Conformational Equilibria Accompanying the Electron Transfer between Cytochrome *c* (P551) and Azurin from *Pseudomonas aeruginosa*†

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ABSTRACT: The redox reaction between cytochrome *c* (Cyt *c*) (P-551) and the blue copper protein azurin, both from *Pseudomonas aeruginosa,* was studied using the temperature-jump technique. Two relaxation times were observed in a mechanism assumed to involve three equilibria. The fast relaxation time ($0.4 < \tau < 8$ ms) was ascribed to the electron exchange step. The slow relaxation time ($\tau \simeq 37$ ms) was assigned to a conformational equilibrium of the reduced azurin that was coupled through the electron exchange step to a faster conformational equilibrium of the oxidized Cyt *c* (P551). But because the Cyt *c* (P551) isomerization, being very rapid, was uncoupled from the two slower equilibria, and was assumed to involve no spectral change, the amplitude of its relaxation time ($\tau \simeq 0.1$ ms) would be zero. At 25 °C and pH 7.0 the rate constants for the oxidation and reduction of Cyt *c* (P551) by azurin were 6.1×10^6 and 7.8×10^6 M^{-1} s^{-1}, respectively; for the formation and disappearance of the reactive conformational isomer of azurin they were 12 and 17 s^{-1}, respectively. The rates for the Cyt *c* (P551) isomerization could only be estimated at $\sim 10^4$ s^{-1}. The thermodynamic parameters of each reaction step were evaluated from the amplitudes of the relaxations and from Eyring plots of the rate constants. Measurements of the overall equilibrium constant showed it to be temperature independent (5–35 °C), i.e. $\Delta H_{tot} = 0$. This zero enthalpy change was found to be compatible with the enthalpies calculated for the individual steps. In the electron exchange equilibrium, the values of the activation enthalpies were two to three times higher than the values published for various low molecular weight reagents in their electron exchange with copper proteins, yet the rate of exchange between Cyt *c* (P551) and azurin was some hundreds of times faster. This was explained in terms of the measured positive or zero entropies of activation that could result from a high level of specificity between the proteins particularly in areas of complementary charges. The mechanism of electron transfer was considered as essentially an outer sphere reaction, of which the rate could be approximated by the Marcus theory.

Small, sequenced, and well characterized, the cytochrome *c* (P551) and the blue copper protein azurin comprise a simple element in the respiratory system of *Pseudomonas aeruginosa*. Studies of the electron transfer between proteins such as these would supplement the large number of investigations that have dealt with individual proteins in which the electrons have been exchanged with low molecular weight reagents. To date, the paths of electrons to and from proteins are generally considered to involve outer sphere mechanisms, disulfide bond adducts, and quantum mechanical tunnelling (Bennett, 1973; Sutin, 1973), while the change in the redox state of the active center is thought to cause the protein to undergo various conformational changes. For example, by reducing horse heart cytochrome *c* with ferrocyanide, a biphasic response was observed (Greenwood and Palmer, 1965). This was explained in terms of a conformational equilibrium of the oxidized cytochrome that formed an inactive isomer predominant at high pH (Brandt et al., 1966). The process was later resolved into a deprotonation ($pK_H = 11$) that preceded the conformational change (Davis et al., 1974). The electron pathway for both reduction (Yandell et al., 1973; Creutz and Sutin, 1973; Hodges et al., 1974) and oxidation (McArdle et al., 1974) is now thought to be via the exposed edge of the porphyrin ring of the heme arriving at or leaving the iron by an outer sphere electron transfer.

The copper proteins that are thought to participate in electron transport have not been so intimately studied as no structural determination has been accomplished. What structural information there is is vague, but points to the copper moiety being coordinated to nitrogens and isolated from any solvent water molecules (Rist et al., 1970). Hemmerich (1964), noting the ease with which cuprous glycine complexes can disproportionate, suggested that for copper proteins a stability, other than structural, could be provided through d–π bonding by mercaptide ions or disulfide bonds. Evidence for an involvement of the mercaptide ion and disulfide bond in electron mediation has come from fluorescence (Finazzi-Agrò et al., 1970), kinetic (Faraggi and Pecht, 1973), binding (Byers et al., 1973), and spectroscopic studies (McMillin et al., 1974).

Although electron transfer between proteins has not been a subject actively pursued, models for its interpretation have. In kinetic terms, a second-order association and dissociation between the species is considered with an intermediate step to form some sort of complex enabling the electron transfer. This has been suggested for the electron exchange in *Pseudomonas* and horse heart Cyt *c*[1] (Morton et al., 1970), between *Ps. azurin* and *Pseudomonas* cytochrome oxidase (Wharton et al., 1973), and between *Ps. azurin* and *Pseudomonas* Cyt *c* (Antonini et al., 1970; Pecht and Rosen, 1973).

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[1] Abbreviations used are: EDTA, disodium ethylenediaminetetraacetate; Cyt(II) and Cyt(III), reduced and oxidized cytochrome *c* (P551), respectively; Az(I) and Az(II), reduced and oxidized azurin, respectively; Cyt *c*, cytochrome *c*.

Table I: Extinction Coefficients (M^{-1} cm^{-1}) for the Various Protein Species.

Protein and Wavelength (nm)	5 °C	15 °C	25 °C	35 °C
Cyt(II), 625	55	64	73	85
Cyt(III), 625	1220	1230	1240	1270
Cyt(II), 551	3.09×10^4	3.06×10^4	3.0×10^4	2.94×10^4
Cyt(III), 551	9280	9280	9280	9280
Cyt(II), 520	1.76×10^4	1.74×10^4	1.73×10^4	1.70×10^4
Cyt(III), 520	1.09×10^4	1.07×10^4	1.06×10^4	1.06×10^4
Az(II), 625	5700	5700	5700	5700
Az(II), 551	1760	1760	1760	1760

The cytochrome *c* (P551) from *Ps. aeruginosa* is acidic (p*I* = 4.7) (Horio et al., 1960) and differs markedly in its sequence (Ambler, 1963) from the mammalian proteins (Margoliash and Schejter, 1966), in common with all the bacterial cytochromes *c*. However, the recent structural analysis of the *Rhodospirillium rubrum* cytochrome c_2 (Salemme et al., 1973) has shown a structural similarity to the horse heart protein, and, as the *Pseudomonas* protein does retain the "invariant" amino acids (Dickerson and Timkovitch, 1976), it is reasonable to argue here for a similar structure. However, the surface charge and distribution are different, because of its low isoelectric point and its high reactivity toward the *Pseudomonas azurin* that is considered to be its natural oxidant (Horio, 1958). In fact, the reaction between this azurin and the Cyt *c* (P551) was found to be 1000 times faster than with horse heart cytochrome *c* (Antonini et al., 1970). The mechanism was regarded as second order but, because of a rate-limiting concentration dependence, modified by a complex formation. The reaction rate was found to be essentially independent of pH (from 4 to 9) in contrast to the marked pH dependence of horse heart Cyt *c* in its reactions. Pecht and Rosen (1973), using the temperature-jump technique (Eigen and DeMaeyer, 1963) to resolve the normal modes of the reaction and increase the time resolution, observed two relaxations. These were explained in terms of the two association steps and an electron transfer between the associated species. A later communication by Brunori et al. (1974) succeeded in repeating these observations but excluded the mechanism of Pecht and Rosen.

The properties of *Pseudomonas* azurin have shown it to be more unusual than the *Pseudomonas* Cyt *c*. The hyperfine splitting in its electron spin resonance (ESR) absorption bands (Mason, 1963) and its tryptophan fluorescence wavelength (Finazzi-Agrò et al., 1970; Grinvald et al., 1975) are both lower than any other blue protein. This, together with studies of the luminescence quenching (Finazzi-Agrò et al., 1973), has suggested that the copper is buried in a hydrophobic pocket closely associated with the sulfhydryl group and the single tryptophan. However, on comparing the differences in the fluorescence lifetime measurements of azurin in D_2O and H_2O, Grinvald et al. (1975) have suggested that water molecules may be able to reach the copper ion. In the same study a variation was observed in the circularly polarized component of fluorescence of the reduced azurin with wavelength. This was interpreted as reflecting at least two different chiralities of the single tryptophan existing in a conformational equilibrium.

The kinetic and thermodynamic studies on the interaction of azurin and Cyt *c* (P551) have been extended over a wide range of concentrations and temperatures and the mechanism of electron transfer is interpreted in the light of the above findings.

Experimental Section

Materials

Cells of *Ps. aeruginosa* (formerly misnamed *Pseudomonas fluorescens* (Pecht and Rosen, 1973)) were grown anaerobically for 48 h in a medium of yeast extract (1800 g), KH_2PO_4 (450 g), trisodium citrate (2250 g), $NaNO_3$ (2250 g), $CuSO_4 \cdot 5H_2O$ (0.4 g), and $MgSO_4 \cdot 7H_2O$ (225 g) at pH 7.0 (adjusted with 1 M NaOH) in a 450-l. fermentor. The temperature was kept at 26–30 °C. After harvesting, the cells were frozen and stored at −20 °C. Azurin and Cyt *c* (P551) were extracted according to the procedures of Ambler (1963; Ambler and Brown, 1967; Ambler and Wynn, 1973). The purified Cyt *c* had an extinction ratio $E_{red}^{551}/E_{ox}^{280} = 1.16$ and azurin had a ratio of $E_{ox}^{625}/E_{ox}^{280} = 0.52$. The proteins were stored at 4 °C in ammonium acetate buffer (pH 3.9).

