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Kinetics and Equilibria of the Electron Transfer between Azurin and the Hexacyanoiron (II/III) Couple†

Michel Goldberg and Israel Pecht*

ABSTRACT: The electron transfer reaction between the "blue" single copper protein azurin (from *Pseudomonas aeruginosa*) and the hexacyanoiron (II/III) couple has been studied. Equilibrium constants for the reduction of azurin were measured spectrophotometrically in the temperature range 5–33 °C ($K = 1.1 \times 10^{-2}$ at 25 °C, $\Delta H° = 10.9$ kcal/mol, 0.1 M potassium phosphate, pH 7.0, $I = 0.22$). The enthalpy change was also determined by microcalorimetry and from the analysis of chemical relaxation amplitudes. Following a temperature-jump perturbation of this equilibrium, only a single relaxation was observed. The reciprocal of the relaxation time increased linearly as oxidized azurin was reacted with increasing amounts of ferrocyanide, yet reached saturation when reduced azurin was titrated with ferricyanide. This behavior as well as the analysis of the relaxation amplitudes led to the following scheme for this system:

$$\mathrm{Az(II)} + \mathrm{Fe(II)} \overset{K_1}{\rightleftharpoons} \mathrm{Az(II)} \cdot \mathrm{Fe(II)} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \mathrm{Az(I)} \cdot \mathrm{Fe(III)} \overset{K_2}{\rightleftharpoons} \mathrm{Az(I)} + \mathrm{Fe(III)}$$

At 25 °C the rate constants for the electron transfer were $k_{+3} = 6.4\ s^{-1}$ and $k_{-3} = 45\ s^{-1}$, the association constants $K_1 = 54\ M^{-1}$ and $K_2^{-1} = 610\ M^{-1}$. The activation and overall thermodynamic parameters as well as the individual thermodynamic values for the different steps were combined to construct a self-consistent energy profile for the reaction.

The azurins, "blue" single-copper proteins, being sequenced (Ambler and Brown, 1967) and readily available, have been used in recent years to study the electron transfer mechanism (Antonini et al., 1970; Pecht and Rosen, 1973), the function of specific amino acid residues (Finazzi-Agrò et al., 1970; McMillin et al., 1974), and the role of conformation (Grinvald et al., 1975) in this unique group of proteins. The exceptional spectroscopic characteristics of azurin from *Pseudomonas aeruginosa* are due to its copper chromophore which gives rise to an intense blue absorption band and to its single tryptophan which exhibits a well-resolved fine structure in the absorption and CD spectra (Tang et al., 1968) and an unusually blue-shifted emission band (Finazzi-Agrò et al., 1970). Though no structural information for azurin is yet available from x-ray crystallography, spectroscopic, magnetic resonance, and chemical studies indicate that the copper is bound at a relatively inaccessible site inside the protein (Finazzi-Agrò et al., 1970; Boden et al., 1974). No direct interaction between the copper ion and external ligands can be found. NMR[1] measurements of water proton relaxation establish a minimum distance of ~5 Å between the protein surface and the metal site (Koenig and Brown, 1973). Still, in terms of electron transfer, an efficient pathway to and from this site must exist as illustrated by the very fast electron exchange of azurin with its biological partner cytochrome-*c* P551 and the ease of its reduction by hydrated electrons (Faraggi and Pecht, 1971).

The electron transfer between azurin and cytochrome-*c* P551 has been investigated by stopped-flow and chemical relaxation methods (Wilson et al., 1975; Rosen and Pecht, 1976). Conformational equilibria of both proteins were found to affect this process. The actual electron-exchange step between the two proteins was evaluated to be some hundred to thousand times faster than observed for the electron transfer between azurin and simple redox reagents. This, together with the zero

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[1] Abbreviations used: Az(II), oxidized azurin; Az(I), reduced azurin; Fe(III), ferricyanide; Fe(II), ferrocyanide; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; phen, 1,10-phenanthroline.

or positive activation entropies found, reflects the unusual features of the electron transfer between the two proteins. The electron transfer to and from the redox site of "blue" single-copper proteins has recently been studied using inorganic redox couples (Wherland et al., 1975). The relative reactivity of azurin, stellacyanin, and plastocyanin in the reduction by $Fe(EDTA)^{2-}$ has been determined and it has been proposed that an "outer sphere" pathway is operative in the electron transfer.

In the present study, the electron transfer between azurin (from *Ps. aeruginosa*) and $Fe(CN)_6^{4-/3-}$, a well-defined redox couple known to react by an outer sphere mechanism, has been investigated by spectrophotometric titrations, temperature-jump relaxation spectroscopy (Eigen and De Maeyer, 1974), and microcalorimetry. The chemical, thermodynamic, and mechanistic properties of the $Fe(CN)_6^{4-/3-}$ couple have been extensively studied (Hanania et al., 1967; Campion et al., 1967) and, therefore, its use enabled a detailed analysis of the reactivity of azurin. The mechanism of electron transfer was found to involve protein–reductant and protein–oxidant binding equilibria, and a detailed energy profile, including thermodynamic and activation parameters, was obtained.

Experimental Procedures

Materials

Azurin was isolated and purified from *Pseudomonas aeruginosa* according to the procedures of Ambler and Brown (1967). The stock solution of azurin was stored at 4 °C in 0.05 M ammonium acetate buffer, pH 3.9. Before experiments, the buffer was exchanged by dialysis against 0.1 M potassium phosphate buffer, pH 7.0 (ionic strength 0.22 M), prepared from doubly glass distilled water and containing 20 μM EDTA. This buffer was used in *all* experiments. When necessary, the protein solution was concentrated by vacuum dialysis before use. Samples used in this study had A_{625}/A_{280} ratios in the range of 0.47 to 0.53.

Platinum black, used for catalytic reduction by hydrogen, was treated with 10% nitric acid, washed with distilled water, and dried under vacuum before use. Potassium ferrocyanide and ferricyanide were of analytical grade. The purity of the former was checked spectrophotometrically and was found to be >99.7%. Solutions of these reactants in oxygen-free phosphate buffer were always prepared just before use and protected from light. The extinction coefficient used for ferricyanide was ϵ_{420} 1020 M^{-1} cm^{-1} (Schellenberg and Hellermann, 1958).

Methods

Reduction of Azurin. Azurin was reduced by hydrogen, using platinum black as catalyst, according to the method developed by Rosen and Pecht (1976). This method led to a product identical with the one obtained by reduction with ascorbate, followed by dialysis, as determined by absorption spectra of the reduced and reoxidized protein (Goldberg and Pecht, to be published).

Spectrophotometric Measurements. All measurements of absorption spectra and titrations were carried out on a Cary 15 spectrophotometer equipped with a thermostated cell compartment. The temperature was monitored by a digital thermometer, the thermoelement of which had been attached to the cuvette.

Static Titrations. Titrations were performed, either by starting with oxidized azurin and titrating with ferrocyanide or starting with reduced azurin and titrating with ferricyanide. Anaerobic control experiments showed that aerobic conditions did not affect the titrations in the required time span (30 min). The starting concentrations of azurin were varied from 4×10^{-5} to 1.6×10^{-4} M. For the series of titrations of oxidized azurin, the temperatures were from 5.7 to 33.0 °C. Each titration usually involved the addition of six to ten aliquots of freshly prepared titrant solution. The absorbance of oxidized azurin at 625 nm was monitored. Corrections were made for dilution.

Microcalorimetry. The calorimetric measurements were carried out on an LKB 10700 batch microcalorimeter by Dr. L. Tumerman and Mr. R. Zhidovetsky. Since the equilibrium lies far on the side of oxidized azurin, only the reaction of reduced azurin with ferricyanide was measured. The concentration of the protein varied from 2×10^{-7} to 5×10^{-7} M; ferricyanide was added in a 2- to 20-fold excess. The values obtained were corrected for the heat of dilution by running separate control experiments with azurin and ferricyanide.

Relaxation Kinetics. Temperature-jump measurements were performed using a double-beam instrument (cf. Rosen and Pecht, 1976), with a cell adapted for anaerobic work. A flow of water-saturated, oxygen-free argon was maintained over the solution throughout the experiment. Temperature jumps of 2.9 or 4.7 °C as calibrated by the method described by Havsteen (1969) were applied to the reaction solutions. The subsequent changes in transmission were monitored at 625 (absorption maximum of oxidized azurin), 550 (azurin), or 420 nm (absorption band of ferricyanide). These relaxation signals were recorded on a Tektronix 549 storage oscilloscope and their amplitudes determined. The relaxation times were evaluated as described by Rosen and Pecht (1976), using an HP 2100 computer.

Relaxation measurements were all performed in 0.1 M potassium phosphate buffer, pH 7.0 (I = 0.22 M) with solutions of different initial composition. Either ferrocyanide was added to oxidized azurin, or ferricyanide to reduced azurin. Since the relative changes in ionic strength due to the added titrant were insignificant in practically all experiments, no compensation was made for the varying amounts of titrant added. The azurin concentrations used ranged from 8×10^{-6} to 8×10^{-4} M. The temperature range extended from 6.5 to 26.2 °C. Each reported value of the relaxation time or amplitude represents the average of at least four measurements.

Data Analysis. Where the parameters of a linear function had to be determined, the data were analyzed by standard least-squares procedures. The standard deviations of the slope or intercept were taken as error estimates of the corresponding parameters.

