THE KINETICS OF THE CYTOCHROME-c-AZURIN REDOX EQUILIBRIUM

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SUMMARY

A preliminary analysis of the temperature-jump relaxation spectrum of the cytochrome-c-azurin redox system from Pseudomonas fluorescens revealed two chemical relaxation times. These are explained in terms of two association steps and an electron transfer between the associated species.

INTRODUCTION

The blue copper protein azurin and the P-551 cytochrome c are electron mediating proteins that have been isolated from species of Pseudomonas (1,2) where they participate in the respiratory system. Their redox potentials were reported to be similar (2,3), and the specificity of the electron transfer reaction between them has been indicated by preliminary kinetic studies using the stopped-flow technique (4).

The application of chemical relaxation methods (5) to the investigation of the redox equilibrium between these proteins would add the advantages of resolving the normal modes of their interaction and increasing the time resolution.

MATERIALS AND METHODS

The P-551 cytochrome-c and azurin were prepared from P. fluorescens using the procedures of Ambler (6,7), and stored at $4^{o}C$ in 0.1 M sodium dihydrogen phosphate (pH 7.0). The cyt-c was reduced by a 2-fold molar excess of sodium hydrosulphite (dithionite) and then removed by gel filtration on Sephadex G-10 at room temperature. The reduced cyt-c was stored under nitrogen as it was found to be slowly oxidized in air over a period of a few days. Concentrations of protein were determined spectrophotometrically using extinction coefficients of 28.3×10^{3} 1 $M^{-1}cm^{-1}$ (at 551 nm) for the cyt-c (2) and 3.5×10^{3} 1 M^{-1} cm^{-1} (at 625nm) for the azurin (8).

Solutions for both kinetic and static measurements were prepared by mixing aliquots of oxidized azurin and reduced cyt-c in 0.1 M sodium dihydrogen phosphate buffer at pH 7.0. Also EDTA (10^{-5} M) was included to avoid traces of metal ions participating in the redox equilibrium.

A double beam, temperature-jump spectrophotometer (9) was used to examine the reaction mechanism. The spectral changes following the temperature perturbation (17 to 25°C) were observed throughout the spectral range from 280-700nm on a storage oscilloscope coupled to an analogue-to-digital converter which then transferred the information to a computer for evaluation. The static absorption spectra were measured at 25°C using a Cary 14 spectrophotometer.

RESULTS AND DISCUSSION

The static titrations revealed a progressive oxidation of the reduced cytochrome-c on addition of oxidized azurin (Fig.1). When evaluating the concentrations of the species from the spectrophotometric data, by assuming an absence of intermediate forms, no constancy in the equilibrium ratio was found. The values for $K = \{Cyt(II)Az(II)\}/\{(Cyt(III))(Az(I))\}$ varied between 0.4 and 2.9 in an apparently regular manner. Therefore, although the presence of complexed species is at least indicated, their nature must be such as to cause no observable spectral changes. So to this effect, computations are now being made that assume finite concentrations of intermediates.

The relaxation spectrum of this system consisted of three parts (Fig. 2), a very fast effect (I) completed within the heating time of the instrument (<2μS), followed by two chemical relaxations, one in the range of 1-5 msec. (II), and the other that of 25-50 msec. (III). The two amplitudes differed by a factor of five in their sizes and were of opposite directions throughout the spectral and concentration ranges studied. The faster time (II) as displayed on the oscilloscope, was found to show a linear dependence on total azurin concentration (Fig. 3a), the slower relaxation (III) displayed an assymptotic dependence (Fig. 3b). The same behaviour was also found to be maintained when the cytochrome-c concentration was varied.

The minimal overall course of the reaction must involve a binding step of the free species and an electron transfer between them. The proposed scheme, modified from the suggestion of Antonini et al. (4) is:

$$Cyt(III)+Az(I) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \{Cyt(III)Az(I)\} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \{Cyt(II)\ Az(II)\} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} Cyt(II)+Az(II)$$

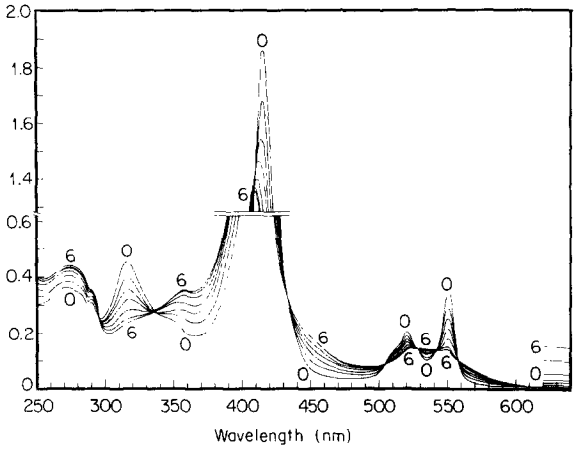

Fig. 1.