Platinum black, obtained from Fluka (Buchs, Switzerland), was washed with 10% nitric acid and distilled water and dried under vacuum before use. All other chemicals used were of analytical grade. The buffer used in all experiments was 0.05 M sodium phosphate at pH 7.0, containing 10 μM EDTA.[1]

Methods

Reduction of Cyt c (P551). The previous method of reducing Cyt *c* (P551) by dithionite followed by gel filtration (Pecht and Rosen, 1973) resulted in a product that contained up to 25% of the oxidized form. A slow atmospheric oxidation (2×10^{-4} l. mol^{-1} min^{-1}) was observed (azurin was insensitive), and so all subsequent experiments were therefore carried out under argon washed with methyl viologen (Sweetser, 1967) to remove traces of oxygen.

The Cyt *c* (P551) was reduced catalytically by hydrogen using the following procedure: sodium phosphate buffer (0.05 M, pH 7.0) was added to a 10-ml Amicon concentration cell fitted with a Millipore filter (0.25 μm) and a serological cap on the top, and containing a small quantity of platinum black. Hydrogen, saturated with water, was passed over the stirred mixture and after 1 h the deoxygenated buffer was forced under slight hydrogen pressure into a test tube flushed with argon (the first milliliter of buffer being discarded). This buffer solution was then used in the subsequent measurements. The oxidized Cyt *c* (P551) was added through the serological cap and then reduced in about 15 min by the resumed flow of hydrogen. It was collected in the manner just described and contained on average <1% of the oxidized protein.

Static Titrations. These (and all optical density measurements) were performed using a Cary 14 spectrophotometer equipped with thermostated cell holders. The oxidized azurin was deoxygenated by passing argon over 0.25 cm^3 of an approximately 4 mM solution for about 30 min. The azurin was then titrated against the reduced Cyt *c* (P551) in a closed cell, the final increase in volume being kept below 2%. For each starting concentration of reduced Cyt *c* the amount of oxidized azurin was varied over approximately 1.5 orders of magnitude beginning at about one-tenth of the Cyt *c* concentration. After the first two or three additions, by keeping the oxidized azurin concentration below 0.5 μM, it was possible to observe the isosbestic points between the oxidized and reduced Cyt *c* (P551) without distortion, as the reduced azurin has no absorption in the visible region. In subsequent additions the absorption at the isosbestic point (5583 Å) was adjusted to its former value by adding oxidized azurin to a second blank which was then used for recording the spectrum below 600 nm.

Extinction Coefficients of the Proteins. The extinction coefficients of cytochrome *c* and azurin used in the calculations are shown in Table I. The value for reduced Cyt *c* at 551 nm was taken from Ambler (1963), and the remainder obtained by comparison. On using an extinction coefficient for azurin of 3500 l. mol^{-1} cm^{-1} (Brill et al., 1968), the calculated concentrations of oxidized Cyt *c* and reduced azurin in the titrations were unequal, a finding untenable by stoichiometry. A new value for the azurin coefficient was determined by titrating potassium ferricyanide against reduced azurin in 0.1 M phosphate buffer (pH 7.0). Starting from the equation for the equilibrium

$$K = \frac{[\mathrm{Az(II)}][\mathrm{Fe(CN)_6^{4-}}]}{[\mathrm{Az(I)}][\mathrm{Fe(CN)_6^{3-}}]}$$

where Az(II) and Az(I) represent the oxidized and reduced azurins, respectively, we have after simple manipulation:

$$\frac{[\mathrm{Fe^{tot}}]}{D^{625}} = \frac{1}{K\epsilon_{\mathrm{Az}}{}^{625}} \frac{D^{625}}{D_\infty - D^{625}} + \frac{1}{\epsilon_{\mathrm{Az}}{}^{625}} \qquad (1)$$

where $[\mathrm{Fe(CN)_6^{4-}}] = [\mathrm{Az(II)}]$, by stoichiometry, and $[\mathrm{Fe^{tot}}]$ = total concentration of iron hexacyanide, D^{625} = optical density at 625 nm, D_∞ = optical density of the fully oxidized azurin, and $\epsilon_{\mathrm{Az}}{}^{625}$ = extinction coefficient (decadic) of oxidized azurin at 625 nm.

A plot of this equation is shown in Figure 1 and from the intercept, $\epsilon_{\mathrm{Az}}{}^{625} = 5700 \pm 100$ l. mol^{-1} cm^{-1}, a value of the same order as those of other blue copper proteins.

Kinetic Titrations. These were carried out using a double beam temperature-jump spectrophotometer (Rigler et al., 1974), with a cell modified for anaerobic work (I. Pecht, unpublished results). The solutions and titrant were added to the cell by syringes through one of two holes that otherwise served as outlets for the argon. The solution was then mixed by placing the cell on a Vortex stirrer. The concentrations of oxidized azurin were increased through an order of magnitude beginning at about one-half of the starting concentration of the reduced Cyt *c* (P551). The apparatus was previously calibrated by the method of Havsteen (1969), and temperature jumps of 2.9 °C so calculated were used for all measurements. The spectral change at 551 nm following a temperature perturbation was recorded on a Tektronix 549 storage oscilloscope to measure its amplitude and stored in a Biomation 802 A/D converter fitted with a buffer memory. The data were then transferred to an HP 2100A computer to evaluate the relaxation times.

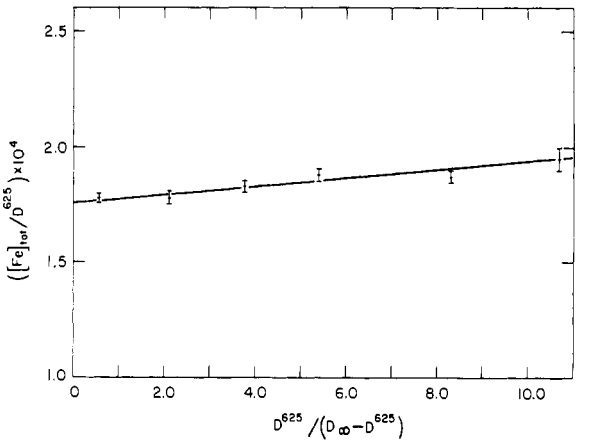

FIGURE 1: Oxidation of reduced azurin by potassium ferricyanide at 25 °C. $[Fe]_{tot}$ is the total concentration of iron hexacyanide, D^{625} is the optical density at 625 nm, and D_∞ is the optical density at 625 nm of the fully oxidized protein. The extinction coefficient at 625 nm of the oxidized azurin is obtained from the reciprocal of the intercept.

An average of five to ten values for each amplitude or relaxation time was made before plotting. All graph lines were drawn using the linear least-squares analysis that assumed no error in the horizontal coordinates.

Nonlinear least-squares analysis of the relaxation spectra were also carried out using the method of Grinvald and Steinberg (1974). However, at the high starting concentrations of cytochrome *c* the separation of the relaxation times increased to more than a factor of 20, and the corrections of the amplitudes and time constants were then reduced to below 10%.

Amplitude Spectra. The variations of the relaxation amplitudes with wavelength for the oxidized and reduced Cyt *c* (P551) and for the reaction mixture were plotted from measurements taken at 5-nm intervals. This was decreased to every 1 nm over rapidly changing regions of the spectrum. A Schoeffel GM 250 grating monochromator was used for recording these spectra.

Results

Static Measurements. A typical titration at 25 °C is shown in Figure 2 and the data leading to the equilibrium constant are summarized in Table II. The new extinction coefficient (cf. Methods) for azurin (5700 l. mol^{-1} cm^{-1}) lowered the difference between the Cyt(III) and Az(I) concentrations to less than 5% which was considered to be satisfactory. An equilibrium constant of 4.2 ± 0.3 was determined from six different starting concentrations (0.6–6.6 × 10^{-5} M) of Cyt *c* (P551) at 25 °C. No temperature dependence of the constant was observed (implying $\Delta H_{tot} = 0$) and was averaged to 3.9 ± 0.3 over all the measurements (at 5, 15, 25, and 35 °C).

