Flavine–Protein Interactions in Flavoenzymes. Thermodynamics and Kinetics of Reduction of *Azotobacter* Flavodoxin†

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ABSTRACT: The redox potentials for *Azotobacter* flavodoxin have been determined to be +50 mV for the oxidized–semiquinone interconversion and −495 mV for the semiquinone–hydroquinone equilibrium. The positive value for the first potential is strikingly different from any of the other flavodoxins and correlates well with the unusual stability to oxygen of the semiquinone form of the *Azotobacter* flavodoxin. A rationale for this correlation is proposed in terms of the extremely large association constant of the flavine semiquinone to the apoflavodoxin. The second potential is more negative than that of the other flavodoxins. The temperature-jump method has also been applied to the semiquinone–hydroquinone equilibrium. A single relaxation is observed which is dependent on pH and reductant concentration in a manner consistent with a previously proposed mechanism [(Edmondson, D. E., and Tollin, G. (1971), *Biochemistry 10*, 133]. The kinetic constants obtained were also used to calculate a reduction potential which is in reasonable agreement with that obtained potentiometrically.

As a means of obtaining further insights into the modification of flavine properties upon incorporation of the coenzyme into a flavoprotein, we have determined the thermodynamic one-electron reduction potentials and the kinetics of reduction by methylviologen of *Azotobacter* flavodoxin (Shethna flavoprotein). Although other flavodoxin redox potentials have been determined previously (*cf.* below), the *Azotobacter* protein has sufficiently unusual properties (*e.g.*, it forms the most stable semiquinone species of all flavoproteins) to make the evaluation of its reduction thermodynamics of particular interest. Furthermore, no previous study of reduction kinetics using relaxation methods (temperature jump) has been reported.

Experimental Section

Materials

Azotobacter flavodoxin (Shethna flavoprotein) was isolated from *Azotobacter vinelandii*, strain Wisconsin "O," using a modified procedure (Edmondson, 1970) originally developed by Hinkson and Bulen (1967). *Peptostreptococcus elsdenii* flavodoxin was a gift from Dr. S. G. Mayhew, University of Michigan, Ann Arbor, Mich. Protein concentrations were determined spectrophotometrically using published extinction coefficients. Methylviologen was obtained from K and K Labs, Inc., Hollywood, Calif. Sodium dithionite (90%) was a product of Eastman Organic Chemicals, Rochester, N. Y.; DEAE-cellulose was purchased from Calbiochem, Los Angeles Calif.

Reduction Potential Measurements. Reduction potentials were determined for *Azotobacter* flavodoxin by coulometric reduction and also by potentiometric titration with sodium dithionite. Coulometric reductions were performed using a small volume "Coulometric Redoxstat" (Swartz and Wilson, 1971). In this method, the reductant is coulometrically generated within the sample by applying current to a generating electrode pair. The potential of the sample is sensed by a platinum indicating electrode and compared to a standard Ag–AgCl electrode. Any desired potential can be chosen and the degree of reduction monitored spectrophotometrically. In the present work, methylviologen (MV)[1] was used as the redox buffer and primary reductant. This method is particularly applicable to slowly established equilibria inasmuch as any given potential within the sample cell can be maintained indefinitely.

Spectra for determination of redox potentials were measured in a 1-cm rectangular Pyrex cell which contained three ground-glass side arms for the insertion of salt bridges and working and/or reference electrodes. Both the Cary 14R and Coleman-Hitachi Model 124 spectrophotometers were used in these measurements. The reduction cell was stirred by a magnetic bar to maintain a uniform potential. All spectra were run at ambient temperatures (23 ± 2°).

Potentiometric titrations were performed in the anaerobic three-arm cuvet described above. Reductant was added from a microsyringe placed in one arm, an indicating platinum electrode occupied another arm, and a Ag–AgCl reference electrode was placed in the third arm. Methylviologen (5 mol %) was added to mediate the reduction of the flavoprotein by dithionite. The cell potential relative to the Ag–AgCl reference electrode (+0.200 V) was monitored using a high impedance digital voltmeter and corrected to the standard hydrogen electrode potential. The degree of reduction of the flavoprotein was monitored spectrophotometrically using the Coleman-Hitachi spectrophotometer. All potential measurements were performed at pH 8.2 in 0.1 M Tris buffer. The degree of reduction was calculated from the appearance and subsequent disappearance of the 580-nm absorbance of the flavodoxin semiquinone. The one- and two-electron potentials were found to be sufficiently separated to permit this procedure. The concentrations of the semiquinone were calculated using a molar absorptivity of 5600 M^{-1} cm^{-1} at 580 nm. The corresponding concentrations of oxidized flavoprotein were

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[1] Abbreviations used are: MV, methylviologen; FP, flavoprotein.

