TEMPERATURE-JUMP STUDIES OF *DESULFOVIBRIO VULGARIS* FLAVODOXIN: KINETICS OF FMN BINDING AND OF REDUCTION OF SEMIQUINONE BY METHYL VIOLOGEN†

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SUMMARY

Temperature-jump relaxation spectrometry has been used with the flavodoxin from *Desulfovibrio vulgaris* to investigate the kinetics of FMN binding to apoprotein and of reduction of half-reduced to fully-reduced protein by methyl viologen. FMN binding was characterized by a single, concentration dependent, exponential relaxation which corresponded to a diminution of flavin fluorescence intensity. This implies the absence of detectable intermediates during coenzyme binding and a positive enthalpy of binding. The latter was confirmed by microcalorimetry and fluorometric titration studies at two temperatures. The reduction of the half-reduced holoprotein also displayed simple exponential kinetics, again implying the absence of intermediates. The results of these studies are compared with earlier work with other flavodoxins.

Previous work from this laboratory has utilized temperature-jump relaxation spectrometry with the flavodoxins from *Azotobacter vinelandii* and *Peptostreptococcus elsdenii* to investigate the binding of flavin analogs to apoprotein (1) and the kinetics of reduction of half-reduced protein by methyl viologen (2). In view of the recent x-ray structure determination of *Desulfovibrio vulgaris* flavodoxin, (3), it seemed of interest to extend these studies to this protein as well. A comparison of the *Azotobacter* and *Desulfovibrio* proteins is of particular significance because of the many similarities which have been found to exist between these two flavodoxins (4).

MATERIALS AND METHODS

All experimental procedures were as previously described (1,2). The

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flavodoxin from *D. vulgaris* was isolated and purified by the method of Dubourdieu and LeGall (5).

RESULTS

FMN Binding

The kinetics of FMN binding to the apoflavodoxin were determined by subjecting the holoprotein to a temperature jump ($\Delta T = 7°C$) and observing the establishment of a new equilibrium by monitoring flavin fluorescence, which is known to be quenched as a consequence of binding (4). Some typical relaxation data are shown in Figure 1. A single, concentration dependent, exponential relaxation process was found which corresponded to a diminution of fluorescence intensity. This is in contrast to what has been previously observed (1) with the *Azotobacter* and *P. elsdenii* flavodoxins, which exhibit biphasic relaxation composed of a concentration independent fluorescence decrease and a concentration dependent fluorescence increase. The latter results were interpreted in terms of the following mechanism:

$$F + P \rightleftharpoons X \rightleftharpoons FP$$

An appreciable concentration of a partially fluorescent intermediate (X) was assumed to be present at equilibrium, and the biphasic nature of the relaxation

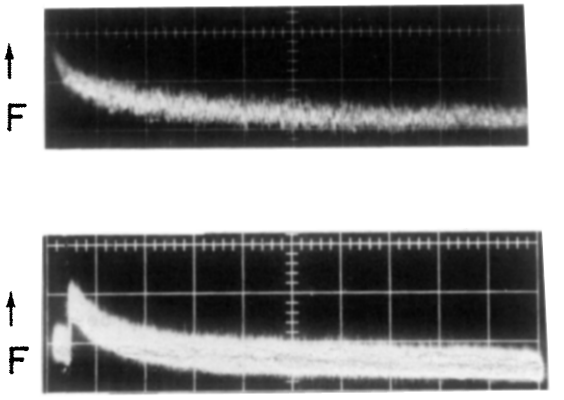

Figure 1. Relaxations obtained upon temperature jump of solutions of holoprotein of *D. vulgaris* flavodoxin. Starting temperature 4°C; 0.05 M phosphate buffer containing 0.1 M KNO_3, pH 7.0. Top trace: protein concentration = 1.0×10^{-4}M; horizontal scale = 0.2 sec per division. Bottom trace: protein concentration = 1.7×10^{-5}M; horizontal scale = 2 sec per division.

curve was presumed to be a consequence of increases in the concentrations of F, P and FP following the temperature jump. If a similar mechanism applies to the *D. vulgaris* flavodoxin, the equilibrium concentration of X must be considerably smaller than is the case for the other flavodoxins. Furthermore, the fluorescence decrease which is obtained upon increasing the temperature implies that the ΔH of binding of flavin to apoprotein is positive, again in contrast with the *Azotobacter* flavodoxin. In order to confirm this, we have measured the association constant for FMN to the apoflavodoxin using a fluorometric titration procedure.* The values obtained were $K_a = 8 \times 10^7 M^{-1}$ at 26°C and $K_a = 2.5 \times 10^7 M^{-1}$ at 15°C. This corresponds to a $\Delta H^\circ = 14.2$ kcal/M and a calculated $\Delta S^\circ_{26^\circ} = 84$ cal/deg mole ($\Delta F^\circ_{26^\circ} = -10.9$ kcal/M). The corresponding values (1) for *Azotobacter* flavodoxin are $\Delta F^\circ_{24^\circ} = -11.1$ kcal/mole, $\Delta H^\circ = -4.1$ kcal/mole and $\Delta S^\circ_{24^\circ} = 23.6$ cal/deg mole. Thus, the binding of FMN to the *D. vulgaris* apoflavodoxin is characterized by a positive enthalpy which is compensated by a very large positive entropy term.