Amplitude Spectra. In Figure 3 are the temperature-jump relaxation spectra at 5, 25, and 35 °C. The variation of the amplitudes of the two relaxations with wavelength is shown in Figure 4. Now it can be seen from Table I that the formation of oxidized Cyt *c* is accompanied by a decrease in optical density, because the sum of the extinction coefficients at 551 nm of oxidized Cyt *c* and reduced azurin is less than the sum of reduced Cyt *c* and oxidized azurin. Therefore, as the direction of the fast relaxation involved a decrease in optical density it represented a net formation of oxidized Cyt *c*. Conversely, the slow relaxation, having an opposite sense, represented a net formation of reduced Cyt

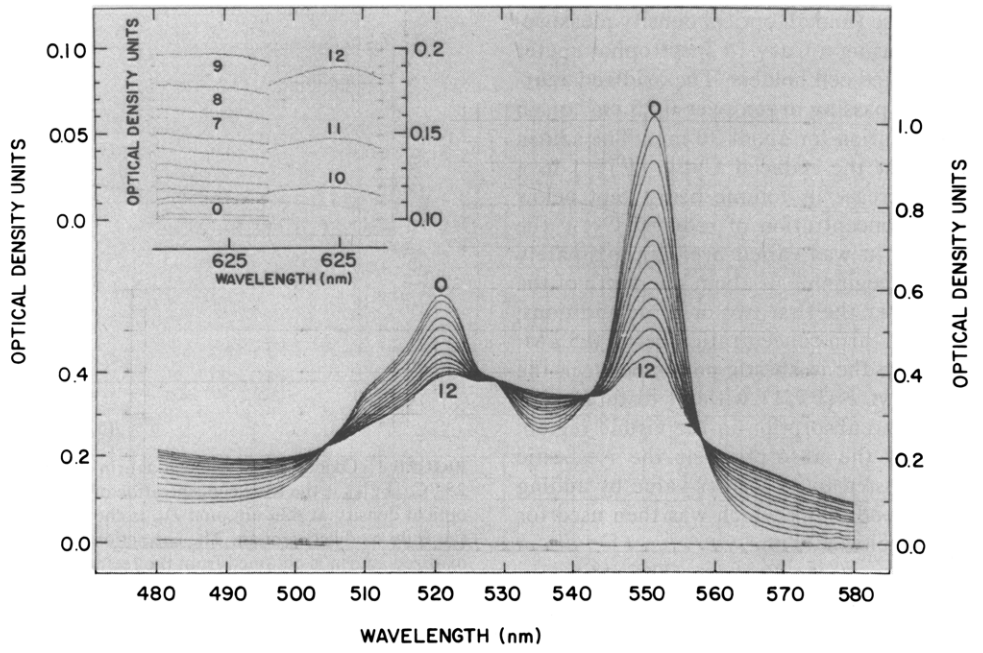

FIGURE 2: Static titration of oxidized azurin (4×10^{-6} to 5.4×10^{-5} M) against reduced Cyt *c* (P551) at 25 °C. The absorption of oxidized azurin below 600 nm was compensated out by adding oxidized azurin to a second blank cell. This reduced the optical density at the isosbestic point (5583 Å) between the oxidized and reduced Cyt *c* to its original value.

Table II: Detailed Calculations for the Overall Equilibrium Constant K_{tot}^{av} (=3.9) at 25 °C.[a]

[Cyt(III)] × 10^5	[Cyt(II)] × 10^5	Net OD (625nm)	[Az(II)] × 10^5	$[Az]_{tot}$ × 10^5	[Az(I)] × 10^5	([Cyt(III)] [Az(I)])/ ([Cyt(II)] [Az(II)])
0.0412	3.36					
0.939	2.50	0.0058	0.104	0.963	0.859	3.10
1.24	2.20	0.0096	0.172	1.34	1.17	3.83
1.46	1.95	0.0160	0.286	1.74	1.44	3.77
1.66	1.72	0.0223	0.397	2.10	1.71	4.16
1.82	1.50	0.0352	0.628	2.55	1.92	3.72
1.97	1.32	0.0488	0.872	2.99	2.11	3.63
2.14	1.14	0.0662	1.18	3.49	2.30	3.66
2.31	0.971	0.0866	1.54	4.04	2.50	3.85
2.49	0.802	0.1132	2.02	4.66	2.64	4.07
2.72	0.631	0.1524	2.72	5.40	2.67	4.25

[a] The first two azurin additions are omitted (concentrations are in molar units).

c. The algebraic sum of the two amplitude spectra would therefore correspond to a difference spectrum of reduced vs. oxidized Cyt *c* (most accurately in the Soret region where azurin has no absorbance) and this is compared in Figure 4 with the same difference spectrum obtained in a Cary 14 spectrophotometer. In contrast to the findings of Brunori et al. (1974), and in confirmation of the earlier results of Pecht and Rosen (1973), there are differences between the two spectra especially within the Soret region. These differences can be overlooked if the resolution of the monochromator is too small and/or the wavelength increments at which they are monitored are too large. As can be seen in the expression for the amplitudes of the two relaxations (eq 13 and 16) their variations with wavelength at fixed concentrations of the proteins depend only upon the stoichiometric sum of the extinction coefficients and are coincident only when that sum, $\Sigma \nu_i \epsilon_i$, is zero.

Spectra of the effect of temperature on the oxidized and reduced forms of Cyt *c* (P551) are shown in Figures 5 and 6. Those in Figure 5 were obtained through a 30 °C difference between the two cuvettes in a Cary 14 spectrophotometer and those in Figure 6 were measured in the temperature-jump apparatus, with a rise in temperature of 2.9 °C. Both methods revealed the same spectral shifts for the reduced Cyt *c* but large differences between the two spectra were observed for the oxidized protein around 420 nm. The changes observed in the T-jump occurred within the heating time of the instrument (~5 μs), and were reproducible for different preparations of the Cyt *c*. No slower changes ($\tau >$ 0.2 s) at 420 nm were observed as might have been expected if the effect of the temperature rise, or of the preceding electric field that accompanied the capacitor discharge, were to induce some reversible instability in the protein.

In the broadest terms these shifts and changes in absorption of the oxidized Cyt *c* (P551) take on the appearance of the reduced protein in the Soret region.[2]

[2] It is interesting that the result of lyophilizing oxidized Cyt *c* (P551) in ammonium acetate (pH 6.5) and then redissolving it in the same buffer produced ~5–6% of the reduced form. Also, it is interesting that the lyophilized, horse heart Cyt *c* commercially available contains about 10% of the reduced form.

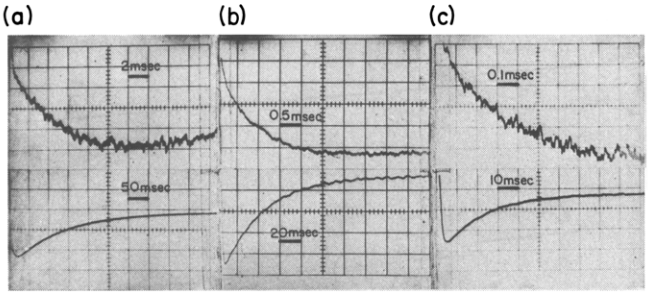

FIGURE 3: Oscilloscope traces of the spectrum of relaxation times at 551 nm for the interaction between Cyt *c* (P551) and azurin: (a) reaction at 5 °C, upper trace, 2 mV/cm; 2 ms/cm; lower trace, 20 mV/cm, 50 ms/cm; (b) reaction at 25 °C, upper trace, 10 mV/cm, 0.5 ms/cm; lower trace, 10 mV/cm, 20 ms/cm; (c) reaction at 35 °C, upper trace, 5 mV/cm, 0.1 ms/cm; lower trace, 10 mV/cm, 10 ms/cm. For the vertical scales, 1 mV ≡ 1.35×10^{-5} optical density units. Total concentrations: azurin, 2.2×10^{-4} M; Cyt *c* (P551), 4.5×10^{-5} M, for all traces.

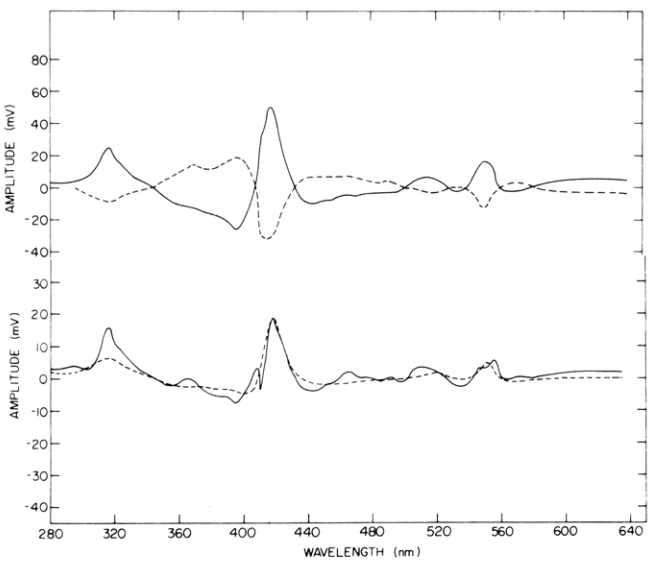

FIGURE 4: Upper trace: The amplitude spectra of the fast (- - -) and slow (—) relaxations. Starting concentration of Cyt(II) = 1.42×10^{-5} M, and the total azurin concentration = 5.8×10^{-5} M; ΔT = 2.9 to 25 °C. Lower trace: The algebraic sum of the fast and slow relaxations (—) compared to the static difference spectrum (- - -) of the reduced against oxidized Cyt *c* (P551) normalized to the temperature-jump difference spectrum at 416 nm.

Kinetic Measurements. Of the two relaxations observed, the slower showed a limiting dependence on increasing azurin concentration and the faster a linear one. In its simplest interpretation this behavior may be explained by a slow monomolecular step coupled to a fast bimolecular one, the former interpreted in terms of a conformational transition of the complexed proteins and the latter, their association–dissociation.