TABLE I: Reduction Potentials of Flavodoxins.

Organism	*Azotobacter*	*Clostridium pasteurianum*	*Clostridium* MP	*P. elsdenii*	*E. coli*	*D. vulgaris*
E°_1' oxidized–semiquinone (mV)[a]	+50 (8.2)	−132 (7.0)	− 92 (7.0)	−115 (7.0)	−240 (7.7)	−150 (7.8)
		−203 (8.2)	−167 (8.2)	−186 (8.2)	−270 (8.2)	−175 (8.2)
E°_2' semiquinone–hydroquinone (mV)[a]	−495 (8.2)	−419 (7.0)	−399 (7.0)	−371 (7.0)	−410 (7.7)	−440 (7.8)
		−490 (8.2)	−480 (8.2)	−442 (8.2)	−440 (8.2)	−462 (8.2)
Reference	This study	*b*	*b*	*c*	*d*	*d*

[a] pH values to which potentials refer are given in parentheses. [b] Mayhew (1971). [c] Mayhew and Massey (1969). [d] Vetter and Knappe (1971). [e] M. Dubourdieu (1972), personal communication.

calculated using the difference between the initial oxidized concentration and the semiquinone concentration. The concentrations of the fully reduced flavoprotein were calculated using the difference between the maximum semiquinone concentration and the concentration of semiquinone upon further reduction. Correction was made for the absorption due to reduced methylviologen (MVH·) at each potential. The formal potentials were then calculated, using the Nernst equation, from a plot of log (red)/(ox) *vs.* cell potential, in which the slope was equal to the constant factor RT/nF and the intercept equal to E°'.

Preparation of Reduced Flavoproteins for Temperature-Jump Studies. The degree of reduction of the *Azotobacter* flavodoxin semiquinone in the pH range above 6.0 is controlled by the ionization of the fully reduced form of the flavoprotein (pK_a = 7.0) (Edmondson and Tollin, 1971). Even in the presence of excess dithionite, the degree of reduction will depend solely upon the pH. Therefore, by using an excess of dithionite and varying the pH in the range of 6.5–8.2, one can obtain equilibrium mixtures with different concentrations of semiquinone and fully reduced flavoprotein.

In order to achieve an equilibrium mixture of the semiquinone and hydroquinone of *P. elsdenii* flavodoxin, excess dithionite (1 mg/ml) together with MV (20 mol %) at pH 5.8 was added to the degassed sample (Mayhew *et al.*, 1969).

Samples were prepared by degassing 2 ml of flavoprotein (3×10^{-5} M) solution in Thunberg cuvets and adding 1 mg of sodium dithionite from the Thunberg side arm. The pH was maintained with 0.1 M KH_2PO_4. Methylviologen (10–40%) was added to increase the reduction rates (Edmondson and Tollin, 1971). Potassium nitrate (0.1 M) was also added to each sample as a conducting electrolyte. Concentrations were determined spectrophotometrically at 580 nm, using an extinction of 5600 M^{-1} cm^{-1} for the semiquinone. Fully reduced flavoprotein concentrations were determined spectrophotometrically as the difference between the semiquinone concentration and the total flavoprotein concentration before reduction.

Kinetics were determined with the temperature-jump spectrophotometer described previously (Barman and Tollin, 1972). Partially reduced flavoprotein solutions were transferred anaerobically (under nitrogen) to the temperature-jump cell. The cell was maintained anaerobic by a positive nitrogen pressure. All kinetics were determined at 9°.

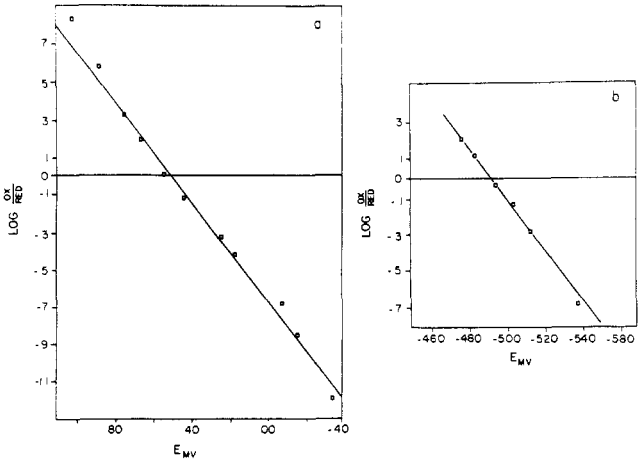


FIGURE 1: Plots of log (ox)/(red) *vs.* *E*. (a) For the determination of the oxidized–semiquinone reduction potential of *Azotobacter* flavodoxin: pH 8.2, 0.1 M Tris buffer. (b) For the determination of the semiquinone–hydroquinone reduction potential of *Azotobacter* flavodoxin. pH 8.2, 0.1 M Tris buffer.