Results and Interpretation

1. Determination of Equilibrium Constants and Extinction Coefficient. The titration of azurin(I) and ferricyanide allowed a simultaneous determination of the equilibrium constant and of the extinction coefficient of the blue absorption band of azurin(II). Assuming a simple equilibrium

$$K = [Az(I)][Fe(III)]/[Az(II)][Fe(II)] \qquad (1)$$

where Az(I), Az(II), Fe(III), and Fe(II) represent the equilibrium concentrations of reduced and oxidized azurin and ferri- and ferrocyanide, respectively, we have the relation

$$\frac{[Fe]_t}{A_{625}} = \frac{K}{\epsilon_{625}}\left[\frac{A_{625}}{A_{625}^{\circ} - A_{625}}\right] + \frac{1}{\epsilon_{625}} \qquad (2)$$

where $[Fe]_t$, A_{625}, A_{625}°, and ϵ_{625} are the total concentration of hexacyanoiron, absorbance of azurin at 625 nm, absorbance

TABLE I: Thermodynamic Parameters.[a]

	Az(II) + $e^- \leftrightarrows$ Az(I)	Az(II) + Fe(II) $\leftrightarrows$ Az(I) + Fe(III)		Az(II) + Fe(II) $\leftrightarrows$ Az(II)Fe(II)	Az(II)Fe(II) $\leftrightarrows$ Az(I)Fe(III)	Az(I)Fe(III) $\leftrightarrows$ Az(I) + Fe(III)
$\Delta H°$ (kcal/mol)	-16.2 ± 1.2[b]	10.9 ± 1.2[c]	10.0 ± 0.9[d]	-5.5 ± 0.4[f]	7.8 ± 2.1[d]	7.7 ± 0.8[e]
		8.9 ± 1.3[e]	5.3 ± 1.1[f]		6.7 ± 0.9[i]	
		8.3 ± 0.3[g]				
$\Delta S°$ (eu)	-30.5 ± 3.1[b]	27.6 ± 3.1[c]		-10.5 ± 1.5[h]	22.3 ± 7.0[d]	13.1 ± 2.7[h]
		24.9 ± 3.0[d]				

[a] Potassium phosphate buffer (0.1 M), pH 7.0, 2×10^{-5} M EDTA, $I = 0.22$ M. [b] On the hydrogen electrode scale. [c] From the van't Hoff isochore. [d] Difference of corresponding activation parameters. Error estimated as sum of the absolute errors of the parameters combined. [e] From relaxation amplitudes, titration of azurin(I) with ferricyanide. [f] From relaxation amplitudes, titration of azurin(II) with ferrocyanide. [g] From microcalorimetry. [h] From $\Delta S° = \Delta H°/T + R \ln K$. Error estimated from $\delta\Delta H°/T + R\delta K/K$. [i] From relaxation amplitudes, combination of results from both types of titration.

of the fully oxidized azurin, and its extinction coefficient, respectively. Titration data plotted according to eq 2 are shown in Figure 1. The value for ϵ_{625} 5700 ± 70 M^{-1} cm^{-1}, obtained from the intercept, differs considerably from the generally accepted value of 3500 M^{-1} cm^{-1} (cf. Fee, 1975). However, the titrations of azurin(II) with ferrocyanide led to a similar result: from the increase of the ferricyanide absorption at 420 nm, being by stoichiometry proportional to the decrease of the azurin absorption at 625 nm, ϵ_{625} was found to be 5500–5900 M^{-1} cm^{-1}. The equilibrium constant, calculated from the ratio of slope to intercept, is $1.10 \pm 0.07 \times 10^{-2}$ at 25 °C.

The equilibrium was now measured by titrating azurin(II) with ferrocyanide. The spectra exhibit an isosbestic point at 449 nm (Figure 1, insert), suggesting the presence of only two absorbing species in this spectral region. The position of the equilibrium, strongly favoring the oxidation of azurin, made it necessary to modify the treatment of these titration data. The apparent equilibrium constant was calculated according to the expression

$$K = \left[\frac{A_{625}° - A_{625}}{A_{625}}\right]\left[\frac{\epsilon_{625}[\mathrm{Fe}]_t}{A_{625}° - A_{625}} - 1\right]^{-1} \qquad (3)$$

derived from eq 1 with the stoichiometric condition [Az(I)] = [Fe(III)], ϵ_{625} being now taken as 5700 M^{-1} cm^{-1}. Values of K, obtained in this way, consistently showed a slight decrease with increasing ferrocyanide concentration. This behavior repeated itself in all experiments. It was first ascribed to an ionic strength effect, as it is well known that the redox potential of the ferro–ferricyanide couple is very sensitive to the ionic strength (O'Reilly, 1973). The change of the redox potential $+\Delta E_I$ due to the increase in ionic strength is expected to affect the "true" equilibrium constant by a factor $\exp(-\mathcal{F}\Delta E_I/RT)$. We calculated values for ΔE_I from the data of O'Reilly (1973) and found that they never exceeded a few millivolts, thus contributing only negligibly to the decreasing trend of K. Therefore, "true" equilibrium constants were determined by extrapolation to zero concentration of ferrocyanide (Figure 2). This led to an excellent agreement with the results obtained from titrations of azurin(I) with ferricyanide.

2. Thermodynamic Parameters Derived from Equilibrium Data. The equilibrium constant was found to increase with temperature, showing that the reduction of azurin by ferrocyanide is endothermic (Table I). From the equilibrium constant and the reduction potential of the $Fe(CN)_6^{4-/3-}$ couple under the conditions used, +421 mV (O'Reilly, 1973), we get a value of +304 mV for the reduction potential of azurin at 25

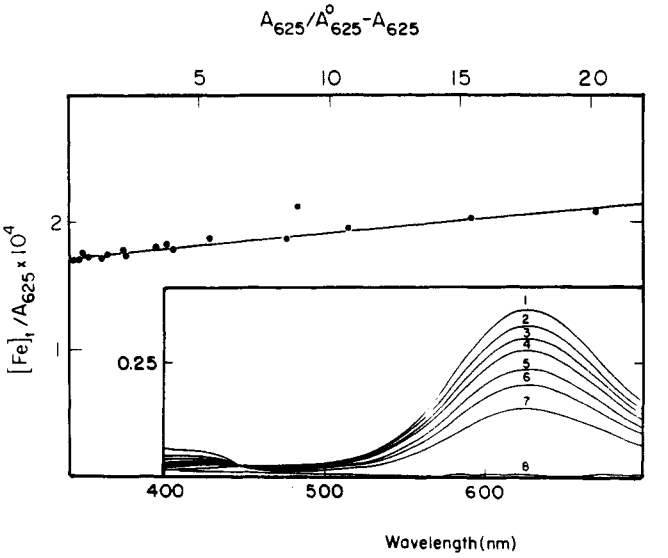

FIGURE 1: The equilibrium constant of the azurin–$Fe(CN)_6^{4-/3-}$ system and the extinction coefficient of the blue band of azurin(II) simultaneous determination. Data from three different titrations of azurin(I) (7×10^{-5} to 1.4×10^{-4} M) with ferricyanide at 25 °C are plotted as indicated by eq 2. (Insert) Spectrophotometric titration of azurin(II) (6.1×10^{-5} M) with ferrocyanide (2×10^{-2} M) at 15.5 °C. Curves are numbered according to the sequence of addition of the titrant. Abscissa: absorbance.

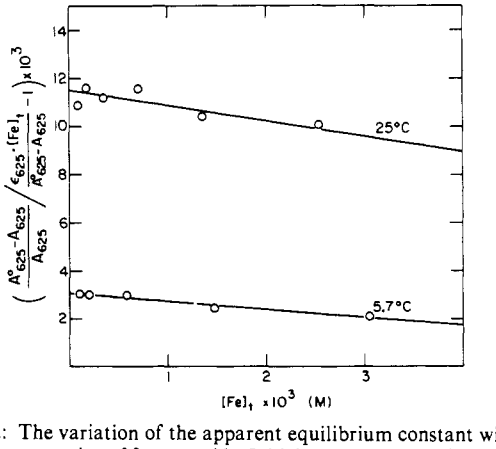

FIGURE 2: The variation of the apparent equilibrium constant with increasing concentration of ferrocyanide. Initial concentrations of azurin(II): 6×10^{-5} M (at 5.7 °C), 4×10^{-5} M (at 25 °C). Abscissa: $[Fe(II)] \simeq [Fe]_t$ (total concentration of hexacyanoiron). Ordinate: apparent equilibrium constants calculated according to eq 3.

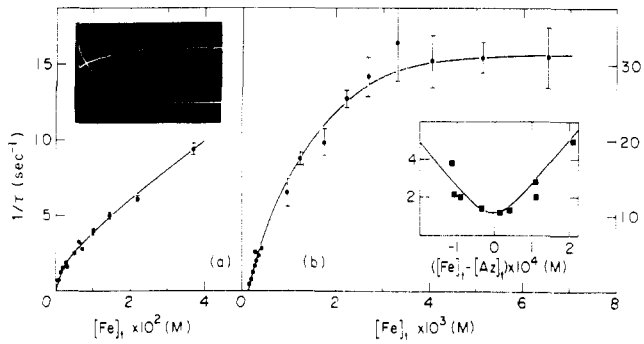

FIGURE 3: The concentration dependence of the reciprocal relaxation time. (a) Azurin(II) (2.6×10^{-5} to 1.6×10^{-4} M) reacted with ferrocyanide (24- to 860-fold excess), 6.5 °C. (Insert) Relaxation following a temperature-jump perturbation of the azurin(II/I)-$Fe(CN)_6^{4-/3-}$ equilibrium. (Upper trace) Formation of ferricyanide, wavelength 420 nm, vertical scale 0.6 mOD/scale unit; (lower trace) reduction of azurin(II), wavelength 625 nm, vertical scale 1.25 mOD/scale unit; (horizontal scale) 200 ms/scale unit. Total azurin 3.3×10^{-5} M, ferrocyanide 7.7×10^{-3} M; temperature rise of 4.7 to 23.5 °C. (b) Azurin(I) (2.8×10^{-4} to 5.3×10^{-4} M) reacted with ferricyanide, 16.8 °C. Points from measurements in the region $[Fe]_t < [Az]_t$ have been omitted for clarity. (Insert) Azurin(I) (2.3×10^{-4} M) reacted with ferricyanide, 23 °C. The curve drawn was calculated on the basis of the parameters given in Tables II and III. The reciprocal relaxation time has a minimal value at $[Fe]_t \simeq [Az]_t$.