In the reaction scheme proposed by Pecht and Rosen (1973) the fast relaxation was considered to be a sum of the two bimolecular association steps, both displaying the same concentration dependence. Because the association steps were much faster than the monomolecular transition their equilibration was independent of it, and so the dependence of the fast relaxation time (τ_{fast}) would derive from an equilibrium of the type:

$$A + B \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} C$$

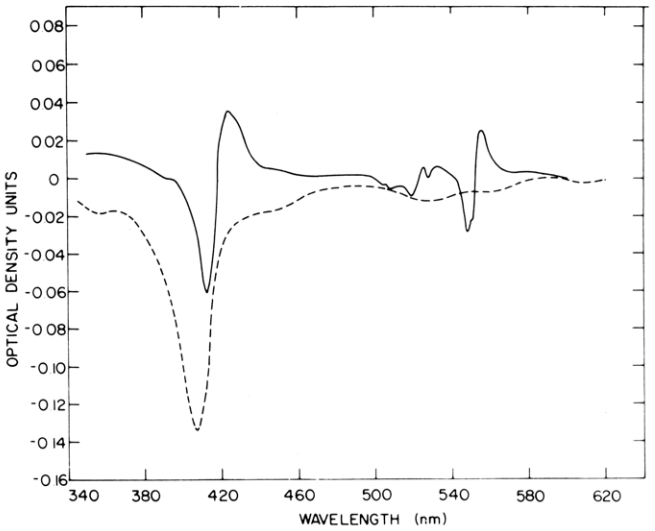

FIGURE 5: The temperature difference spectrum (35–5 °C) of reduced (—) and then oxidized (- - -) Cyt *c* (P551) measured in a Cary-14 spectrophotometer; [Cyt(II)] = [Cyt(III)] = 1.3×10^{-5} M; 0.05 M sodium phosphate buffer, (pH 7.0) containing 10 μM EDTA.

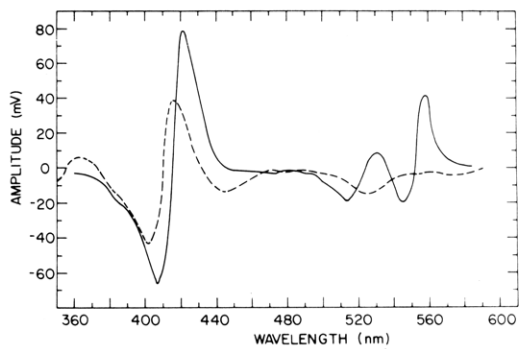

FIGURE 6: The temperature difference spectrum of reduced (—) and then oxidized (- - -) Cyt *c* (P551) measured in the temperature-jump. The difference in the voltage signal before and after the jump represents the difference in extinction of Cyt(II) or Cyt(III) between the two temperatures at the particular wavelength: scaling factor, 1 mV ≡ 1.35×10^{-5} optical density units; [Cyt(II)] = [Cyt(III)] = 1.42×10^{-5} M; 0.05 M sodium phosphate buffer (pH 7.0) containing 10 μM EDTA; ΔT = 2.9 to 17.9 °C.

In a plot of $1/\tau_{fast}$ against [Cyt(II)] + [Az(II)], (where the brackets denote equilibrium concentrations), the intercept would be positive (Eigen and DeMaeyer, 1963). Figure 7 shows the relaxation times for five different starting concentrations of reduced Cyt *c*. It demonstrates, very clearly, a funnelling behavior of the relaxation times toward the origin in the family of curves expressing the dependence of $1/\tau_{fast}$ with [Cyt(II)] + [Az(II)]. This trend is incompatible with the scheme of Pecht and Rosen (1973), but a scheme where the fast relaxation would be the result of a reaction of the type:

$$A + B \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} C + D$$

can explain this behavior, as the expression of the concentration dependence of its relaxation time (eq 2) is zero when [Cyt(II)] = [Az(II)] = 0. With complex formation excluded as a thermodynamically or kinetically distinct step, the slow relaxation was considered to result from an isomerization of one of the protein species, coupled to the faster electron exchange reaction.

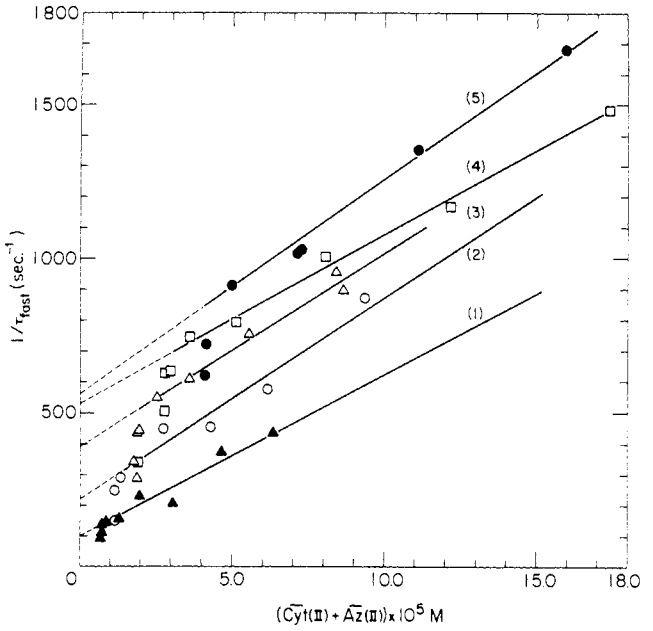

FIGURE 7: Plots of the fast relaxation time at 25 °C for five starting concentrations of Cyt(II). Assumes a reaction mechanism that involves a bimolecular association of the protein species to form a stable complex before electron transfer: [Cyt c] × 10^5 M, (▲) 1.0; (○) 1.9; (△) 2.9; (□) 4.5; (●) 6.6.

$$\frac{1}{\tau_{fast}} = k_{12}([Cyt(II)] + [Az(II)]) + k_{21}([Cyt(III)] + [Az(I)]) \quad (2)$$

Evidence for the existence of an equilibrium between two species of reduced azurin in solution has come from measurements showing a wavelength variation of the circularly polarized component of the fluorescence of its single tryptophan (Grinvald et al., 1975). The variation, not seen in the apo- or oxidized azurin, was indicative of the presence of two conformers in solution. The slow relaxation was therefore considered to arise from this equilibrium between two species of reduced azurin, i.e.

$$\begin{array}{ccc} Cyt(II) + Az(II) & \underset{k_{21}\ (fast)}{\overset{k_{12}}{\rightleftharpoons}} & Cyt(III) + Az(I) \\ & & k_{32} \downarrow\uparrow k_{23}\ (slow) \\ & & Az(I)^* \end{array} \quad (3)$$

where Az(I) is the active and Az(I)* the nonactive conformer.

The relaxation times for this system are given by eq 2 for the fast relaxation and by eq 4 for the slow, where $K_e = k_{12}/k_{21}$, and we have assumed that $k_{12}([Cyt(II)] + [Az(II)])$, $k_{21}([Cyt(III)] + [Az(I)]) \gg k_{23}, k_{32}$.

$$\tau_{slow}^{-1} = k_{23} \times \frac{1 + [Az(I)]/\{([Cyt(II)] + [Az(II)])K_e\}}{1 + ([Az(I)] + [Cyt(III)])/\{([Cyt(II)] + [Az(II)])K_e\}} + k_{32} \quad (4)$$

By letting [Cyt(II)] + [Az(II)] = $\bar{c}$ and [Cyt(III)] + [Cyt(II)] + [Az(I)] + [Az(II)] + [Az(I)*] = c_0 we have for eq 2:

$$\tau_{fast}^{-1} = \bar{c}(k_{12} - k_{21}) + k_{21}c_0 - k_{21}[Az(I)^*]$$

In all the titrations after the first three additions of oxidized azurin, ([Cyt(II)] + [Az(II)]) ≳ 5Az(I)* and so, after dividing by c_0, we have for eq 2 the expression shown in eq 5.