Results and Discussion

Determination of Reduction Potentials of Azotobacter Flavodoxin. The course of reduction of flavodoxin at pH 8.2 is described by eq 1 and 2 (Edmondson and Tollin, 1971). The

$$FP + [H\cdot] \overset{E^{\circ}_1'}{\rightleftharpoons} FPH\cdot \quad (1)$$

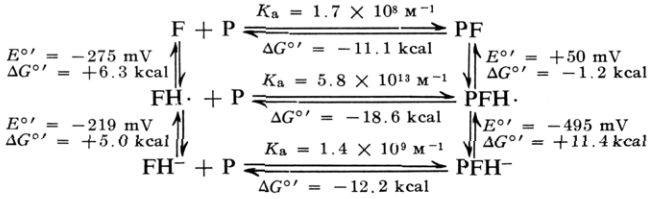
$$FPH\cdot + [H\cdot] \overset{E^{\circ}_2'}{\rightleftharpoons} FPH^- + H^+ \quad (2)$$

reduction process upon going from the oxidized to the semiquinone flavoprotein was monitored by absorption spectra. The absorption at 450 nm was decreased while a broad absorption (due to the semiquinone) with peaks at 580 and 615 nm appeared as the potential became increasingly negative. A well-defined isosbestic point occurred at 500 nm, showing that only two species were present in equilibrium. In Figure 1a is shown a plot of log ox/red *vs.* *E* for the determination of E°_1'. The intercept at log ox/red = 0 yielded a value of E°_1' of +50 mV. The slope corresponds to a one-electron process.

The course of reduction in going from the semiquinone to the hydroquinone flavoprotein was also monitored by absorption spectra. Because of the interference in absorption at these negative potentials by MVH· (5 mol %), no clear isosbestic point was seen. As the cell potential became increasingly negative, the 580-nm absorption disappeared. The plot of log ox/red *vs.* *E* is linear (Figure 1b) and corresponds to a one-electron reduction potential (E°_2') of −495 mV.

Since *Azotobacter* flavodoxin has properties which are similar to the flavodoxins from other bacteria, it is of interest to compare the reduction potentials for this protein to those of the various flavodoxins. Inspection of Table I shows that *Azotobacter* flavodoxin possesses by far the most positive first reduction potential of all of the flavodoxins. Indeed, this potential ranges from 225 to 320 mV more positive than any of the other flavodoxins and is consistent with the unusually high stability of the semiquinone form of this protein to oxidation by oxygen (see below for further discussion). On the other hand, the second reduction potential is the most negative of all the flavodoxins. These results agree well with the reduction of the *Azotobacter* protein by hydrogen and hydrogenase as determined by Van Lin and Bothe (1972). They noted that the reduction potential must be very negative, because of the small amount of fully reduced protein formed upon hydrogen reduction, even more negative than that reported for *Escherichia coli* flavodoxin (−410 mV) and for phytoflavine (−470 mV).

Calculation of the Association Constants for the Various Flavine Redox Forms with the Azotobacter Apoprotein. By combining the reduction potentials for *Azotobacter* flavodoxin with the published reduction potentials for free flavines (Draper and Ingraham, 1968) and with the previously determined association constant of the apoprotein with oxidized FMN (Barman and Tollin, 1972), it was possible to calculate the association constants for the semiquinone and hydroquinone forms of FMN with the apoprotein. The calculations are based on the fact that the free energy is path independent. These results are shown below. It is seen that there is a



large increase in the binding of flavine in going from the oxidized ($K_a^F = 1.7 \times 10^8$ M^{-1}) to the semiquinone ($K_a^{FH\cdot} = 5.8 \times 10^{13}$ M^{-1}) form of FMN which partly reverses in going from the semiquinone to hydroquinone form ($K_a^{FH_2} = 1.4 \times 10^9$ M^{-1}). A similar pattern, although not as marked, has been calculated for *P. elsdenii* flavodoxin[2] where $K_a^F = 2.3 \times 10^9$ M^{-1}, $K_a^{FH\cdot} = 2.8 \times 10^{11}$ M^{-1} and $K_a^{FH_2} = 1.1 \times 10^8$ M^{-1} (Mayhew, 1971). The magnitude of this enhancement in the *Azotobacter* flavodoxin would suggest the possibility of extensive rearrangement of the flavine binding site upon reduction to the semiquinone,[4] perhaps due to the fact that charge is generated in the flavine molecule upon formation of this species. The decrease in affinity upon further reduction could be due to the loss of one charge upon forming FH$^-$ or to the fact that fully reduced flavine has a bent conformation (Dudley *et al.*, 1964), or both. It is also interesting that free flavine semiquinones show an enhancement in complexation with indoles over that of oxidized flavines (Draper and Ingraham, 1970).