°C. This is in excellent agreement with the value of +300 mV measured potentiometrically under similar conditions (pH 7.0, phosphate buffer) by Horio (1958). A frequently cited value, +328 mV, has been measured under different conditions (cf. Fee, 1975). The temperature coefficient of the azurin redox potential was found to be −1.3 mV/°C.

By subtracting the contribution of the $Fe(CN)_6^{4-/3-}$ couple from the thermodynamic parameters of the whole reaction system, we can get information which is independent of this redox couple and directly relevant to azurin. The data obtained will also be helpful in predicting the behavior of azurin with other redox reagents. Fortunately, a large body of information is available on the thermodynamic properties of the $Fe(CN)_6^{4-/3-}$ system (Eaton et al., 1967; Hanania et al., 1967, O'Reilly, 1973). From the temperature dependence of $E^{\circ\prime}$ of $Fe(CN)_6^{4-/3-}$, as given for conditions similar to ours (O'Reilly, 1973), we calculated the enthalpy (ΔH_{Fe}°) and entropy (ΔS_{Fe}°) of the $Fe(CN)_6^{4-/3-}$ half reaction relative to the normal hydrogen electrode, using the Gibbs–Helmholtz equation:

$$\Delta H_{Fe}^\circ = \mathcal{F}\left[-E^{\circ\prime}(T) + T\left(\frac{\partial E^{\circ\prime}(T)}{\partial T}\right)_P\right] \quad \text{and} \quad \Delta S_{Fe}^\circ = \mathcal{F}\left(\frac{\partial E^{\circ\prime}(T)}{\partial T}\right)_P \tag{4}$$

The results, $\Delta H_{Fe}^\circ = -27.1$ kcal/mol and $\Delta S_{Fe}^\circ = -58.1$ eu, are very similar to values derived for infinite dilution, $\Delta H_{Fe}^\circ = -26.7$ kcal/mol and $\Delta S_{Fe}^\circ = -62.1$ eu (Hanania et al., 1967). Combining these data with the reaction enthalpy (ΔH°) and entropy (ΔS°) of the azurin-$Fe(CN)_6^{4-/3-}$ system, we get the enthalpy ΔH_{Az}° and entropy ΔS_{Az}° of the azurin half reaction relative to the normal hydrogen electrode (Table I).

$$\Delta H_{Az}^\circ = \Delta H^\circ + \Delta H_{Fe}^\circ \text{ and } \Delta S_{Az}^\circ = \Delta S^\circ + \Delta S_{Fe}^\circ \tag{5}$$

Finally, the partial molar entropy change of the reduction of azurin was determined according to

$$\bar{S}_{Az(I)}^\circ - \bar{S}_{Az(II)}^\circ = \Delta S_{Az}^\circ - \Delta S_H^\circ = -11.6 \pm 3.0 \text{ eu} \tag{6}$$

where $\Delta S_H^\circ = -18.9$ eu (Noyes, 1962) is the entropy change of the normal hydrogen electrode. The value obtained, −11.6 eu, shows that the reduction of the azurin copper site is entropically unfavorable.

3. Relaxation Times and Amplitudes: Characteristic General Features. The temperature-jump relaxation spectrum consists of a single relaxation mode, expressed in a decrease in optical density at 625 nm, and an increase at 420 nm (Figure 3a, insert). It represents a net formation of reduced azurin and ferricyanide upon heating. This is expected, because the reduction of azurin was found to be endothermic (Table I). The relaxation curves showed the same behavior in all measurements done at both wavelengths, 625 and 420 nm, displaying similar relaxation times with an amplitude ratio of 5.5, as expected from the ratio of the extinction coefficients. Most of the measurements were carried out at 625 nm since the optical density change was largest at this wavelength and the light sensitivity of both ferro- and ferricyanide is much lower in this spectral region.

The relaxation time τ was measured over a wide range of protein and hexacyanoiron concentrations. Two characteristic forms of variation of τ^{-1} with concentration were found (Figures 3a and 3b). When azurin(II) was titrated with increasing amounts of ferrocyanide, τ^{-1} showed a monotonous increase up to very high concentrations (4×10^{-2} M). When azurin(I) was titrated with ferricyanide, a leveling-off with increasing concentrations of ferricyanide was observed. These features indicate that the electron transfer between azurin and $Fe(CN)_6^{4/3-}$ is not a simple bimolecular process as the observed single relaxation might suggest. The limiting dependence on ferricyanide concentration points toward a fast preequilibrium followed by slow electron transfer which becomes rate limiting under certain conditions.

In addition to the relaxation time, the method of chemical relaxation spectroscopy provides further information, in the form of the relaxation amplitude. The amplitude data must be consistent with any mechanism suggested by relaxation time data (Thusius et al., 1973). Sometimes, even additional mechanistic insight can be gained from the analysis of relaxation amplitudes.

The expression for the amplitude of a relaxation mode, induced by a temperature jump and detected by spectrophotometry, is given by

$$-\frac{1}{2.3d}\frac{\Delta V}{V_0} = \tilde{\Gamma}\Delta\tilde{H}\Delta\tilde{\epsilon}\frac{\Delta T}{RT^2} \tag{7}$$

provided that $\Delta V \ll V_0$ (Thusius et al., 1973; Jovin, 1975). Here are d (cm) the optical path length of the temperature-jump cell, ΔV (mV) the observed change of photovoltage ("amplitude"), and V_0 (mV) the total photovoltage. The normal gamma factor $\tilde{\Gamma}$ (M) gives the dependence on the equilibrium concentrations. $\Delta\tilde{H}$ (kcal mol^{-1}) and $\Delta\tilde{\epsilon}$ (M^{-1} cm^{-1}), the normal enthalpy and extinction changes of the relaxation mode, respectively, are linear combinations of the net enthalpy and extinction changes of the individual reaction steps. ΔT (K) is the temperature rise, T (K) the temperature after jump, and R (kcal mol^{-1} K^{-1}) the gas constant.

Generally speaking the expressions for $\tilde{\Gamma}$, $\Delta\tilde{H}$ and $\Delta\tilde{\epsilon}$, respectively, are concentration dependent and determined by the mechanism of reaction. Therefore the concentration dependence of the relaxation amplitude can be used as a diagnostic tool in the elucidation of the reaction scheme.

Using this approach we found the simplest scheme compatible with the behavior of both relaxation time and amplitude to be as shown in eq 8. At first, one would expect three relax-

ations for such a mechanism. But if we assume that the association equilibria involve no spectral changes, then their relaxations will not be detectable, since assumed to be much faster, they will be uncoupled from the slower electron transfer equilibrium which exhibits a spectral change. Thus the scheme will display only one relaxation, in agreement with our observation.

$$\mathrm{Az(II)} + \mathrm{Fe(II)} \underset{\text{(fast)}}{\overset{K_1}{\rightleftharpoons}} \{\mathrm{Az(II)} \cdot \mathrm{Fe(II)}\} \underset{k_{-3}\ \text{(slow)}}{\overset{k_{+3}}{\rightleftharpoons}} \{\mathrm{Az(I)} \cdot \mathrm{Fe(III)}\} \underset{\text{(fast)}}{\overset{K_2}{\rightleftharpoons}} \mathrm{Az(I)} + \mathrm{Fe(III)} \quad (8)$$

$$K_1 = [\{\mathrm{Az(II)} \cdot \mathrm{Fe(II)}\}][\mathrm{Fe(II)}]^{-1}[\mathrm{Az(II)}]^{-1}$$
$$K_2 = [\mathrm{Az(I)}] \cdot [\mathrm{Fe(III)}][\{\mathrm{Az(I)} \cdot \mathrm{Fe(III)}\}]^{-1}$$

4. Kinetic Results. The expression for the concentration dependence of the relaxation time is given by (see Appendix)

$$\frac{1}{\tau} = k_{+3}K_1 \frac{[\mathrm{Az(II)}] + [\mathrm{Fe(II)}]}{K_1([\mathrm{Az(II)}] + [\mathrm{Fe(II)}]) + 1} + k_{-3}\frac{[\mathrm{Az(I)}] + [\mathrm{Fe(III)}]}{K_2 + [\mathrm{Az(I)}] + [\mathrm{Fe(III)}]} \quad (9)$$

Using this equation we should be able to understand why the concentration dependence of the relaxation time may be different for the two types of titrations and why only one of the two fast steps can be kinetically observed.

Azurin (I) is oxidized to a large extent upon addition of *ferricyanide.* At $[\mathrm{Fe}]_t > [\mathrm{Az}]_t$ its concentration is very small, so that $[\mathrm{Az(II)}] = [\mathrm{Fe(II)}] \simeq [\mathrm{Az}]_t$ and $[\mathrm{Fe(III)}] \simeq [\mathrm{Fe}]_t - [\mathrm{Az}]_t$. The first term on the right-hand side of eq 9, representing the overall rate of azurin(II) reduction and designated h, will be practically constant. Thus, in the range $[\mathrm{Fe(III)}] \ll K_2$ the relaxation time changes linearly according to

$$\frac{1}{\tau} = h + \frac{k_{-3}}{K_2}[\mathrm{Fe(III)}] \quad (10)$$

In the intermediate region, where $[\mathrm{Fe(III)}] \sim K_2$, the reciprocal relaxation time will show typical saturation behavior

$$\frac{1}{\tau} = h + k_{-3}\frac{[\mathrm{Fe(III)}]}{K_2 + [\mathrm{Fe(III)}]} \quad (11)$$

and at $[\mathrm{Fe(III)}] \gg K_2$ a constant value will be reached, given by

$$\frac{1}{\tau} = h + k_{-3} \quad (12)$$

In Figure 4a we have plotted an experiment which corresponds to eq 10. Since, from results presented below, $h \ll 1/\tau$ eq 10 reduces to $1/\tau = (k_{-3}/K_2)([\mathrm{Fe}]_t - [\mathrm{Az}]_t)$, an expression containing only stoichiometric concentrations. This formula is not only convenient but also allows an independent evaluation of k_{-3}/K_2 without the prior knowledge of the overall equilibrium constant.