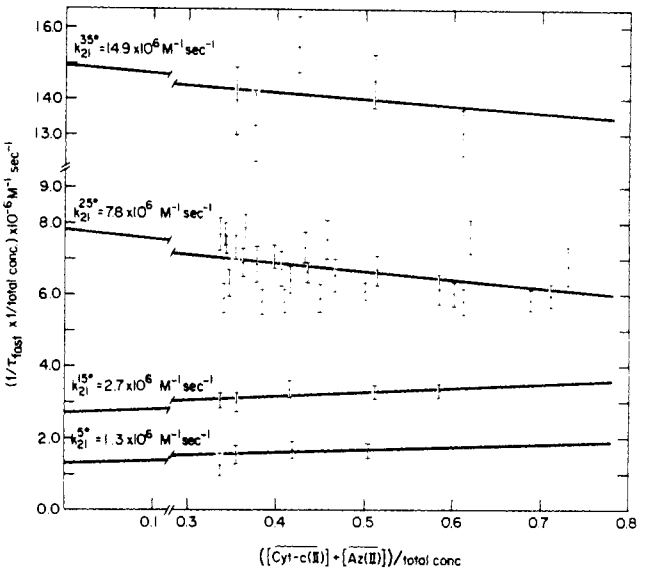

FIGURE 8: Plots of the fast relaxation time. The mechanism assumed involves an electron transfer in the scheme of eq 10 for the fast step. The concentration of Cyt c at temperatures other than 25 °C was 4.5 × 10^{-5} M, and at 25 °C they were the same as for Figure 7.

$$(1/c_0)(1/\tau_{fast}) \simeq (\bar{c}/c_0)(k_{12} - k_{21}) + k_{21} \quad (5)$$

This is an equation of a straight line of slope $(k_{12} - k_{21})$, and it is plotted for titrations of Cyt(II) by Az(II) at the different temperatures in Figure 8. As the slopes expressed the difference between two rate constants of similar value the error in measuring k_{12} would have been correspondingly large. Therefore, data plotted in Figure 7 were used where k_{12} was measured directly from the slopes. Under these conditions of excess Az(II), we have [Az(II)] + [Cyt(II)] ≃ [Az(II)] and [Cyt(III)] ≃ [Cyt c]$_{tot}$. Thus, [Az(I)] ≃ [Cyt c]$_{tot}$ − [Az(I)*] = [Cyt c]$_{tot}$ − K_i[Az(I)], where K_i = [Az(I)*]/[Az(I)]. Substituting these approximations into eq 2 we have:

$$\tau_{fast}^{-1} \rightarrow k_{12}[Az(II)] + k_{21}[Cyt\ c]_{tot}\left(1 + \frac{1}{1 + K_i}\right) \quad (6)$$

where the limiting slope gives k_{12}. This plot is shown in Figure 9 at four different temperatures. The values of the rate constants obtained from both plots (eq 5 and 6) are presented in Table III. For evaluating the equilibrium constant K_e (Table IV), the intercept of eq 5 and slope of eq 6 were used.

As shown in Figure 3 the rates displayed a marked increase with temperature, with k_{21} now seen in Table III to have the steeper dependence. Thus, K_e decreased with increasing temperature which meant that the production of oxidized Cyt c was exothermic. Therefore, on heating, this equilibrium should be driven toward the formation of reduced Cyt c. But in the observed relaxation spectra (Figure 3) the direction of the fast relaxation resulted in a lowering of the optical density, indicating a net formation of oxidized Cyt c.

A discrepancy also arose from the analysis of the slow relaxation. A plot of eq 4 yielding k_{23} and k_{32} gave a K_i (= k_{23}/k_{32}) of 0.5 at 25 °C. Now in the calculation of the static equilibrium constant the concentrations of reduced azurin could not be directly measured (as the reduced protein has no absorption in the visible region) but were calculated from the difference between the concentrations of total azu-

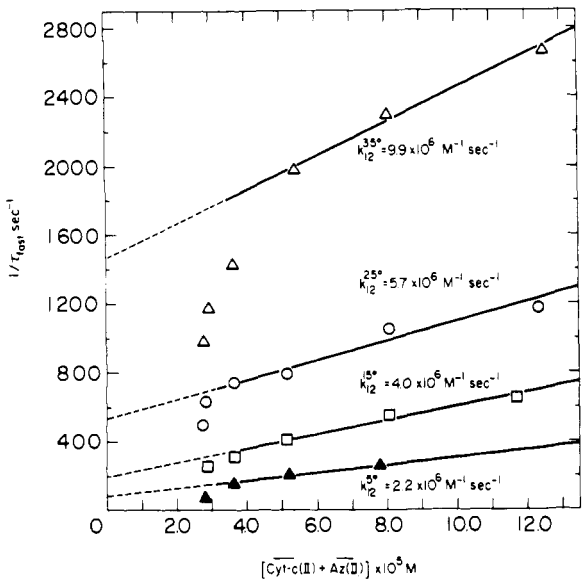

FIGURE 9: Plots of the fast relaxation time at four temperatures used to measure k_{12} from the limiting slope.

rin and oxidized azurin. From the reaction scheme of eq 3 we have:

$$K_{tot} = \frac{[Cyt(III)]([Az(I)] + [Az(I)^*])}{[Cyt(II)][Az(II)]} = K_e(1 + K_i) \quad (7)$$

where $K_e = ([Cyt(III)][Az(I)])/([Cyt(II)][Az(II)])$ and $K_i = [Az(I)^*]/[Az(I)]$. Substituting a value of K_e at 25 °C of 0.78 (Table III), a value of $K_i = 4$ was then calculated, compared with 0.5 from the slow relaxation in eq 4.

For these two reasons (the contradiction between the observed direction of the fast relaxation and that expected from the change in K_e, and the inconsistency between the value of K_i evaluated from the slow relaxation time and that from eq 7 using K_{tot}) it was found necessary to assume that an additional endothermic equilibrium was involved in this system. In eq 7 (because of the small value of K_i) the value of $K_e(1 + K_i)$ could only be increased by assuming an isomerization of the oxidized Cyt *c*. This additional equilibrium would be of the same form as for the reduced azurin, but of a rate ($\sim 10^4$ s^{-1}) much faster than the rate of the electron exchange. Equation 7 then becomes:

$$K_{tot} = K_e(1 + K_i)(1 + K_0) \quad (8)$$

where $K_0 = [Cyt(III)^*]/[Cyt(III)]$. The new reaction scheme is shown in reaction 9.

$$\begin{array}{ccc} & & Cyt(III)^* \\ & & k_{10}\uparrow\downarrow k_1 \quad \text{(very fast)} \\ Cyt(II) + Az(II) & \underset{k_{21}\ \text{(fast)}}{\overset{k_{12}}{\rightleftharpoons}} & Cyt(III) + Az(I) \\ & & k_{32}\uparrow\downarrow k_{23} \quad \text{(slow)} \\ & & Az(I)^* \end{array} \quad (9)$$

If we assume that this new equilibrium involves no spectral change then, being uncoupled from the two slower equilibria, its relaxation will have no amplitude, and so the new scheme will still display only two relaxation times. The direction of the fast relaxation can now be explained by the coupling of the exothermic electron exchange equilibrium to the endothermic conformational transition of the oxidized Cyt *c*. The relaxation expression for the electron exchange is now given by eq 10. As $K_0 \sim 1$ then, because k_{21} and k_{12} were previously calculated (eq 5 and 6) under conditions of excess oxidized azurin, their values remained unaffected, although eq 6 was altered to eq 11. This did change the values of k_{21} by ~15%. Calculations of k_{21} according to eq 5 remained essentially unchanged by the additional equilibrium.

$$\tau_{fast}^{-1} = k_{12}([Cyt(II)] + [Az(II)]) + k_{21}\left([Cyt(III)] + [Az(I)]\left(\frac{1}{1 + K_0}\right)\right) \quad (10)$$

$$\tau_{fast}^{-1} \rightarrow k_{12}[Az(II)] + k_{21}[Cyt\ c]_{tot}\left(\frac{1}{1 + K_0} + \frac{1}{1 + K_i}\right) \quad (11)$$

For the slow relaxation the relaxation expression of eq 4 becomes eq 12. To calculate the values of k_{23} and k_{32} a simple iteration procedure was used. A value of K_i was guessed and substituted into eq 8 to obtain K_0. The values of k_{23} and k_{32} estimated from eq 12 were then used to obtain a new value of K_i. A good convergence ($\Delta K_i < 0.001$) was obtained at all temperatures. The values of k_{23} and k_{32} evaluated from Figure 10 are presented in Table III and the values of K_i, K_e, and K_0 are summarized in Table IV. The values of K_i decreased with increasing temperature and, like the electron exchange equilibrium, were therefore exothermic.

Table III: Temperature Dependence of the Rate Constants Assuming the Three-Step Mechanism.[a]

	5 °C	15 °C	25 °C	35 °C
k_{12}(Fig. 7) × 10^{-6} M^{-1} s^{-1}	2.2	4.0	6.1	9.9
	(2.0)	(3.8)	(5.4)	(13)
k_{21}(Fig. 8) × 10^{-6} M^{-1} s^{-1}	1.3	2.7	7.8	15
	(0.9)	(2.6)	(9.2)	(27)
k_{23}, s^{-1}	4.9	7.5	12	18
k_{32}, s^{-1}	4.3	9.2	17	35

[a] Those constants in parentheses are taken from Figures 7 and 8 for k_{12} and k_{21}, respectively.

Table IV: Equilibrium Constants for the Three-Step Reaction at the Four Temperatures.

	5 °C	15 °C	25 °C	35 °C
K_e	1.7	1.5	0.8	0.6
K_i	1.1	0.8	0.7	0.5
K_0	0.1	0.4	1.9	2.9

Table V: Activation Parameters.