Assuming that the binding of FMN to the *D. vulgaris* apoprotein follows simple second order kinetics, i.e. $F + P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} FP$, the concentration dependence of the relaxation time should be as follows:

$$\frac{1}{\tau} = k_2[(\bar{F}) + (\bar{P})] + k_1$$

where $(\bar{F})$ and $(\bar{P})$ are the new equilibrium concentrations of free flavin and apoprotein. A plot of this equation is shown in Figure 2. Although the data are not good enough to allow an accurate determination of k_1 and k_2, the approximate values ($k_2 \sim 10^6 M^{-1}sec^{-1}$ and $k_1 \sim 0.1\ sec^{-1}$ at a temperature of 11°C) are consistent with the abovementioned equilibrium constant measurements and a previous stopped-flow determination of k_2 ($7 \times 10^5 M^{-1}sec^{-1}$) at 26°C and pH = 7.0 (4).

Reduction of Semiquinone

We have also utilized temperature-jump spectrometry to determine the pH

*Preliminary microcalorimetric experiments also indicate a positive enthalpy of binding.

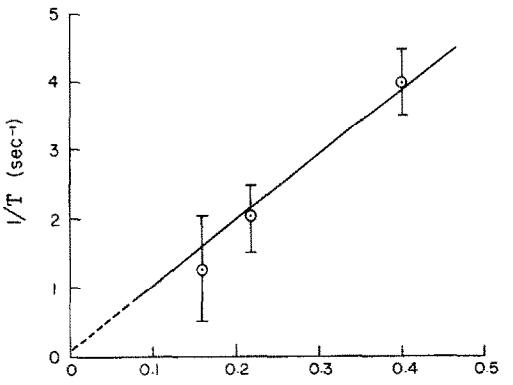

Figure 2. Plot of reciprocal of relaxation time vs. sum of free flavin and apoprotein concentrations. Conditions as in Figure 1.

dependence of the rate of reduction of the flavodoxin semiquinone by dithionite-reduced methyl viologen. By varying the pH in the presence of reduced methyl viologen, we have determined that the degree of reduction of the semiquinone is controlled by a pK = 6.6. In the case of *Azotobacter* flavodoxin (6), this pK = 7.0 and has been interpreted in terms of an ionization occurring in the fully reduced form of the flavoprotein. As a consequence of this pH dependence, it is possible to vary the equilibrium amounts of semiquinone and fully reduced protein by adding excess dithionite to flavodoxin in the presence of a small amount of methyl viologen (10 mole %) at several pH values between 5 and 7 (2). The application of a temperature jump results in a perturbation of this equilibrium and this can be easily followed at 580 nm (a wavelength at which only the semiquinone absorbs). A typical relaxation is shown in Figure 3. A single exponential was obtained. The pH dependence of the relaxation time is shown in Figure 4. These results are exactly analogous to those obtained with *Azotobacter* flavodoxin and thus imply that the following mechanism is operative (2):

$$PFH\cdot + [H\cdot] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} PFH_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} PFH^- + H^+$$