Measurements carried out in the saturation region are presented in Figure 3b. They offer the opportunity for partially checking the self-consistency of the proposed model. From the initial slope we estimate $k_{-3}/K_2 \simeq 3 \times 10^4\ \mathrm{M^{-1}\ s^{-1}}$, from the saturation level $k_{-3} \simeq 33\ \mathrm{s^{-1}}$ (keeping in mind that $h \ll 1/\tau$) and from half-saturation $K_2 \simeq 1 \times 10^{-3}$ M. Clearly, the mutual agreement is satisfactory.

Exact values of k_{-3} and K_2 may be obtained via a rearranged form of eq 11. Neglecting h again we transform it into

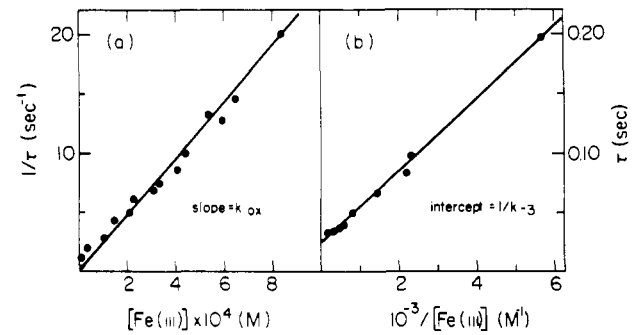

FIGURE 4: (a) Azurin(I) reacted with a small excess of ferricyanide—linear variation of the reciprocal relaxation time with ferricyanide concentration (eq 10). The plot includes the data from two sets of experiments. Total azurin, 2.3×10^{-4} and 5.2×10^{-4} M, 26.2 °C; $[\mathrm{Fe(III)}] = [\mathrm{Fe}]_t - [\mathrm{Az}]_t$. (b) Dependence of the relaxation time on the *reciprocal* concentration of ferricyanide (eq 13) for azurin(I) reacted with saturating amounts of ferricyanide. Total azurin, 5.3×10^{-4} M, 16.8 °C; $[\mathrm{Fe(III)}] = [\mathrm{Fe}]_t - [\mathrm{Az}]_t$.

$$\tau = \frac{K_2}{k_{-3}}\frac{1}{[\mathrm{Fe(III)}]} + \frac{1}{k_{-3}} \quad (13)$$

Data from measurements at 16.8 °C are plotted accordingly in Figure 4b. From the reciprocal intercept we get $k_{-3} = 38\ \mathrm{s^{-1}}$ and from the ratio of slope to intercept $K_2 = 1.1 \times 10^{-3}$ M.

In the titrations of *azurin(II)* with *ferrocyanide,* the concentrations [Az(I)] and [Fe(III)] generally remained small, due to the position of the overall equilibrium. In almost all cases, the relation $K_2 \gg [\mathrm{Az(I)}] + [\mathrm{Fe(III)}]$ was maintained. This simplifies eq 9 to

$$\frac{1}{\tau} = k_{\mathrm{red}}\frac{[\mathrm{Az(II)}] + [\mathrm{Fe(II)}]}{K_1([\mathrm{Az(II)}] + [\mathrm{Fe(II)}]) + 1} + k_{\mathrm{ox}}([\mathrm{Az(I)}] + [\mathrm{Fe(III)}]) \quad (14)$$

where $k_{\mathrm{red}} = k_{+3}K_1$ and $k_{\mathrm{ox}} = k_{-3}/K_2$.

In cases where $1/K_1 \gg [\mathrm{Az(II)}] + [\mathrm{Fe(II)}]$, eq 14 becomes

$$\frac{1}{\tau} = k_{\mathrm{red}}([\mathrm{Az(II)}] + [\mathrm{Fe(II)}]) + k_{\mathrm{ox}}([\mathrm{Az(I)}] + [\mathrm{Fe(III)}]) \quad (15)$$

an expression corresponding to a simple bimolecular reaction.

The analysis of relaxation time measurements was performed using a simplified and rearranged form of eq 14 (Appendix), namely

$$\frac{1}{\tau[\mathrm{Fe}]_t}(1 + K_1[\mathrm{Fe}]_t) = k_{\mathrm{red}} + 2k_{\mathrm{ox}}\frac{[\mathrm{Az(I)}]}{[\mathrm{Fe}]_t}(1 + K_1[\mathrm{Fe}]_t) \quad (16)$$

In Figure 5 several titrations of azurin(II) with ferrocyanide are plotted as suggested by eq 16. Values of K_1 at different temperatures were obtained from the results of the amplitude measurements (see below). The relation $1/K_1 \gg [\mathrm{Fe}]_t$ was valid for a large fraction of the experiments. This is consistent with the fact that in analyzing the experiments of Figure 5 according to eq 15 instead of eq 16, we got a very similar goodness of fit (regression coefficient). It is noteworthy that in experiments where ferrocyanide is present in large excess

TABLE II: Overall Rate and Equilibrium Constants Measured at Various Temperatures.[a]

T(°C)	$k_{red} = k_{+3}K_1$ (10^{-2} M^{-1} s^{-1})	$k_{ox} = k_{-3}K_2^{-1}$ (10^{-4} M^{-1} s^{-1})	k_{red}/k_{ox} ($\times 10^2$)	K^e ($\times 10^2$)
6.5	1.7 ± 0.5[b]	4.0 ± 0.3[b]	0.42 ± 0.18	0.32 ± 0.04
13.7	2.2 ± 0.4[b]	3.4 ± 0.2[b]	0.64 ± 0.14	0.56 ± 0.06
16.8		3.35 ± 0.18[c]		
18.7	2.7 ± 0.4[b]	3.1 ± 0.3[b]	0.87 ± 0.22	0.77 ± 0.08
20.0		1.2[d]		
23.5	3.30 ± 0.24[b]	2.75 ± 0.10[b]	1.20 ± 0.13	1.05 ± 0.12
26.2		2.4 ± 0.2[c]		

[a] Potassium phosphate buffer (0.1 M), pH 7.0, 2×10^{-5} M EDTA, $I = 0.22$ M. [b] Azurin(II) titrated with ferrocyanide; values evaluated according to eq 18. [c] Azurin(I) titrated with ferricyanide; values evaluated according to the simplified eq 12, as explained in the text. [d] From Antonini et al. (1970), measured by stopped flow. [e] Values calculated from interpolation of van't Hoff plot.

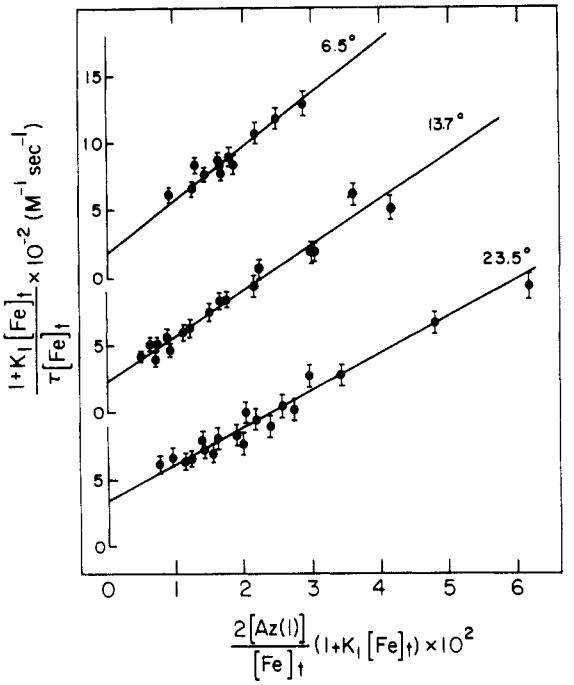

FIGURE 5: The variation of the relaxation time with concentration plotted according to eq 16 [azurin(II) titrated with ferrocyanide]. [Az(I)] and K_1 were evaluated as explained in the text. At 6.5 °C: data from two sets of measurements, total azurin, 2.6×10^{-5} and 1.1×10^{-4} M. At 13.7 °C: data from three sets of measurements, total azurin 8.9×10^{-6} to 7.3×10^{-5} M. At 23.5 °C: data from four sets of measurements, total azurin 2.6×10^{-5} to 7.2×10^{-5} M.

over other species, no direct kinetic evidence for any of the two postulated complex formations can be found. The reaction kinetics appears "simple bimolecular", as already reflected in Figure 3a.

Values for the overall rate constants k_{ox} and k_{red} are presented in Table II. "Kinetic" overall equilibrium constants were calculated from their ratio. In general, the agreement with the values obtained from static titrations is satisfactory (Table II) which constitutes support for the proposed mechanism. Further evidence comes from the temperature dependence of the rate constants. As can be seen from the slopes of the straight lines in Figure 5, the overall rate constant of azurin(I) oxidation, k_{ox}, decreases significantly with increasing temperature. This suggests that the process described by k_{ox} is not an elementary step and must be of a complex nature (Poerschke and Eigen, 1971). The activation parameters $\Delta H_{red}^{\ddagger} = 5.9$ kcal/mol and $\Delta S_{red}^{\ddagger} = -27.1$ eu for the overall reduction, and $\Delta H_{ox}^{\ddagger} = -4.1$ kcal/mol and $\Delta S_{ox}^{\ddagger} = -52$ eu for the overall oxidation were obtained from Eyring plots.