Rate Const.	$\Delta H^\ddagger$ (kcal mol^{-1})	$\Delta S^\ddagger$ (cal deg^{-1} mol^{-1})
k_{12}	7.8	−1.1
k_{21}	13.7	+18.8
k_{23}	6.7	−30.1
k_{32}	11.1	−15.6

$$\tau_{slow}^{-1} = k_{23}\frac{1 + [Az(I)]/\{[Az(II)] + [Cyt(II)])(1 + K_0)K_e\}}{1 + ([Cyt(III)](1 + K_0) + [Az(I)])/\{([Az(II)] + [Cyt(II)])(1 + K_0)K_e\}} + k_{32} \quad (12)$$

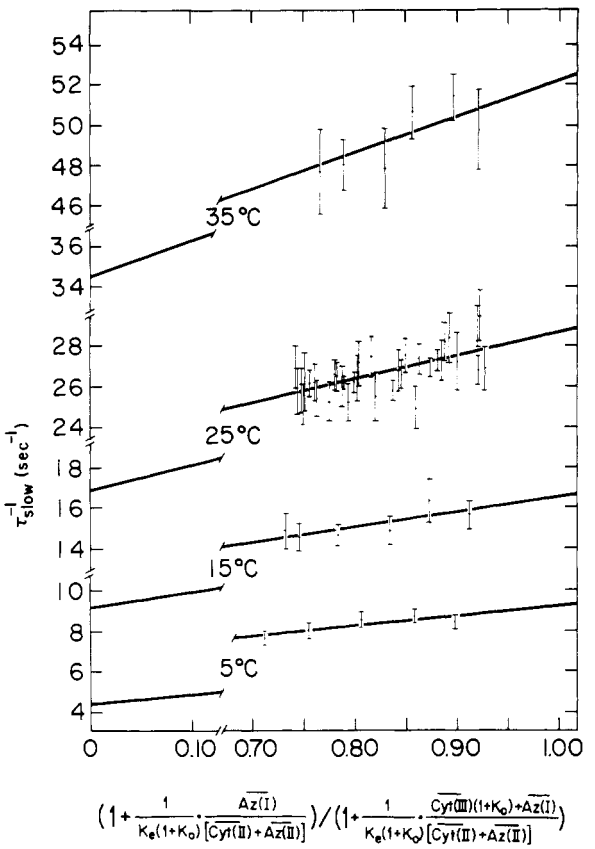

FIGURE 10: Variation of the slow relaxation time with temperature. The plot assumed the mechanism of eq 9. Concentrations of Cyt *c* were identical with those in Figure 8.

Thermodynamic Measurements. The activation enthalpies and entropies for the two slower equilibria were obtained from Eyring plots and the values are summarized in Table V. The equations relating the enthalpy changes for each step to the amplitudes of the relaxations were derived according to the guidelines laid down by Castellan (1963) and Jovin (1976), and are summarized in the Appendix. For the fast relaxation amplitude the relation is:

$$\frac{-\Delta I_{fast}}{2.303 I_0 d} = (\Gamma_e^{-1} - \Gamma_0[\text{Cyt(III)}]^{-2})^{-1}\frac{\Delta T}{RT^2}\times (\Delta H_e + (1 + 1/K_0)^{-1}\Delta H_0)\times (\Delta\theta_e + \Delta\theta_0(1 + 1/K_0)^{-1}) \quad (13)$$

where Γ_0^{-1} = [Cyt(III)*]$^{-1}$ + [Cyt(III)]$^{-1}$; Γ_e^{-1} = [Cyt(III)]$^{-1}$ + [Cyt(II)]$^{-1}$ + [Az(II)]$^{-1}$ + [Az(I)]$^{-1}$; ΔT = temperature rise; d = optical path length of the T-jump cell; ΔH_0 and ΔH_e = enthalpy changes for the Cyt *c* isomerization and electron exchange, respectively; ΔI_{fast} = the observed amplitude change of the fast relaxation (for small changes in concentration); I_0 = the total light intensity; and $\Delta\theta_0$ and $\Delta\theta_e$ = the net change in extinction for the Cyt *c* isomerization and electron exchange, respectively, where each extinction coefficient (ϵ_i) is multiplied by the stoichiometric coefficient (ν_i) of that species ($\Delta\theta_0 = 0$).

Both the amplitudes of the fast and slow relaxations went through a maximum with increasing concentration of oxidized azurin. The plot of the fast relaxation amplitude at 25 °C, according to eq 13, is shown in Figure 11. As $\Delta\theta_0 = 0$, the slope (of 0.7) is given by:

$$0.7 = \Delta H_e + \Delta H_0(1 + 1/K_0)^{-1} \quad (14)$$

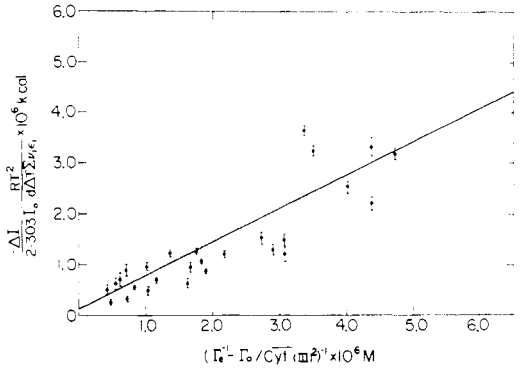

FIGURE 11: The amplitude variation of the fast relaxation at 25 °C plotted as a function of concentration terms (see Appendix) according to eq 13. The starting concentrations of Cyt(II) are the same as for Figure 8. The error bars for each point were calculated assuming an error only in the amplitudes of the relaxations.

Substituting the value of ΔH_e (−5.9 kcal mol^{-1}) obtained from the activation enthalpies in Table V, and the value of K_0 from Table IV, we have $\Delta H_0 = (5.9 + 0.7) \times 1.5 = 10$ kcal mol^{-1}. If we now substitute eq 8 into the van't Hoff relation we have:

$$\frac{\partial \ln [K_e(1 + K_i)(1 + K_0)]}{\partial T} = \frac{\Delta H_{tot}}{RT^2}$$

Differentiating we obtain:

$$\frac{\partial \ln K_e}{\partial T} + \frac{K_i}{1 + K_i}\frac{\partial \ln K_i}{\partial T} + \frac{K_0}{1 + K_0}\frac{\partial \ln K_0}{\partial T} = \frac{\Delta H_{tot}}{RT^2}$$

Thus, on substituting $\Delta H_{tot} = 0$, eq 15 is obtained. Inserting the values of K_i and K_0 from Table IV, and ΔH_e and ΔH_i from Table V, we obtain $\Delta H_0 = 12$ kcal mol^{-1}, which compares favorably with the value obtained from the fast relaxation.

$$\Delta H_e + \frac{K_i}{1 + K_i}\Delta H_i + \frac{K_0}{1 + K_0}\Delta H_0 = 0 \quad (15)$$

The expression for the slow relaxation amplitude is given by:

$$\frac{-\Delta I_{slow}}{2.303 I_0 d} = \Gamma_{slow}\frac{\Delta T}{RT^2}\times (\alpha\Delta H_0 + \beta\Delta H_e + \Delta H_i)(\alpha\Delta\theta_0 + \beta\Delta\theta_e + \Delta\theta_i) \quad (16)$$

where $\Gamma_{slow} = (\Gamma_0^{-1}\Gamma_e^{-1} - [\text{Cyt(III)}]^{-2})/[\Gamma_0^{-1}(\Gamma_e^{-1}\Gamma_i^{-1} - [\text{Az(I)}]^{-2}) - \Gamma_i^{-1}[\text{Cyt(III)}]^{-2}]$, $\Gamma_i^{-1} = [\text{Az(I)}]^{-1} + [\text{Az(I)*}]^{-1}$, $\alpha = \{[\text{Cyt(III)}][\text{Az(I)}](\Gamma_0^{-1}\Gamma_e^{-1} - [\text{Cyt(III)}]^{-2})\}^{-1}$, $\beta = (1 + 1/K_0)\alpha$, $\Delta\theta_i$ = net change of extinction in the azurin isomerization equilibrium (= 0), and ΔH_i = the enthalpy change for the azurin isomerization.

Rearranging eq 16 we have:

$$\frac{-\Delta I_{slow}}{2.303 I_0 d}\frac{RT^2}{\Delta T}\Gamma_{slow}^{-1}\frac{1}{(1 + 1/K_0)\alpha\Delta\theta_e} = \alpha[\Delta H_0 + (1 + 1/K_0)\Delta H_e] + \Delta H_i \quad (17)$$

This equation is plotted in Figure 12 for the amplitude changes at 25 °C. The slope was 14.9 kcal mol^{-1}, and according to eq 17 we have:

$$14.9 = \Delta H_0 + (1 + 1/K_0)\Delta H_e$$

Substituting K_0 and ΔH_e as before we have:

$$\Delta H_0 = 14.7 + 8.9 = 23 \text{ kcal mol}^{-1}$$

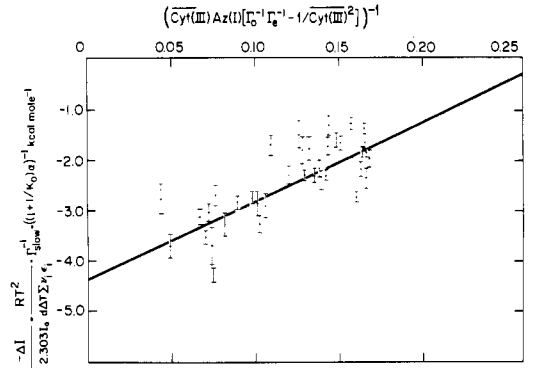

FIGURE 12: The variations of the amplitudes of the slow relaxation time plotted according to eq 17 using a linear least-squares analysis. Starting concentrations of Cyt(II) are given in the legend to Figure 8. The error bars assume an error in the amplitude measurements only.