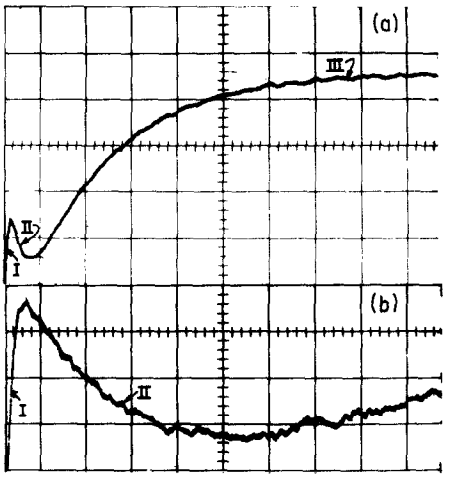

Fig. 2.

Figure 1. Titration of oxidized azurin against reduced cyt-c at 25°C in 0.1M NaH_2PO_4 buffer at pH 7.0. The concentration of oxidized azurin was determined at 625 nm and this amount was added to a second cell which was then used as reference. Curves are in numerical sequence which is reversed at each isosbestic point.

Total concentrations (Cyt-c, Azurin; 10^5 M), 0-1·20, 0·0; 1 - 1·19, 0·49; 2 - 1·19, 0·93; 3 - 1·18, 1·39; 4 - 1·17, 2·30; 5 - 1·15, 3·62; 6 - 1·14, 4·92.

Figure 2. T-jump (17-25°C) relaxation spectra of the cyt-c (1·15 x 10^{-5} M)-azurin (3·62 x 10^{-5} M) reaction in 0·1M NaH_2PO_4, pH 7.0 followed at 416 nm. (a) y-axis, 20mV/cm; x-axis, 20msec/cm. (b) y-axis, 10mV/cm; x-axis, 2 msec/cm.

That the transfer occurs within a complex of the two proteins is confirmed by the appearance of the same relaxation spectrum at 625nm and at 416nm which are the unique absorption bands of the azurin and cytochrome-c respectively. The mechanism proposed is that upon binding of either of the pairs of free protein species a structural change is induced in them that mediates the electron transfer within the complex. Indeed, preliminary measurements of the amplitude spectrum have shown it to differ from that of the static difference spectrum of reduced against oxidized cytochrome-c. This not only demonstrates the presence of intermediate forms but also that they appear to be in different conformations than the free species.

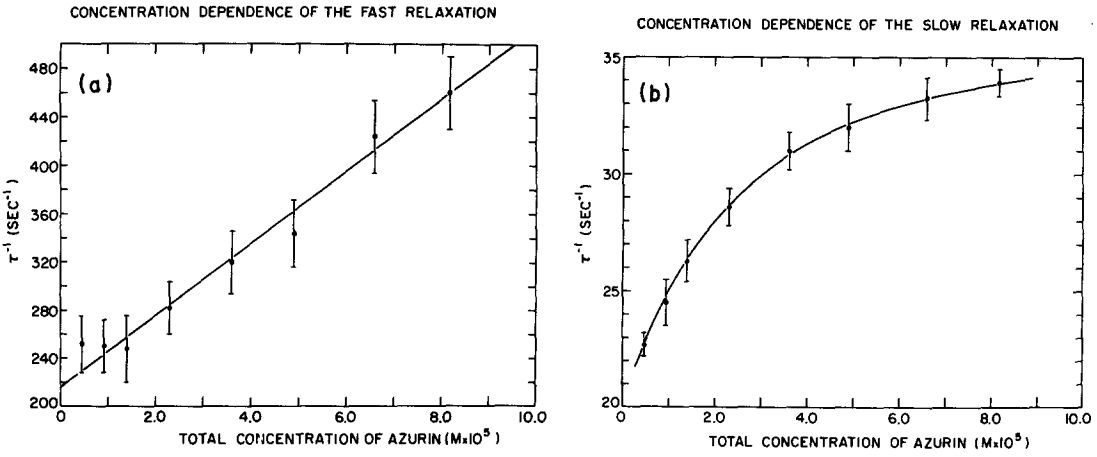

Figure 3. Concentration dependence of relaxation times, conditions as in Figure 2. (a) Fast relaxation (II). (b) Slow relaxation (III).