5. Analysis of Relaxation Amplitudes. The amplitude of relaxation is related to the enthalpy changes of the individual reaction steps. The equation corresponding to the proposed mechanism (eq 8) is given by

$$\Delta\tilde{H}\left(\frac{\Delta V}{V_0}, \tilde{\Gamma}\right) = \left(\frac{1}{2.3d}\frac{\Delta V}{V_0}\right)\frac{RT^2}{\epsilon_{625}\Delta T}\tilde{\Gamma}^{-1} = \frac{1}{K_1([Az(II)] + [Fe(II)]) + 1}\Delta H_1^\circ + \frac{K_2}{K_2 + [Az(I)] + [Fe(III)]}\Delta H_2^\circ + \Delta H_3^\circ \quad (17)$$

where

$$\tilde{\Gamma}^{-1} = \left(\frac{1}{[Az(II)]} + \frac{1}{[Fe(II)]}\right)\frac{1}{K_1([Az(II)] + [Fe(II)]) + 1} + \left(\frac{1}{[Az(I)]} + \frac{1}{[Fe(III)]}\right)\frac{K_2}{K_2 + [Az(I)] + [Fe(III)]}$$

and ΔH_1°, ΔH_2°, and ΔH_3° are the individual reaction enthalpies (Appendix). The other symbols are as defined above.

Here again, we have to distinguish between the two types of titrations. In those of azurin(I) with ferricyanide, the concentrations of azurin(II) and ferrocyanide produced were always small enough so that $(K_1[Az(II)] + K_1[Fe(II)] + 1)^{-1} \simeq 1$, simplifying accordingly the form of eq 17. This means that the extent of complex formation between azurin(II) and ferrocyanide was too small to be observed. A similar situation was present in experiments involving addition of ferrocyanide to azurin(II). Under the conditions employed, the concentrations of azurin(I) and ferricyanide never reached a level sufficient for significant complex formation between them. Formally, this corresponds to $K_2/(K_2 + [Az(I)] + [Fe(III)]) \simeq 1$.

Amplitude data from the set of measurements analyzed kinetically in Figure 4b were plotted in Figure 6 according to

$$\Delta\tilde{H}\left(\frac{\Delta V}{V_0}, \tilde{\Gamma}\right) = \frac{K_2}{K_2 + [Fe(III)]}\Delta H_2^\circ + \Delta H_1^\circ + \Delta H_3^\circ \quad (18)$$

taking $K_2/(K_2 + [Fe(III)])$ as abscissa and $\Delta\tilde{H}(\Delta V/V_0, \tilde{\Gamma})$ as ordinate. The evaluation of $\tilde{\Gamma}$ in $\Delta\tilde{H}(\Delta V/V_0, \tilde{\Gamma})$ was greatly facilitated by the fact that all terms *not* containing the factor

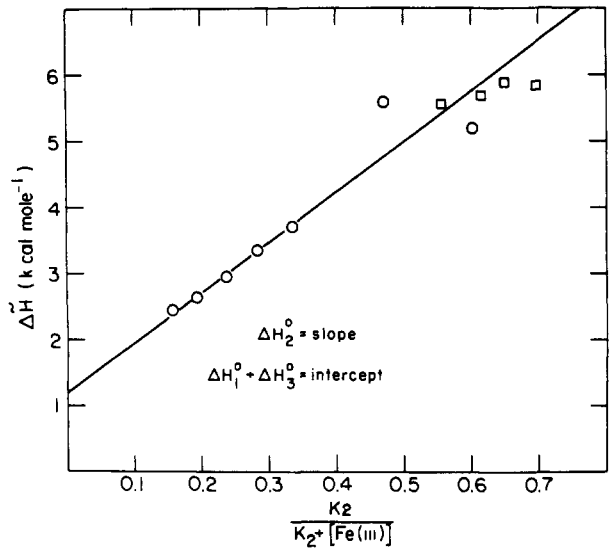

FIGURE 6: Relaxation amplitude data plotted according to eq 18 [azurin(I) titrated with ferricyanide]. $\Delta\tilde{H}$, the normal enthalpy of the electron transfer step, is given by eq 17. (○) Data from the set of measurements analyzed kinetically in Figure 4b, 16.8 °C, used for calculating the linear regression line. The value for K_2 was obtained from the slope to intercept ratio of Figure 4b. (□) Data from measurements at 23.0 °C, total azurin 3.9×10^{-4} M. K_2 (23 °C) = 1.44×10^{-3} M was evaluated from K_2 at 16.8 °C and ΔH_2° (slope of the regression line).

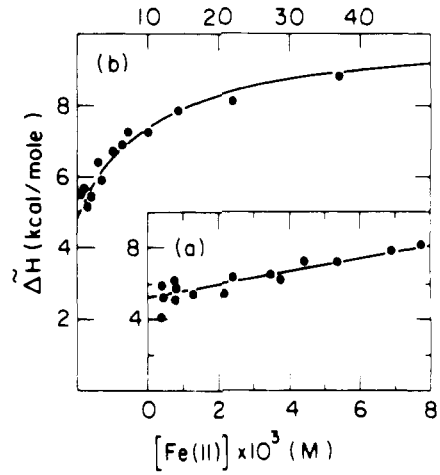

FIGURE 7: Relaxation amplitude data [azurin(II) reacted with ferrocyanide]—variation of $\Delta\tilde{H}$ with ferrocyanide concentration. (a) At 23.5 °C, plot according to eq 20 includes data from three different titrations. Total azurin 2.6×10^{-5} to 7.2×10^{-5} M. (b) At 6.5 °C, plot according to eq 19, includes data from three different titrations. The line drawn is the best fit to the experimental points with $K_1 = 1.0 \times 10^2$ M^{-1} and $\Delta H_1^\circ = -5.5$ kcal/mol. Total azurin 2.6×10^{-5} to 1.6×10^{-4} M.

1/[Az(I)] could be neglected due to experimental conditions. The plot shows a good linear fit and the values obtained, ΔH_2° = 7.7 kcal/mol and $\Delta H_1^\circ + \Delta H_3^\circ$ = 1.2 kcal/mol, seem reasonable with respect to sign and magnitude. The fact that a second set of data collected at a different temperature fits the line obtained (Figure 6) reflects the internal consistency of the analysis.

In Figure 7 amplitude data from titrations of azurin(II) with ferrocyanide are analyzed according to the simplified equation:

$$\Delta\tilde{H}\left(\frac{\Delta V}{V_0}, \tilde{\Gamma}\right) = \frac{1}{1 + K_1[\mathrm{Fe(II)}]}\Delta H_1^\circ + \Delta H_2^\circ + \Delta H_3^\circ \tag{19}$$

If K_1[Fe(II)] is significantly smaller than 1, but still large enough so that its variation will be expressed in $\Delta\tilde{H}$, then eq 19 may be approximated by

$$\Delta\tilde{H}\left(\frac{\Delta V}{V_0}, \tilde{\Gamma}\right) = (1 - K_1[\mathrm{Fe(II)}])\Delta H_1^\circ + \Delta H_2^\circ + \Delta H_3^\circ \tag{20}$$

It is evident that in Figure 7a ΔH varies in a manner which conforms to this function. From the slope we evaluate $K_1\Delta H_1^\circ = -365$ kcal mol^{-2} (23.5 °C). Figure 7b shows the saturation effect predicted by eq 19 for large concentrations of ferrocyanide. The curve drawn is the best fit to the experimental points with $K_1 = 1.0 \times 10^2$ M^{-1} (at 6.5 °C) and $\Delta H_1^\circ = -5.5$ kcal/mol. Thus, the binding of ferrocyanide ion to azurin(II) is also exothermic and the magnitude of its enthalpy change is similar to ΔH_2°. The association constant K_1 at 23.5 °C can be evaluated in two ways: either from the results of Figure 7b, using van't Hoff's formula, or by dividing $K_1\Delta H_1^\circ$ from Figure 7a by the value of ΔH_1° obtained from Figure 7b. The first method yields 57 M^{-1}, the second 66 M^{-1}, in good agreement taking into account the approximations involved.

Whereas the association equilibrium between azurin(I) and ferricyanide was already indicated by the rate saturation at high ferricyanide concentration (Figure 3b), the evidence for the association of ferrocyanide to azurin(II) came so far only from the amplitude measurements. It therefore appeared important to exclude experimental factors as the cause for the observed variation of $\Delta\tilde{H}$. Among others, we considered and rejected the influence of systematic errors in the amplitude measurements, photochemical changes in the $Fe(CN)_6^{4-/3-}$ system, autoxidation of ferrocyanide or azurin(I), effects of ferrocyanide concentration on the partial molar enthalpy difference of the $Fe(CN)_6^{4-/3-}$ couple, and ionic strength effects. Before adopting the mechanism proposed, we also considered and analyzed further mechanistic alternatives such as abortive binding of ferrocyanide to azurin(I) or of ferricyanide to azurin(II), the isomerization of free or complexed azurin (Rosen and Pecht, 1976), or binding of potassium cations to one of the reacting species. The data could not be fit to any of these alternatives.