From a van't Hoff plot of the values of K_0 from Table IV a value of 21 kcal mol^{-1} was obtained. Reasonable agreement was found between the evaluations of ΔH_0 from the amplitudes of the slow relaxation and the van't Hoff plot, but not from the amplitudes of the fast relaxation. However, the value for ΔH_0 of 10–20 kcal mol^{-1} for the isomerization is comparable to a ΔH of 15 kcal mol^{-1} for the heat-induced isomerization of horse heart ferricytochrome *c* (Schejter and George, 1964).

There is good agreement between the value of ΔH_i measured from the intercept of the slow relaxation amplitudes ($\Delta H_i = -4.2$ kcal mol^{-1}) and the value of $\Delta H_i = -4.4$ kcal mol^{-1} obtained from the activation enthalpies of Table V. These comparisons are summarized in Table VI.

Discussion

The two types of reaction schemes could be distinguished by comparing the different concentration dependencies of the fast relaxation time. The increase in the intercept for different starting concentrations of reduced Cyt *c* (P551) shown in Figure 7 demonstrated that the fast relaxation was of the A + B ⇌ C + D type, ruling out the other mechanism. This implied that the complex formation in the reaction before the electron transfer was not a significant step. The slow relaxation time displayed a limiting dependence on increasing azurin or cytochrome concentrations and was therefore considered to be the result of a monomolecular transition, between reactive and nonreactive forms of one of the protein species, coupled to the electron transfer. Independent evidence that the reduced azurin exists in two conformers in solution has been presented by Grinvald et al. (1975) from the wavelength variation of the circularly polarized component of its tryptophan fluorescence.

To account for the contradiction between the temperature dependence of the equilibrium constant of the electron transfer step, and the observed direction of the amplitude of this relaxation, it was necessary to assume an endothermic, conformational equilibrium of the oxidized Cyt *c* (P551), uncoupled from the electron exchange ($\tau \simeq 10^{-4}$ s). As the titrations were generally carried out under conditions of an excess of oxidized azurin the rate constants were found to be unaffected. However, this additional step is problematical as it assumes the existence of a species for which we have no direct evidence. Though a similar isomerization has been assumed for horse heart Cyt *c* (Brandt et al., 1966; Davis et al., 1974) the isomer was only observed (at 695 nm) at alkaline pH and the rate of equilibrium ($\tau \simeq 0.2$ s)

Table VI: Comparison of the Thermodynamic Data from Amplitude and van't Hoff Measurements.[a]

		Amplitude Data	
	van't Hoff Data	Fast Relaxn.	Slow Relaxn.
ΔH_i	−4.4		−4.2
ΔH_e	−5.9		
ΔH_0	+21	+10	+23

[a] Subscripts: 0, Cyt(III) isomerization; e, electron exchange; i, Az(I) isomerization. All units are kilocalories per mole.

Table VII: Comparison of Kinetic Data at 20°C.

	This paper[a]	Wilson et al. (1975)
K_{tot}	3.9	3.7
k_{12} (M^{-1} s^{-1})	5.0×10^6	6.25×10^6
k_{21} (M^{-1} s^{-1})	4.7×10^6	3.38×10^6
k_{23} (s^{-1})	9.7	40
k_{32} (s^{-1})	13	40

[a] Values measured from the Eyring plots.

was very much slower. It is a moot point whether the differences between the cytochromes *c* from horse heart and *Pseudomonas* would be realized in this way.

In a recent paper, Wilson et al. (1975) using T-jump and stopped-flow measurements on the same system have also suggested an isomerization of the reduced azurin. Since their measurements were conducted at twice our ionic strength and at one temperature only, comparison is limited, but generally there is good agreement between the two sets of measurements, which are compared in Table VII. The reaction scheme they proposed for explaining their data is in agreement, in part, with the one we are suggesting. Their criticism of the mechanism proposed by us earlier (Pecht and Rosen, 1973) is justified insofar as the step involving the spectral charge must be expressed in the faster relaxation, which is not the case for that mechanism.

The stopped flow measurements of Wilson et al. (1975) contrasted with the earlier results of Antonini et al. (1970). In that earlier paper it was demonstrated that the pseudo-first-order rate constants for the oxidation and reduction of Cyt *c* (P551) tended to limiting values, though for the oxidation of Cyt *c* the trend was far less noticeable. However, the same plots in Wilson et al. (1975) under identical conditions were linear (though a limiting value *was* observed in the reduction of Cyt(III) when Tris-HCl buffer at pH 7.4 was used instead of phosphate at pH 7.0).

The effect of the Tris-HCl buffer was explained in terms of a shift in the reduced azurin equilibrium in favor of the inactive form. As this would require the equilibrium constant to change from 1 to ~100, the explanation appears spurious. Rather, a cause for the marked curvature observed by Antonini et al. (1970) can be suggested by assuming the isomerization equilibrium of the oxidized Cyt *c*. Using our rate constant of 8×10^6 at 25 °C for the reduction of Cyt(III), and substituting their concentrations, we have:

$$\frac{-d[\text{Cyt(III)}]}{dt} = 8 \times 10^6[\text{Az(I)}][\text{Cyt(III)}]$$
$$= (8 \times 10^6)(5 \times 10^{-5})(5 \times 10^{-7})$$
$$= 2 \times 10^{-4}\ \text{M s}^{-1}$$

Table VIII: Comparison of the Rates and Activation Parameters for the Reduction of Various Proteins.[a]

Protein	Reductant	Specific Rate (M^{-1} s^{-1})	$\Delta H^{\ddagger}$ (kcal mol^{-1})	$\Delta S^{\ddagger}$ (eu)
Stellacyanin	Fe^{2+}(EDTA)	4.3×10^5	3.0	−21
Plastocyanin	Fe^{2+}(EDTA)	8.2×10^4	2.2	−29
Laccase (blue)	Fe^{2+}(EDTA)	2.6×10^2	13.0	−5.1
Azurin	Fe^{2+}(EDTA)	1.3×10^3	2.0	−37
Azurin	$Fe(CN)_6^{4-}$	2.7×10^2	7.3	−23[b]
Plastocyanin	Cyt *f*	3.6×10^7	10.5	+11[c]

[a] The parameters for the reduction of the various copper proteins with Fe^{2+} (EDTA) were obtained from Wherland et al., 1975. [b] Goldberg and Pecht, 1975. [c] Wood, 1974.

If we now substitute a value of 2 for the equilibrium constant (K_0) of the Cyt(III) isomerization (Table IV), and therefore a value of 3×10^3 s^{-1} ($\tau \sim 10^4$ s^{-1}) for the rate constant k_{01} (eq 9) we have:

$$\frac{-d[Cyt(III)^*]}{dt} = k_{01}[Cyt(III)^*]$$
$$= (3 \times 10^3)(3 \times 10^{-7})$$
$$= 9 \times 10^{-4}\ M\ s^{-1}$$

With these approximate calculations we can bring the rates to within an order of magnitude of each other, so that the rate of the electron transfer would become dependent upon the rate of isomerization of the Cyt *c*.

The rates for the reduction and oxidation of Cyt *c* (Table III) by azurin are about 100 times faster than the rates measured when inorganic reagents have been used to oxidize or reduce redox proteins. McArdle et al. (1974), assuming an outer-sphere mechanism, found good theoretical agreement for the rate of oxidation of horse heart Cyt *c* using the Marcus theory (Marcus, 1963), as have Ewall and Bennett (1974) for the reduction of the same Cyt *c* using hexaammineruthenium(II). Using this theory then to estimate the rate of oxidation of Cyt *c* (P551) by azurin at 25 °C the rate, k_{12}, is given by:

$$\log k_{12} = 0.5[\log k_{11} + \log k_{22} + 16.9\Delta E]$$

where k_{11} and k_{22} are rates of the self-exchange electron transfer of the reductant and oxidant, and ΔE is the difference in their redox potentials (negative for $K_{eq} < 1$). Using the data of Morton et al. (1970) for the electron exchange between *Pseudomonas* and horse heart Cyt *c*, we obtain for the rate of the self-exchange reaction[3] of the *Pseudomonas* Cyt *c* (k_{Cyt}):

$$\log 5 \times 10^4 = 0.5[\log k_{Cyt} + \log (0.2 \rightarrow 1) \times 10^3]$$

where the exchange rate for the horse heart Cyt *c* at pH 7.0 is taken from Gupta (1973). A value of $k_{Cyt} < 1 \times 10^7$ M^{-1} s^{-1} was calculated. For the self-exchange rate of azurin the data of Goldberg and Pecht (1975) on the oxidation of azurin by ferricyanide in 0.1 M potassium phosphate were used to calculate a value of $k_{Az} = 6.3 \times 10^2$ M^{-1} s^{-1} (using an exchange rate of 1×10^4 M^{-1} s^{-1} for the hexacyanide couple). The redox potential of the iron hexacyanide couple in 0.1 M phosphate buffer at pH 7.0 is +421 mV (O'Reilly, 1973) and that of azurin, +314 mV. Thus, the rate of oxidation of Cyt *c* (P551) by azurin can be expressed as:

$$\log k_{12} = 0.5[\log 1 \times 10^7 + \log 6.3 \times 10^2 + 16.9 \times 0.028]$$

i.e. $k_{12} = 1.4 \times 10^5$ M^{-1} s^{-1}, where E_0'(Cyt *c* P551) = +286 mV (Horio et al., 1960). This is an order of magnitude less than the observed rate, but in terms of the accuracy of the rates that have been predicted for much simpler reactions, the discrepancy is not too large. Also, the Marcus theory assumes that there are no interactions between the reactants and this condition is perhaps not applicable to this system. The azurin and Cyt *c* (P551), though having similar isoelectric points, contain areas of positive and negative charge at pH 7.0 that would enhance the rate if they were complementary.