To evaluate the relaxation equations for this three-step equilibrium it is necessary to solve a cubic equation. So a simplification that involved the decoupling of the electron-transfer process was made. This was justified by the nature of the observed relaxation spectrum, viz. the appearance of 'discrete' times. This treatment is normally applied when the relevant relaxations are separated in time by an order of magnitude. Here the two relaxations meet this requirement superficially, at least. The simultaneitey of the perturbation to each of the normal modes of the reaction foreshortens the faster relaxation, but the time constant of the slower relaxation is only marginally affected because of its larger amplitude. This reduces their separation in time to approximately a factor of five, which is regarded by some authors as too small (10). However, the simplification had to be made in order that the relaxation equations, shown below, should be tractable. The fast relaxation is thought to be a multiple relaxation time that represents a superposition of the two binding steps {Cyt(III)+Az(I), and Cyt(II)+Az(II)} which are

$$\frac{1}{\tau_1} = k_1 \{\overline{Cyt(III)} + \overline{Az(I)}\} + k_{-1}, \text{ and } \frac{1}{\tau_2} = k_{-3} \{\overline{Cyt(II)} + \overline{Az(II)}\} + k_{+3},$$

where the bars denote equilibrium concentrations. The theory for such multiple relaxations is described by Schwarz (11). Here a mean reciprocal time is envisaged, i.e. $1/\tau_* = \sum_r^{\beta r}/\tau_r$ where the β_r values are weight factors. As there are only two relaxations that are considered similar,

their resultant will still appear to be a single, exponential decay and evaluated as such. Thus we may write

$$\frac{1}{\tau_o} = \frac{1}{\tau_1} + \frac{1}{\tau_2} = k_1 \{Cyt(III)+Az(I)\} + k_{-1}+k_{-3}(Cyt(II)+Az(II)\} + k_3.$$

Indeed we may further assume that the rate constants for the association of the free species are approximately equal, i.e., $k_1 \simeq k_{-3}$, thus

$$\frac{1}{\tau_o} \simeq k_1 \{Cyt(III)+Cyt(II)+Az(I)+Az(II)\} + k_{-1} + k_3.$$

From the slope of this plot (Fig. 3a) a value of $k_1 = 3\times10^6\ M^{-1}\ sec^{-1}$ is obtained.

For the slow relaxation times involving the electron transfer, the expression is:

$$\frac{1}{\tau_3} = \frac{k_2 \{k_1 k_3(\overline{Cyt(III)}+\overline{Az(I)})+k_1 k_{-3}(\overline{Cyt(III)}+\overline{Az(I)})(\overline{Cyt(II)}+\overline{Az(II)})\} + k_{-2}\{k_{-1} k_{-3}(\overline{Cyt(II)}+\overline{Az(II)}) + k_1 k_{-3} (\overline{Cyt(III)}+\overline{Az(I)})(\overline{Cyt(II)}+\overline{Az(I)})\}}{\{k_1 k_3(\overline{Cyt(III)}+\overline{Az(I)})+k_1 k_{-3} (\overline{Cyt(III)}+\overline{Az(I)})(\overline{Cyt(II)}+\overline{Az(II)}) + k_{-1} k_{-3}(\overline{Cyt(II)}+\overline{Az(II)})+k_{-1} k_{+3}\}}$$

Furthermore it can be seen that by dividing each term by $k_1 k_{-3} \{(\overline{Cyt(III)}+\overline{Az(I)})(\overline{Cyt(II)}+\overline{Az(II)})\}$ that $1/\tau_3 \rightarrow k_2 + k_{-2}$ as the concentrations of $\overline{Cyt(II)}$, $\overline{Cyt(III)}$, $\overline{Az(I)}$, and $\overline{Az(II)}$ become large, as is observed for the slow relaxation.

By employing the T-jump technique the assumption of a particular multi-step equilibrium has been demonstrated as basically correct. The resolution of the intermediary complexes and their conformational transitions will be of great significance for the understanding of the electron transfer mechanism between redox proteins and of its implications to the energy conversion process.

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