Support for the postulated mechanism was provided by the static titrations of azurin(II) with ferrocyanide. Recall that the apparent equilibrium constant varied linearly with increasing ferrocyanide concentration (Figure 2). This behavior can easily be explained in terms of the mechanism proposed. The general expression for the apparent equilibrium constant is given by

$$K_{app} = K_1K_2K_3 \times \frac{1 + ([\mathrm{Az(I)}] + [\mathrm{Fe(III)}])/K_2 + [\mathrm{Az(I)}][\mathrm{Fe(III)}]/K_2^2}{1 + ([\mathrm{Az(II)}] + [\mathrm{Fe(II)}])K_1 + [\mathrm{Az(II)}][\mathrm{Fe(II)}]K_1^2} \tag{21}$$

Since under our experimental conditions [Fe(II)] $\gg$ [Az(II)], $K_2 \gg$ [Az(I)] = [Fe(III)] and K_1[Az(II)] $\ll 1$ we get

$$K_{app} = K_1K_2K_3\frac{1}{1 + K_1[\mathrm{Fe(II)}]} \simeq K_1K_2K_3 \times (1 - K_1[\mathrm{Fe(II)}]) \tag{22}$$

(compare derivation of eq 20). Plotting K_{app} vs. [Fe(II)], one obtains the "true" overall equilibrium constant (intercept) and the association constant K_1 (ratio of negative slope to inter-

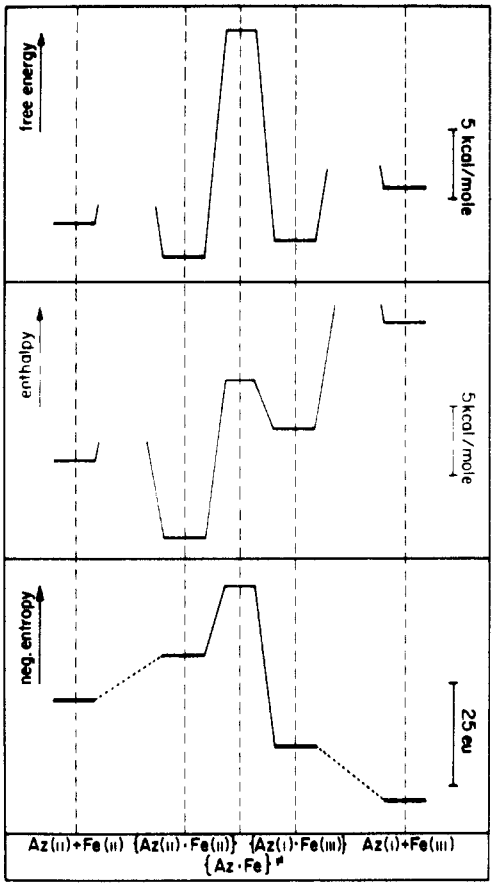

FIGURE 8: Free energy, enthalpy, and entropy profiles of the azurin(II/I)-$Fe(CN)_6^{4-/3-}$ reaction. The free energy has been taken at 25 °C. The standard state is 1 M reactants, in 0.1 M potassium phosphate buffer, pH 7.0, I = 0.22. The entropy scale has been chosen to be identical with the energy scales for 298 K.

cept). The titrations presented in Figure 2 yielded results in close agreement with the data derived from relaxation amplitude measurements: K_1 = 115 M^{-1} at 5.7 °C and 57 M^{-1} at 25.0 °C. In titrations at other temperatures we got less satisfactory agreement (138 M^{-1} at 15.5 °C and 87 M^{-1} at 33.0 °C); still the variation of K_{app} corresponded basically to eq 22.

6. Enthalpy and Entropy Profile of the Reaction. The additivity of thermodynamic reaction parameters permits a further independent check of the mechanism, for thermodynamic data obtained by different methods relating to the same or to different parts of the reaction should form a self-consistent set. A detailed summary is presented in Table I.

In the present case, one simple approach to examine internal consistency is to compare the different values for the overall enthalpy changes $\Delta H°$ as determined by the different methods used. These include: (1) study of the temperature dependence of overall equilibrium constants (van't Hoff isochore); (2) study of the temperature dependence of rate constants (Eyring equation); (3) analysis of relaxation amplitudes; and (4) microcalorimetry. Microcalorimetric experiments were undertaken because they offer the possibility of measuring $\Delta H°$ directly, while the other techniques yield $\Delta H°$ only as a derived value, the physical change primarily observed being a spectral change.

Considering the given error limits, the agreement between the various values for $\Delta H°$ is quite satisfactory (Table I), with one exception. $\Delta H°$ as evaluated from relaxation amplitudes in experiments involving titration of azurin(II) with ferrocyanide is about 3 to 5.5 kcal/mol lower than the other values. The reason for this discrepancy is not known. Nevertheless, it does not seem to us large enough to impair significantly the mechanistic model developed up to here.

The microcalorimetric value for $\Delta H°$ is slightly outside the error range of $\Delta H°$ obtained from a van't Hoff plot. Varying the excess of ferricyanide reacting with azurin(I) had no effect on the heat of reaction, showing that this difference cannot be explained by nonreactive binding of ferricyanide or similar processes. Such differences between calorimetric and van't Hoff data are well-known observations in protein chemistry (Lumry, 1971). They arise from small nonspecific heat effects that cannot be eliminated or appropriately corrected, or to additional slow conformational changes not expressed in the spectral changes observed.

We conclude that on the whole the enthalpy data support the mechanism proposed. Moreover, the combination of various experimental methods enabled us to construct the complete enthalpy and entropy profile of the reaction studied. It is presented in Figure 8.

Discussion

The majority of redox reactions between metalloredox proteins and inorganic agents studied to date has been characterized as simple bimolecular (Bennett, 1973). In several cases the involvement of intermediates has been considered even if direct kinetic evidence was lacking. However, in some redox reactions of cytochrome *c*, complex reaction patterns have actually been observed (Creutz and Sutin, 1974). The kinetics of the cytochrome *c*-$Fe(CN)_6^{4-/3-}$ system have been studied extensively for many years, but only recently was it found that the mechanism involves the formation of relatively stable "outer sphere" complexes between protein and hexacyanoiron (Stellwagen and Shulman, 1973; Miller and Cusanovich, 1975). The results of the present study show that a similar behavior characterizes the azurin-$Fe(CN)_6^{4-/3-}$ system. Its reaction pattern is presented in Figure 8 in terms of free energy, enthalpy, and entropy profiles that are plotted along the formal reaction coordinate (cf. Tables I and III for a summary of the essential parameters). Electron transfer within the complex is clearly the rate-limiting step (at a standard state of 1 M reactants, 25 °C). The entropy barrier largely controls the rate of the overall reaction in both directions, to the extent of becoming the only control in the overall oxidation, overcoming the favorable enthalpy term. In terms of free energy, the reaction is approximately symmetrical about the electron-transfer transition state. This is because the widely varying enthalpies and entropies of the stable states along the reaction path compensate for each other to a large extent. The general course of the entropy profile is not different from that expected for an elementary bimolecular reaction, namely a steady decrease of entropy from the separated reactants to the transition state and a steady increase from the transition state to the separated products. The complexity of the reaction is due to the enthalpy profile. The enthalpy of the transition state for the electron transfer is about midway between reactants and products, which is quite unusual. The electron transfer transition state is closer to the azurin(I)-ferricyanide complex in terms of enthalpy. This suggests that the electronic structure and the bond energies of the transition state resemble more the azurin(I)-ferricyanide complex than the azurin(II)-ferrocyanide complex.

The overall thermodynamic changes accompanying the change of the redox state of azurin (Table I) deserve some

TABLE III: Azurin and *Rhus rubrum* Cytochrome c_2 in Their Interaction with $Fe(CN)_6^{4-/3-}$.

		Overall Reaction		Association of $Fe(CN)_6^{4-/3-}$			Electron Transfer		
		Reduction	Oxidation		$Fe(CN)_4^{4-}$ + Ox. Protein	$Fe(CN)_6^{3-}$ + Red. Protein		Reduction	Oxidation
Ps. aeruginosa azurin[a]	k^c	3.45×10^2	2.71×10^4	$K_{ass}{}^f$	54	610	k^g	6.4	45
	$\Delta H^{\ddagger d}$	5.9	−4.1	$\Delta H°$	−5.5	−7.7	$\Delta H^{\ddagger}$	11.4[h]	3.6[i]
	$\Delta S^{\ddagger e}$	−27.1	−52.0	$\Delta S°$	−10.5	−13.1	$\Delta S^{\ddagger}$	−16.6[j]	−38.9[k]
R. rubrum cytochrome c_2[b]	k^c	2.1×10^4	1.8×10^6	$K_{ass}{}^f$	67	3900	k^g	310	460
	$\Delta H^{\ddagger}$	11.4	5.2	$\Delta H°$	3.5	−8.8	$\Delta H^{\ddagger}$	7.9	14.0
	$\Delta S^{\ddagger}$	−0.5	−12.5	$\Delta S°$	20.1	−13.1	$\Delta S^{\ddagger}$	−20.6	0.6

[a] Potassium phosphate buffer (0.1 M), pH 7.0, I = 0.22 M, 25 °C. [b] Reinterpreted results from Wood and Cusanovich (1975),[2] 0.02 M potassium phosphate buffer, 0.2 M NaCl, pH 7.0, I = 0.235 M, 25 °C. [c] In M^{-1} s^{-1}. [d] All enthalpy values given in kcal/mol. [e] All entropy values given in eu. [f] In M^{-1}. [g] In s^{-1}. [h] From $\Delta H_{red}{}^{\ddagger} - \Delta H_1°$. [i] From $\Delta H_{ox}{}^{\ddagger} + \Delta H_2°$. [j] From $\Delta S_{red}{}^{\ddagger} - \Delta S_1°$. [k] From $\Delta S_{ox}{}^{\ddagger} + \Delta S_2°$.

comments. Its high redox potential, compared with most simple copper complexes, is due to a favorable enthalpy change (−16.2 kcal/mol on the hydrogen electrode scale). In contrast, the Cu(II)/Cu(I) aquo couple ($E°'$ = 0.16 V), for example, has an unfavorable enthalpy change (+3.6 kcal/mol) and its reduction is entirely entropy driven (Latimer, 1952). It is noteworthy that the enthalpy change of azurin and of the "blue" copper of Rhus laccase are practically the same at 25 °C (Goldberg and Pecht, to be published), in spite of their widely differing functions and kinetic reactivity. This result is in line with the current concepts of the coordination of the "blue" copper, which is proposed to be distorted tetrahedral or trigonal-bipyramidal (McMillin et al., 1974; Miskowski et al., 1975), favoring the Cu(I) over the Cu(II) state due to more favorable bonding interactions between the copper and its ligands.