The large enthalpy change observed for the Cyt *c* isomerization (and to a lesser extent for the other two steps) and the low value of ΔG for the equilibrium indicate that the entropy change for the reaction is itself large. This compensation phenomenon between entropy and enthalpy changes has been observed in many proteins (Lumry and Rajender, 1970). It can be interpreted as being caused by the displacement of water molecules in the solvation spheres and this could occur during the isomerization (and to some extent the association) of the proteins.

Values of the activation parameters for the reduction of a number of copper proteins by an inorganic reductant and a protein are shown in Table VIII. In general, we can see that the activation enthalpy is two to three times greater for the natural reductant and that the activation entropy becomes positive. This argues for specific interactions between each of the pairs of proteins that are absent when other reagents are used.

In an electron transfer reaction the height of the energy barrier to the transition state is determined by the potential levels of the products and reactants. Upon interaction these split into upper and lower levels and if the interaction is strong then they will be separated to such an extent that the transition state remains in the lower levels and is able to change continuously from reactant to product with a transition probability essentially equal to one. This is the adiabatic transfer and it has been suggested (Reynolds and Lumry, 1966) that all electron transfers in solution are of this type. In practice, electron tunnelling through the transition state may be resolved only when the kinetic measurements are sufficiently accurate to account for the zero-point energy of the transition state (Bell, 1973), particularly so at low temperatures. Indeed, from studies on the cytochrome *c* from *Chromatium* a tunnelling mechanism has been suggested (De Vault et al., 1967) on the basis of an essentially zero activation enthalpy below 100 K.

But at physiological temperatures the theory developed by Marcus for outer sphere reactions has been shown to be adequate to explain the rate of oxidation and reduction of horse heart Cyt *c* (McArdle et al., 1974; Ewall and Bennet, 1974), and it has provided a reasonable approximation for the oxidation of Cyt *c* (P551) by azurin. The large differences observed in ΔH (Table VIII), when inorganic reductants are used, do mitigate against electron tunnelling mechanisms. For although these increases can be argued as

[3] The redox potential of the Cyt *c* (P551) has been measured as +250 mV (Horio, 1958) and as +286 mV (Horio et al., 1960), the latter value obtained with a purer preparation. Although horse heart Cyt *c* potential was measured as +254 mV (Rodkey and Ball, 1950), Morton et al. (1970) found the same reaction rate for the forward and backward reactions in the electron exchange. For this reason the redox potentials of the *Pseudomonas* and horse heart Cyt *c* were taken as equal (i.e., +286 mV).

greater barriers to the electron transfer they cannot be squared with the 100-fold rise in the rate that is observed with the natural reductant.

That this increased rate can be predicted using the Marcus theory suggests that the mechanism can be ascribed to a measure of outer sphere transfer. The order of magnitude difference might be viewed as additional contributions arising from charge interactions, as the sequences of azurin and cytochrome *c* (P551) have complementary areas of positive and negative charges at neutral pH.

Acknowledgments

The authors wish to thank Michel Goldberg, Doron Lancet, and Dr. T. Jovin for their invaluable help in many discussions.

Appendix

The expressions for the amplitude changes were derived according to the principles laid down by Castellan (1963) and Jovin (1976).

In order to relate the amplitudes of each relaxation time to the enthalpy changes of each reaction step it is necessary to describe each step in terms of its reaction advancements, and the affinity to be used as the force to describe the equilibration of each step. The relationship of the affinity to the reaction advancement for each step and the coupling between equilibria is expressed in the diagonal and off-diagonal terms, respectively, of the **g** matrix of Castellan (1963). For the scheme of eq 9 it is given by:

$$\mathbf{g} = \begin{vmatrix} \Gamma_0^{-1} & -[\text{Cyt (III)}]^{-1} & 0 \\ -[\text{Cyt(III)}]^{-1} & \Gamma_0^{-1} & -[\text{Az(I)}]^{-1} \\ 0 & -[\text{Az(I)}]^{-1} & \Gamma_i^{-1} \end{vmatrix} \quad \text{(A-1)}$$

where the Γ terms have been defined for eq 13 and 16.

The factor relating the change in affinity with the advancement in the electron exchange equilibrium, Γ_2, is given explicitly by:

$$\Gamma_2 \equiv \Gamma_{\text{fast}} = g_{1,1}/g_{2,2} = (\Gamma_e^{-1} - \Gamma_0[\text{Cyt(III)}]^{-2})^{-1} \quad \text{(A-2)}$$

and for the azurin isomerization equilibrium, Γ_3, it is given by:

$$\Gamma_3 \equiv \Gamma_{\text{slow}} = g_{2,2}/g_{3,3} = [\Gamma_0^{-1}\Gamma_e^{-1} - [\text{Cyt(III)}]^{-2}]/(\Gamma_0^{-1}(\Gamma_e^{-1}\Gamma_i^{-1} - [\text{Az(I)}]^{-2}) - \Gamma_i^{-1}[\text{Cyt(III)}]^{-2}) \quad \text{(A-3)}$$

where $g_{i,i}$ is that principal sub-matrix of **g**.

From the **g** matrix may be derived column vectors, Q, corresponding to the coupling between the reaction advancements which are numbered in order of decreasing equilibration rates. These multiply the enthalpies and net extinction coefficients of each step. The general amplitude expression (eq 49 of Jovin, 1976) for the αth equilibrium step is then given by:

$$\frac{-\Delta I_\alpha}{2.303 I_0 d} = \Gamma_\alpha \frac{\Delta T}{RT^2} \sum_{j=1}^{\alpha} Q_\alpha{}^T \Delta H_j \sum_{j=1}^{\alpha} Q_{\alpha j} \Delta\theta_j \quad \text{(A-4)}$$

where $Q_{\alpha\alpha} = 1$, and the remaining symbols have been defined in eq 13 and 16 in the Results section.

Thus the fastest ($\alpha = 1$) equilibrium, the Cyt *c* isomerization, will have no amplitude as its net change in extinction $\Delta\theta_0 = 0$. The electron exchange equilibrium ($\alpha = 2$), however, has, at 551 nm:

$$\Delta\theta_e = \Sigma \nu_i \epsilon_i = \epsilon_{\text{Cyt(III)}} + \epsilon_{\text{Az(I)}} - \epsilon_{\text{Cyt(II)}} - \epsilon_{\text{Az(II)}}$$
$$= 9280 + 0 - 30\,000 - 1760 = 22\,480 \text{ M}^{-1} \text{ cm}^{-1}$$

The first term of the Q vector (Q_{21}) for this equilibrium is given by:

$$Q_{21} = -g_{2,1}/g_{1,1} = \Gamma_0[\text{Cyt(III)}]^{-1} = (1 + 1/K_0)^{-1} \quad \text{(A-5)}$$

Thus, on substituting eq A-2 and A-5 into eq A-4 we obtain eq 13. For the slow relaxation ($\alpha = 3$) we have $\Delta\theta_i = 0$, and:

$$Q_3 = -\mathbf{g}_3{}^T \mathbf{g}_{2,2}{}^{-1}$$

where $\mathbf{g}_3{}^T$ is the transpose of the third column vector of the matrix $\mathbf{g}_{2,3}$ and $\mathbf{g}_{2,2}{}^{-1}$ is the inverse of the principal (2,2) matrix of **g**. So for the first two elements of Q_3 we have:

$$Q_3 = -(0, -[\text{Az(I)}]^{-1}) \begin{vmatrix} \Gamma_e^{-1} & [\text{Cyt(III)}]^{-1} \\ [\text{Cyt(III)}]^{-1} & \Gamma_0^{-1} \\ (\Gamma_0^{-1}\Gamma_e^{-1} & -[\text{Cyt(III)}]^{-2})^{-1} \end{vmatrix}$$

Thus

$$Q_{31} = (\Gamma_0^{-1}\Gamma_e^{-1} - [\text{Cyt(III)}]^{-2})^{-1}([\text{Az(I)}][\text{Cyt(III)}])^{-1} \quad \text{(A-6)}$$

and

$$Q_{32} = Q_{31}(1 + 1/K_0) \quad \text{(A-7)}$$

where we have substituted

$$\Gamma_0^{-1}[\text{Az(I)}]^{-1} = (1 + 1/K_0)([\text{Cyt(III)}][\text{Az(I)}])^{-1}$$

Thus, on substituting eq A-3, A-6, and A-7 into eq A-4 we obtain eq 16.

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