In the case that a steady-state exists with respect to the concentration of

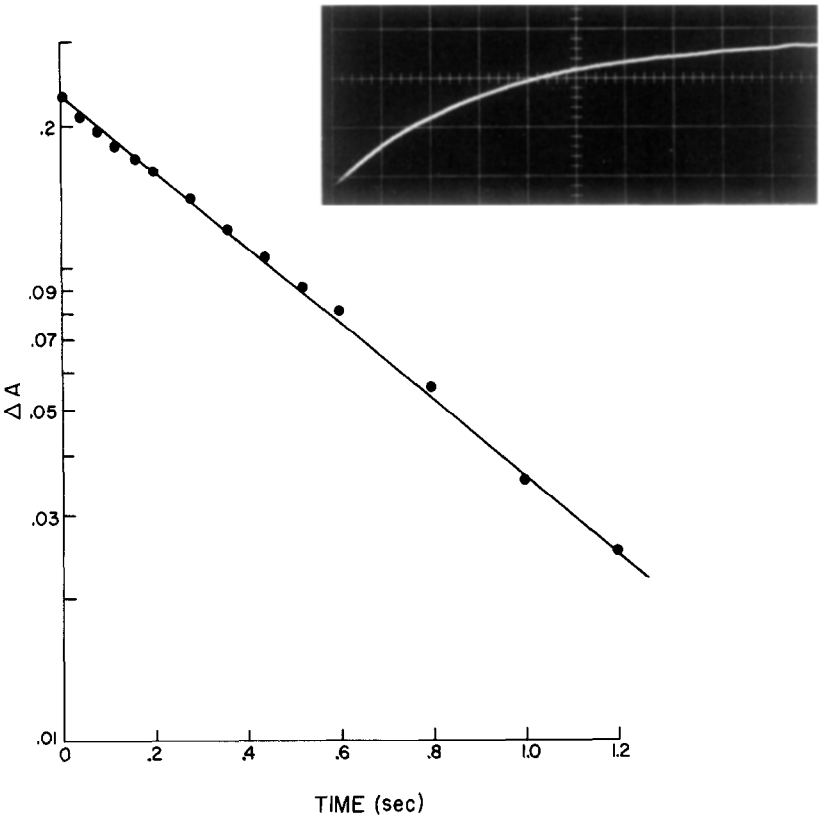

Figure 3. Plot of log ΔA (580 nm) vs. time for relaxation obtained upon temperature jump of equilibrium mixture of semiquinone and fully reduced forms of *D. vulgaris* flavodoxin. Inset shows actual experimental curve (horizontal scale = 0.2 sec per division). pH = 5.1, 0.05 M phosphate-acetate buffer containing 0.1 M KNO_3; protein concentration = 9 x 10^{-5} M; methyl viologen concentration = 9 x 10^{-6}M; sodium dithionite present in excess.

PFH_2 (as is indicated by the observation of only a single exponential relaxation), this mechanism simplifies to:

$$PFH\bullet + [H\bullet] \underset{k''}{\overset{k'}{\rightleftharpoons}} PFH^- + H^+$$

where

$$k' = \frac{k_1 k_3}{k_2 + k_3}$$

$$k'' = \frac{k_2 k_4}{k_2 + k_3}$$

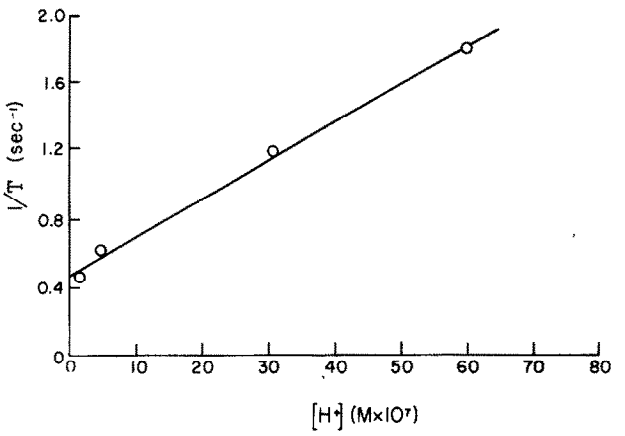

Figure 4. Plot of reciprocal of relaxation time vs. $[H^+]$ for equilibrium of semiquinone and fully reduced forms of *D. vulgaris* flavodoxin. Conditions as in Figure 3.

According to this mechanism,

$$\frac{1}{\tau} = k'[H\cdot] + k''[H^+]$$

Thus, a plot of $1/\tau$ vs. $[H^+]$, at constant methyl viologen concentration, should be linear (cf. Figure 4). The values of the constants obtained from this plot are: $k' = 5.0 \times 10^4 M^{-1} sec^{-1}$ and $k'' = 2.3 \times 10^5 M^{-1} sec^{-1}$. These can be compared with $k' = 3.5 \times 10^4 M^{-1} sec^{-1}$ and $k'' = 6.5 \times 10^6 M^{-1} sec^{-1}$ for *Azotobacter* flavodoxin (2). As a check on the kinetic measurements, the data can be used to calculate $E^{\circ\prime}$, the reduction potential for the semiquinone-fully reduced couple. This gives $E^{\circ\prime} = -480$ mV, which is in good agreement with a value ($E^{\circ\prime} = -438$ mV) previously obtained by potentiometric titration (7).

From the pK for the ionization of PFH_2, it is possible to calculate k_1/k_2. This quantity is equal to $5.5 \times 10^5 M^{-1}$ (compared to $1.2 \times 10^5 M^{-1}$ for *Azotobacter* flavodoxin (2)).

DISCUSSION

From the analysis of the data concerning the binding of FMN to *D. vulgaris* apoflavodoxin, it is apparent that considerable differences exist between this protein and the *Azotobacter* flavodoxin. Although it is not possible to interpret these fully in molecular terms, they may reflect differences in the

nature of the changes in protein conformation which occur upon coenzyme binding (2,4), and possibly also in the details of the flavin-protein interaction. On the other hand, the kinetics of reduction of the two proteins by methyl viologen are quite similar. In both cases, only a single relaxation is observed (implying the absence of significant protein conformational changes upon reduction) and the rate parameters have similar values. This suggests that the reduction mechanisms are probably identical. Since this also seems to be the case for the *P. elsdenii* flavodoxin (2), and since these proteins function enzymically by shuttling between the half reduced and fully reduced forms, such a conclusion is clearly of interest with respect to biological activity.

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