[2] Using a value for K_a^F of 8.2×10^7 M^{-1} for *D. vulgaris* flavodoxin (Dubourdieu, 1972),[3] $K_a^{FH\cdot} = 5 \times 10^9$ M^{-1} and $K_a^{FH_2} = 5 \times 10^5$ M^{-1}.

[3] Personal communication.

[4] Far-uv CD spectral measurements (Edmondson and Tollin, 1971) provide no indication of large conformational changes involving the protein backbone. Thus, if such changes occur, they must be relatively localized.

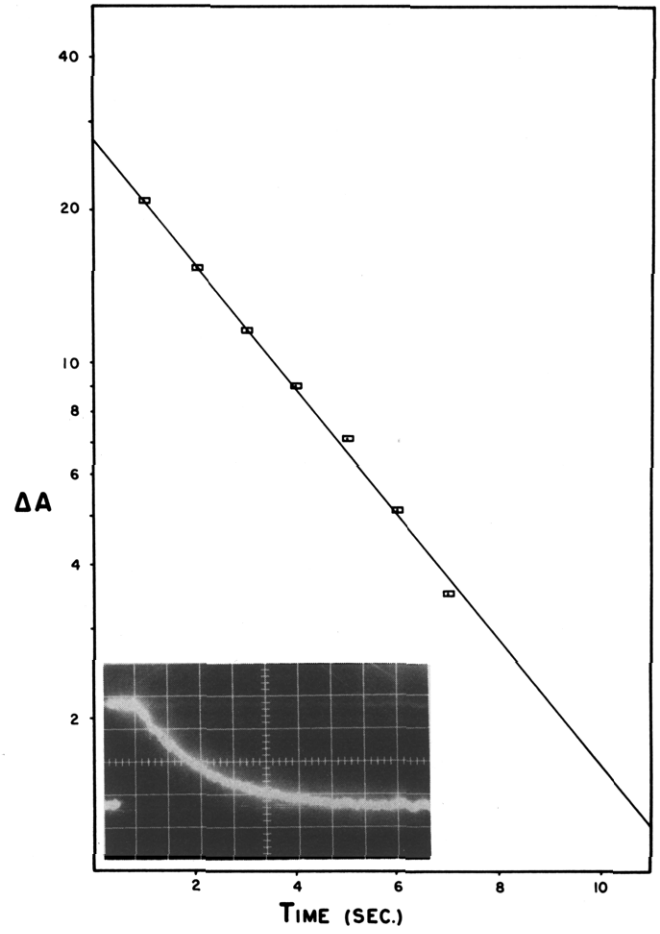


FIGURE 2: Plot of log ΔA *vs.* time for the determination of the relaxation time for the *Azotobacter* flavodoxin semiquinone–hydroquinone equilibrium. Inset shows actual experimental trace; 1 sec/division: pH 7.8, 0.1 M Tris buffer; 8.8 mol % methylviologen, 3×10^{-5} M protein.

Determination of the Kinetics of Reduction of Azotobacter Flavodoxin. The kinetics of the semiquinone to hydroquinone conversion of *Azotobacter* flavodoxin were determined by temperature jump methods. The mechanism below (3) was

$$PFH\cdot + [H\cdot] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} PFH_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} PFH^- + H^+ \qquad (3)$$

shown to be operative, in agreement with the results of Edmondson and Tollin (1971). Under the conditions of the experiment, only a single relaxation was observed indicating that a steady state existed with respect to the concentration of PFH_2 (Figure 2). Therefore the mechanism can be abbreviated using apparent rate constants as shown in reaction 4.