The partial molar entropy of azurin, measured in 0.1 M phosphate buffer, pH 7.0, decreases upon reduction. Various factors may contribute to this effect, the most important to be considered being changes of charge, conformation, and solvation. In view of the low dielectric constant of protein (ϵ 2, McGuire et al., 1972) one may expect significant interactions between the copper site charge and charges on the surface of the azurin molecule. Alternatively, the copper site may interact with polar residues in its immediate surroundings. Reducing the copper must, of course, affect such interactions and this will be reflected in the entropy balance. For the former, it can be shown that the entropy will always decrease upon reduction. Such an effect could easily account for the entropy difference observed. The change in the Born entropy of charging, on the other hand, is estimated to be negligible. Due to limited experimental information, it is difficult to assess the importance of conformation and solvation effects on the entropy. However, the finding that reduced azurin exists as two conformers in solution (Rosen and Pecht, 1976; Grinvald et al., 1975) and the indications that "water compensation" may be an important factor in the interconversion of the two conformers (Rosen and Pecht, 1976) suggest that these effects are not negligible.

The binding of hexacyanoiron to azurin is weak as shown by the low association constants (Table III). It is comparable in strength to the association of hexacyanoiron to *c*-type cytochromes. From NMR and equilibrium dialysis experiments, it has been found that horse heart cytochrome *c* binds hexacyanoiron with constants in the range from 100 to 3000 M^{-1} depending, among other parameters, on the ionic strength (Stellwagen and Shulman, 1973; Stellwagen and Cass, 1975). Similar values (Table III) can be calculated for *Rhodospirillum rubrum* cytochrome c_2 from the analysis of stopped-flow experiments reported by Wood and Cusanovich (1975).[2] Considering this range of values, relatively high concentrations of hexacyanoiron are necessary to make the complex formation kinetically detectable. This could explain why, in a preliminary study of the oxidation of azurin(I) with excess ferricyanide, the reaction was found to be simple bimolecular (Antonini et al., 1970). Based on information accumulated about the binding of small molecules and ions to proteins (Hammes and Schimmel, 1970), we assume that the difference between the association constants K_1, binding of ferrocyanide to azurin(II), and $K_2{}^{-1}$ binding of ferricyanide to azurin(I) mainly arises from different lifetimes of the complexes, whereas the specific rates of association are similar.

The formation of thermodynamically stable precursor complexes may be somewhat surprising in the case of azurin. The binding of the highly charged hexacyanoiron anions is expected to be primarily electrostatic. Yet the overall net charge of azurin at pH 7.0 is negative, its isoelectric point being 4.9 (Blatt and Pecht, unpublished results). This apparent discrepancy can be explained, if one assumes the existence of a cluster of positively charged groups (e.g., lysyl and arginyl residues) on the surface of the protein, where reactive binding can take place (azurin from *Ps. aeruginosa* contains 11 lysine and 1 arginine residues (Ambler and Brown, 1967)). Such an arrangement has been shown to be operative in the case of horse heart cytochrome *c* (Stellwagen and Cass, 1975) and has been postulated for *R. rubrum* cytochrome c_2 (Wood and Cusanovich, 1975). The comparison with cytochrome c_2 is especially interesting because, like azurin, it also is negatively charged at pH 7.0 (pI = 6.2). The overall behavior of the two redox proteins is quite similar in terms of rate and association constants, but less so in terms of the enthalpy and entropy

[2] We have reinterpreted the data presented by Wood and Cusanovich. Their assignments of experimentally obtained rate constants to the various reaction steps imply that the formation and dissociation of cytochrome c_2–hexacyanoiron complexes are rate determining. However, an interpretation where the *electron-transfer step* is rate determining seems to us more plausible. The limiting rates are therefore assigned to the electron-transfer step within the complex and not to the decay of the successor complex. The initial slopes of the second-order plots are identified with $K_c k_e$ (K_c is the formation constant of precursor complex; k_e is the first-order rate constant of the electron transfer) and not with the second-order rate constants for the combination of the separated reactants, as proposed by Wood and Cusanovich.

TABLE IV: Characteristic Data for the Reaction of Azurin with Inorganic Redox Agents and for the Reaction of Some "Blue" Copper Proteins with Ferrocyanide.

	I (M)	k (25 °C) (M^{-1} s^{-1})	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (eu)	$\Delta E^{\circ\prime}$[a] (V)
Azurin(I) + $Fe(CN)_6^{3-}$ [b]	0.22	2.7×10^4	−4.1	−52.0	+0.12
Azurin(I) + $Co(phen)_3^{3+}$ [c]		3.2×10^3	14.3	7.0	+0.12
Azurin(II) + $Fe(CN)_6^{4-}$ [b]	0.22	3.4×10^2	5.9	−27.1	−0.12
Azurin(II) + Fe EDTA^{2-} [d]	0.20	1.3×10^3	2	−37	+0.19
Plastocyanin + $Fe(CN)_6^{4-}$ [e]	0.20	1.9×10^4	8.4	−10.6	−0.04
Fungal laccase B + $Fe(CN)_6^{4-}$ [f]	0.10	1.5×10^6			0.36
Rhus laccase + $Fe(CN)_6^{4-}$ [g]	0.20	24.9	18.4	9.8	~0

[a] $E^{\circ\prime}$(azurin) = 0.304 V. [b] This paper. Values for the overall reaction. [c] S. Wherland and H. Gray, personal communication. [d] Wherland et al. (1975). [e] Plastocyanin from bean; Fensom and Gray, cited in Holwerda and Gray (1975). [f] Andréasson et al. (1973); reduction of "blue" copper. [g] Holwerda and Gray (1975); reduction of "blue" copper.

contributions (Table III). Whereas the complex formation with azurin is exclusively enthalpy driven for both ferro- and ferricyanide, the binding of ferrocyanide to cytochrome c_2 (III) is due to a favorable entropy change. The first-order rate of oxidation of the protein redox center is controlled by an entropy barrier in the case of azurin, whereas an enthalpy barrier is dominant for cytochrome c_2. We conclude that, although defined loci of electron transfer are expected to exist for the two proteins, the details of their interaction with hexacyanoiron are quite different.

Ferrocyanide displays a marked tendency to form electrostatic outer sphere complexes. However, in addition to the electrostatic interaction, hydrogen bonding may also contribute to the binding of hexacyanoiron to azurin. It should be noted that under the experimental conditions used (0.16 M K^+, I = 0.22 M) both ferro- and ferricyanide ions exist largely as ion pairs with potassium ions (at 25 °C: 50% $KFe(CN)_6^{3-}$, 32% $K_2Fe(CN)_6^{2-}$; ~40% $KFe(CN)_6^{2-}$; Eaton et al., 1967). It is not known what species participate in the reaction with azurin. But because the potassium concentration was always kept in constant large excess and as its ion pairing equilibrium is almost temperature independent, no specific potassium effect could be detected in our measurements.

In the absence of information about the three-dimensional structure of azurin, any detailed proposal concerning the electron pathway within the azurin–hexacyanoiron complex will be largely speculative. An estimate of the minimum distance between the two metal centers in the complex is 9–10 Å (radius of $Fe(CN)_6^{4-/3-}$ = 4.4 Å, Nightingale, 1959; copper–protein surface distance ~5 Å, Koenig and Brown, 1973). The actual electron-transfer distance, however, is probably shorter than the separation of the metal centers because a considerable degree of covalency exists in both coordination spheres involved, including substantial delocalization of the redox orbitals onto the ligand atoms. But based on the available information (nonaccessibility of the copper, bond distance Cu–ligand 1.9–2.2 Å, extremely slow autoxidation of azurin), it is not probable that ligand atoms of the copper are freely accessible on the surface. This would, therefore, exclude direct contact between the two redox sites and strongly diminish the possibility of direct interactions between metal-centered redox orbitals.

The activation parameters of the electron transfer within the complex (Table III) can conceptually be partitioned into "intrinsic" contributions from each of the partners plus a reaction specific component. A rough estimate of the contribution from $Fe(CN)_6^{4-/3-}$ is obtained from the parameters of its self-exchange reaction (Campion et al., 1967), after subtraction of Coulombic and encounter terms.[3] Under the assumption that the reaction specific component is of the order of $\frac{1}{2}\Delta H_3^\circ$ and $\frac{1}{2}\Delta S_3^\circ$ (Jacks et al., 1974), we find a highly negative "intrinsic" entropy contribution (−25 to −30 eu) and a low "intrinsic" enthalpy contribution (2–4 kcal/mol) of azurin to the activation barrier. (Note that these estimates do not contain electrostatic terms.) Several mechanistic explanations are conceivable.

It is tempting to regard a large negative value for the entropy of activation of a unimolecular electron-transfer step as indicative for a nonadiabatic reaction. Such reactions are characterized by a very low probability p of electron transfer in the transition state. The apparent activation entropy becomes largely negative because it contains the term $R \ln p$, $p \ll 1$. (A factor of ten in the magnitude of p lowers $\Delta S_{app}^{\ddagger}$ by −4.6 eu.) A small value of p is the result of very weak electronic interaction between the reactants, for reasons such as excessive distance between the redox centers. This would be consistent with the steric situation outlined above for our case.

Alternatively, resonant (adiabatic) electron transfer may occur if the deeply buried redox site is connected to the protein surface by a system of bonds or overlapping orbitals which allow delocalization of the redox orbital so that the required interaction with the external redox partner becomes possible. This could be attained through imidazole, guanidino, or disulfide groups involving the redox site, a part of the protein and the solvation shell. The spatial reorganization processes necessary to establish such a "conduction pathway" and to satisfy Franck–Condon requirements are a priori not expected to be strongly "coupled". Weak "coupling", i.e, low probability of all these distortions from equilibrium positions to occur simultaneously, however, would be reflected by a large negative activation entropy.

