.
4. An optimum exists in the rate versus ionic strength relationship (see Section 6.2 above).

As to criterion 1, this amplitude ratio also saturates at <1.0 in Eq. 2 (my simulation) provided the reverse rate in the e-t step is finite. Criterion 2 may be difficult to apply; as the concentration of Pc is increased, the rate k_2 increases to be comparable with k_1, so reliable separation of exponentials becomes difficult, even in the simulations, and more so since data are never noise-free.

In analyses of experimental data, k_{ret} has mostly been ignored. If it is a small fraction of k_{et}, the error may be small. As a general comment, it is difficult to separate the rate-coefficients of two reversible reactions in series, especially if the rates are similar. Independent estimates of k_{rearr} and k_{et} are desirable.

Appendix B. The relation between k_{so} and I

We start with a common theoretical relationship with which to illustrate the possible effects of ionic strength on reaction rate, the Debye–Marcus equation [99]:

$$\ln k_{so}(I) = \ln k_{so}^{\infty} - (z_D z_A e^2/8\pi\varepsilon\varepsilon_0 k_B T R_{DA}) \left[\frac{\exp(-\kappa R_A)}{1+\kappa R_D} + \frac{\exp(-\kappa R_D)}{1+\kappa R_A}\right] \quad \text{(B1)}$$

where k_{so}^{∞} is the rate at infinite ionic strength, when electrostatic influence is negligible; Eq. B1 also contains the charges and radii of donor and acceptor, the radius of the transition state, ε the dielectric constant of the space separating D and A, and standard physical constants. Ionic strength is hidden in κ which in SI units is

$$= \left(\frac{2000\, N_A e^2}{\varepsilon\varepsilon_0 k_B T}\right)^{1/2} I^{1/2}$$

;strictly, I is in molal, not molar, but the difference is usually small. If the charges have the same sign, the expected relationship is k_{so} increasing with $I^{1/2}$, as shown in Fig. B1. The trace for oppositely charged reactants appears as a decrease with ionic strength or its square root.

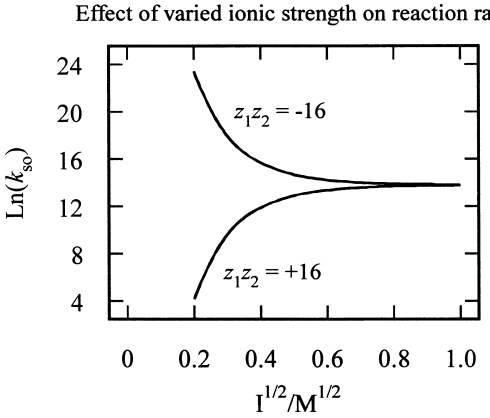

Fig. b1. The general shape of the relation between rate coefficient and ionic strength for reactions between oppositely charged redox partners (top) or those with the same sign (bottom) was computed from the Debye–Marcus (Eq. B1) with $k_{so}^{\infty} = 10^6$, $R_D = 0.6$ nm, $R_A = 0.7$ nm, $\varepsilon = 10$, and the product of charges as indicated. The curves converge on ln (k_{so}^{∞}).

The use of relationships such as the Brønsted–Debye–Hückel [8] or the Debye–Marcus equations (for others see [53,100]) is thought to be poorly justified since they were developed for uniformly charged spheres. By restricting the charge effects to local regions where electrostatic interaction was thought to occur (e.g. the acidic areas of Pc with the basic ones of cyt *f*), and adopting an approximation to simplify the physics, Watkins et al. [101] developed a 'parallel-plate' model which has been extensively used to analyse ionic strength data. It applies to the diffusion-controlled situation and is expressed in Eq. B2:

$$\ln k_{so}(I) = \ln k_{so}^{\infty} - V_{ii}\exp(-\kappa\rho)/(1+\kappa\rho) \quad \text{(B2)}$$

where V_{ii} is energy of interaction expressed as a multiple of $k_B T$, and equals $\alpha z_D z_A r_{DA}/\varepsilon\rho^2$ with the constant $\alpha = e^2/8\pi^2\varepsilon_0 k_B T = 9.0\times10^{-9}$ at 295 K, not 128.5 (Å) as given in [101]. The other parameters are: r_{DA}, the separation of the model parallel plates, ε the dielectric constant in that space, and ρ the radius of the parallel plates. The energy term above is for ion–ion interactions only. Contributions from ion–dipole or dipole–dipole interactions [22] could be added; such interactions were suspected as contributing to the failure of conventional theories to ac-

count for the complex shape of ionic strength plots (Fig. 4), but the addition of more parameters to improve the fit to a single curve has not proved useful [101].

To avoid the approximations associated with Eq. B2, it should be possible to use Eq. B3, representing the 'charge configuration' model [101], with the known, individual charges and their positions in the interacting proteins, to calculate the relation between k_{so} and I, taking into account all electrostatic interactions:

$$\ln k_{so}(I) = \ln k_{so}^{\infty} - \alpha \left\{ \sum_{n=1}^{N} \sum_{m=1}^{M} z_n z_m \exp(-\kappa R_{n,m}) / [\varepsilon_{n,m} R_{n,m} (1 + \kappa R_{n,m})] \right\} / k_B T \quad \text{(B3)}$$

However, the complexity introduced by the many values of ε and deciding the cut-off distance beyond which interaction is negligible, has also not made this approach successful (in our hands).

Fitting data with the Watkins equation involves optimising the parameters r_{DA}, ε and ρ or adopting reasonable values. Then, usually, $\ln k_{so}^{\infty}$ and V_{ii} will be returned by the curve-fitting routine; ρ has often been fixed at 0.7 nm, r_{DA} at 0.35 nm, and ε at 10; ρ may be an optimised parameter when, as in the case of mutated charged residues, the radius of the charge interaction area might vary. The analyses referred to in the text have universally neglected ion–dipole and dipole–dipole energies for the reason already given.

In some reports, enough confidence in the assumed dimensions and dielectric constant has led to extraction of $z_1 \cdot z_2$ from V_{ii}. The maximum, or optimum in the observed k_{so} means that the choice of points to which to fit the Watkins equation is somewhat arbitrary, possibly leading to large errors in estimation of V_{ii}.

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