The low "intrinsic" enthalpy barrier of azurin reduction and oxidation indicates low energetic requirements for the Franck–Condon rearrangement of its redox site. This is in accord with the proposition that the activation process of the transition between the bivalent and the monovalent state involves only small changes of the coordination geometry around the "blue" copper center (Miskowski et al., 1975).

We have compiled in Table IV the overall rate constants and

[3] The "intrinsic" contribution of $Fe(CN)_6^{4-/3-}$ was estimated from the expression $\frac{1}{2}(\Delta X^{\ddagger} - \Delta X_{encounter}^{\ddagger} - \Delta X_{Coulomb}^{\ddagger})$, where X denotes enthalpy and entropy, respectively, using the values given by Campion et al. (1967).

overall activation parameters for the reaction of azurin with different redox couples as well as for the reduction of several other "blue" copper proteins by ferrocyanide. Ferricyanide oxidizes azurin(I) at a rate about one order of magnitude faster than $Co(phen)_3^{3+}$ (J. McArdle and H. B. Gray, personal communication), although the net driving force in terms of free energy is the same for both oxidants. This rate difference seems at least qualitatively explainable; the reactivity of ferricyanide is higher than that of $Co(phen)_3^{3+}$, as concluded from their self-exchange rates, corrected for Coulombic factors (Campion et al., 1967; Baker et al., 1959). However, a comparison of the activation parameters of the two oxidation reactions suggests that they follow different pathways. Even if we take into account that in redox reactions of $Fe(CN)_6^{3-/4-}$ the activation barrier tends to be mainly entropic in nature, while for $Co(phen)_3^{3+/2+}$ it is generally enthalpic, the differences in the activation parameters are too large to be explained in terms of these tendencies alone.

The relative similarity of the activation parameters for the reduction of azurin(II) by $Fe(EDTA)^{2-}$ (Wherland et al., 1975) and by ferrocyanide is interpreted as indicating that these two reactions proceed along similar paths. The very low (overall) $\Delta H^{\ddagger}$ and the largely negative (overall) $\Delta S^{\ddagger}$ of the $Fe(EDTA)^{2-}$ reaction suggest that complex formation followed by slow electron transfer may be a distinct step also for $Fe(EDTA)^{2-}$. The low value of $\Delta H^{\ddagger}$ would then be a consequence of the combination of the activation enthalpy of the actual electron-transfer step with a negative association enthalpy. In fact, the value of 2 kcal/mol measured for $\Delta H^{\ddagger}$ is at the lower limit of the activation enthalpy predicted for a purely diffusion-controlled reaction. Note that the lower rate of the reduction by ferrocyanide compared with $Fe(EDTA)^{2-}$ is due to the significantly higher redox potential of the former.

The reactivity of "blue" copper proteins toward ferrocyanide varies over several orders of magnitude (Table IV). A meaningful assessment of the reactivity of azurin as compared with the other proteins listed can be made by calculating *relative* apparent self-exchange rates (Wherland et al., 1975), using the formalism of the relative Marcus theory. Contributions of the net driving force can thereby be "subtracted". Moreover, there is no need to know the $Fe(CN)_6^{4-/3-}$ self-exchange rate. Relative Marcus theory in its usual form (Bennett, 1973) is difficult to apply to reactions involving $Fe(CN)_6^{4-/3-}$ because its self-exchange rate is very sensitive to the identity and concentration of cations like potassium (Campion et al., 1967) and, although the concentration dependence is accurately known, it is not quite clear what is the correct value of the self-exchange rate constant to be used for each case. The apparent self-exchange rates relative to azurin are 2.4×10^2, 0.2×10^{-5}, and 6×10^{-5} for bean plastocyanin, fungal laccase B and Rhus laccase, respectively. The ratio for plastocyanin is remarkable since it is very similar to the ratio of self-exchange rates obtained from the reaction with $Fe(EDTA)^{2-}$ (Wherland et al., 1975). This is a further indication that the two reductants follow a similar path in their reaction with azurin. The lower reactivity of azurin compared with plastocyanin is primarily due to the higher entropy barrier. As suggested, it may reflect the degree to which the blue center is "insulated" from the reductant. The value for fungal laccase B shows that it is the thermodynamic driving force which makes its reduction by ferrocyanide fast compared with azurin.

In the study of the reaction between azurin and its physiological partner, cytochrome *c* P551, the electron transfer was found to be affected by a conformational equilibrium between two isomers of azurin(I) (Rosen and Pecht, 1976). This equilibrium is not expressed in the azurin(I)–ferricyanide reaction, probably because ferricyanide reacts with both conformers to the same extent and is not as sensitive to this conformational difference as ferricytochrome *c* P551. This may be a reflection of the specificity of the azurin(I)–ferricytochrome *c* P551 interaction, which is further illustrated by an electron-transfer rate more than 200-fold faster than with ferricyanide, in spite of the significantly higher redox potential of the latter reagent. The large positive activation entropy (+18.8 eu) of the azurin(I)–ferricytochrome *c* P551 reaction suggests that it is not only the markedly higher intrinsic reactivity of the ferricytochrome which enhances the rate compared with ferricyanide, but again specific protein–protein interactions. In conclusion, the comparison of the reactivity of azurin toward these two types of iron-containing redox partners illustrates the specificity existing in the reaction between redox proteins. This specificity involves a variety of protein residues and extends beyond the immediate coordination sphere of the redox center.

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Appendix

(1) The expressions for relaxation times and amplitudes can be derived in a straightforward way using the formalism developed by Castellan (1963) and Jovin (1975). The starting point is the **g** matrix as defined by Castellan. For the scheme proposed in eq 10, it is given by:

$$\mathbf{g} = \begin{pmatrix} \Gamma_1^{-1} & 0 & -[P]^{-1} \\ 0 & \Gamma_2^{-1} & -[Q]^{-1} \\ -[P]^{-1} & -[Q]^{-1} & \Gamma_3^{-1} \end{pmatrix}$$

where $[P] = [\{Az(II)\cdot Fe(II)\}]$, $[Q] = [\{Az(I)\cdot Fe(III)\}]$, $\Gamma_1^{-1} = [Az(II)]^{-1} + [Fe(II)]^{-1} + [P]^{-1}$, $\Gamma_2^{-1} = [Az(I)]^{-1} + [Fe(III)]^{-1} + [Q]^{-1}$, and $\Gamma_3^{-1} = [P]^{-1} + [Q]^{-1}$.

(2) The slow relaxation time is then calculated from

$$1/\tau_3 = r_3|\mathbf{g}_{3,3}||\mathbf{g}_{2,2}|^{-1}$$

where

$$r_3 = k_3[P] = k_{-3}[Q],\ \mathbf{g}_{3,3} = \mathbf{g},\ \mathbf{g}_{2,2} = \begin{pmatrix} \Gamma_1^{-1} & 0 \\ 0 & \Gamma_2^{-1} \end{pmatrix}$$

and the vertical bars denote determinants. Using $[P] = K_1[Az(II)][Fe(II)]$ and $[Q] = K_2^{-1}[Az(I)][Fe(III)]$, eq 9 is obtained.

(3) The amplitude expression for a relaxation process induced by a temperature perturbation and monitored by a transmission change is given by eq 7. For the slow step $\tilde{\Gamma}$, $\Delta\tilde{H}$, and $\Delta\tilde{\epsilon}$ are given by the following equations:

$$\tilde{\Gamma} = |\mathbf{g}_{2,2}||\mathbf{g}_{3,3}|^{-1}$$

$$\Delta\tilde{H} = \Delta H_3^{\circ} - g_3\mathbf{g}_{2,2}^{-1}\begin{pmatrix} \Delta H_1^{\circ} \\ \Delta H_2^{\circ} \end{pmatrix}$$

where

$$g_3 = \left(-\frac{1}{[P]}, -\frac{1}{[Q]}\right)$$

$$\mathbf{g}_{2,2}{}^{-1} = |\mathbf{g}_{2,2}|^{-1} \begin{pmatrix} \Gamma_2{}^{-1} & 0 \\ 0 & \Gamma_1{}^{-1} \end{pmatrix}$$

and $\Delta\tilde{\epsilon} = \epsilon_Q - \epsilon_P = -\epsilon_{625}$ (assumptions: transmission change monitored at 625 nm; $\epsilon_P = \epsilon_{Az(II)} + \epsilon_{Fe(II)}$; $\epsilon_Q = \epsilon_{Az(I)} + \epsilon_{Fe(III)}$). Equation 17 is derived using the substitutions for [P] and [Q] mentioned before.

(4) The evaluation of rate constants following eq 14 requires, in principle, the computation of free equilibrium concentrations for each of the participating species. This is a mathematically involved problem, if it has to be solved "exactly". However, several simplifications can be made. (1) In all experiments under consideration, we had $[Fe]_t \gg [Az]_t$, so that $[Az(II)] + [Fe(II)] \simeq [Fe(II)] \simeq [Fe]_t$. (2) By stoichiometry [Az(I)] = [Fe(III)].

Thus, only [Az(I)] remains to be calculated. We make use of the fact that the condition $K_2 \gg [Az(I)] + [Fe(III)]$ is equivalent to the condition $[Q] \ll \frac{1}{2}[Az(I)]$; i.e., the concentration of the complex {Az(I)·Fe(III)} is negligible. Therefore $[Az]_t \simeq [Az(II)] + [Az(I)] + [P]$. With $[P] = K_1[Az(II)]\cdot[Fe(II)]$ and the approximations mentioned, the overall equilibrium quotient assumes the form

$$K = \frac{[Az(I)]^2(1 + K_1[Fe]_t)}{([Az]_t - [Az(I)])[Fe]_t}$$

leading to

$$[Az(I)] = \frac{K}{2}\left(\frac{[Fe]_t}{1 + K_1[Fe]_t}\right) \times \left\{-1 + \left[1 + \frac{4[Az]_t}{K[Fe]_t}(1 + K_1[Fe]_t)\right]^{1/2}\right\}$$

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