$$PFH\cdot + MVH\cdot \underset{k''}{\overset{k'}{\rightleftharpoons}} FPH^- + H^+ + MV \qquad (4)$$

where

$$k' = \frac{k_1 k_3}{k_2 + k_3} \qquad (5)$$

$$k'' = \frac{k_2 k_4}{k_2 + k_3} \qquad (6)$$

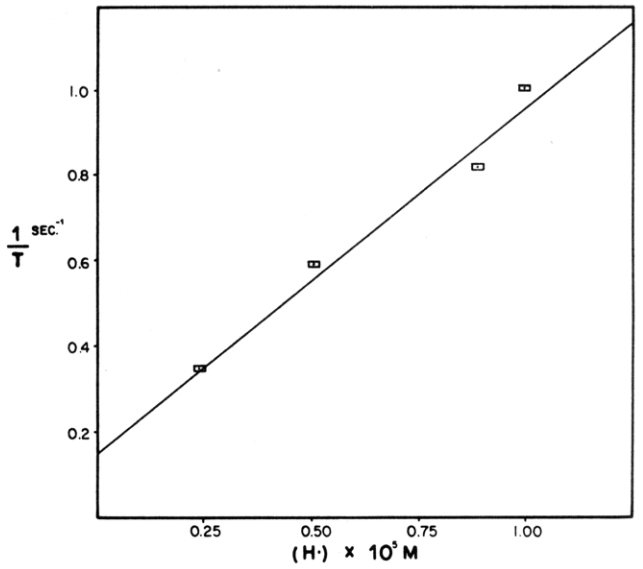


FIGURE 3: Plot of $1/\tau$ *vs.* [MVH·] for the *Azotobacter* flavodoxin semiquinone–hydroquinone equilibrium. $k' = 8.1 \times 10^4$ sec^{-1}; $k'' = 3.1 \times 10^6$ M^{-1} sec^{-1}.

With the large excess of dithionite (1 mg/ml) and strong buffer concentration (0.1 M), both [MVH·] and pH were effectively buffered such that the relationship between the relaxation time and the apparent rate constants was greatly simplified as shown in eq 7. Therefore, by holding the pH constant, a plot

$$\frac{1}{\tau} = k'[\text{MVH}\cdot] + k''[\text{H}^+] \qquad (7)$$

of $1/\tau$ *vs.* [MVH·] should be linear with a slope of k' and intercept of k''[H$^+$] (Figure 3). Similarly, by holding the concentration of reduced methylviologen constant, a plot of $1/\tau$ *vs.* [H$^+$] should be linear with a slope of k'' and an intercept of k'[MVH·] (Figure 4). Both methods yielded linear plots and reasonable agreement in the values of k' and k'', indicating that the assumed mechanism was correct. The values of k' and k'' show that the conversion of hydroquinone to semiquinone protein by methylviologen is two orders of magnitude faster than the conversion of semiquinone to hydroquinone.

Using average values of k' (5.8×10^4 M^{-1} sec^{-1}) and k'' (4.8×10^6 M^{-1} sec^{-1}) obtained from the two series of experiments, the apparent equilibrium constant for the reduction process was calculated (eq 8), where K_{app} is equal to k'/k''.

$$K_{app} = 1.2 \times 10^{-2} \text{ M} \qquad (8)$$

Using this equilibrium constant, the potential for the reduction of the flavoprotein by methylviologen can be calculated ($E^{\circ\prime} = -110$ mV). Combining this information with the reduction potential for methylviologen (Clark, 1960), the potential for the conversion from the semiquinone to the hydroquinone form of the flavoprotein can also be calculated as illustrated:

$$\text{PFH}\cdot + \text{MVH}\cdot \rightleftharpoons \text{PFH}^- + \text{H}^+ + \text{MV} \quad E^{\circ\prime} = -110 \text{ mV}$$
$$\text{MV} + [\text{H}\cdot] \rightleftharpoons \text{MVH}\cdot \quad E^{\circ\prime} = -440 \text{ mV}$$

Sum: $\text{PFH}\cdot + [\text{H}\cdot] \rightleftharpoons \text{PFH}^- + \text{H}^+ \quad E^{\circ\prime} = -550$ mV

This reduction potential is in reasonable agreement with that determined by potentiometric titration (−495 mV). This provides further support for the assumed mechanism. It would appear, then, that temperature-jump relaxation provides no indication of any conformational changes accompanying the conversion of semiquinone to fully reduced protein.

A temperature-jump experiment was also performed using the *P. elsdenii* flavodoxin under conditions similar to those involving the *Azotobacter* protein. The relaxation behavior (Figure 5) was qualitatively the same as that observed for the *Azotobacter* flavodoxin semiquinone–hydroquinone interconversion. Although a complete concentration analysis of the relaxation behavior was not performed, it is most probable that the mechanism of the interconversion is the same for the two proteins.

It was also of interest to determine the kinetics upon going from the oxidized flavoprotein to the semiquinone form.

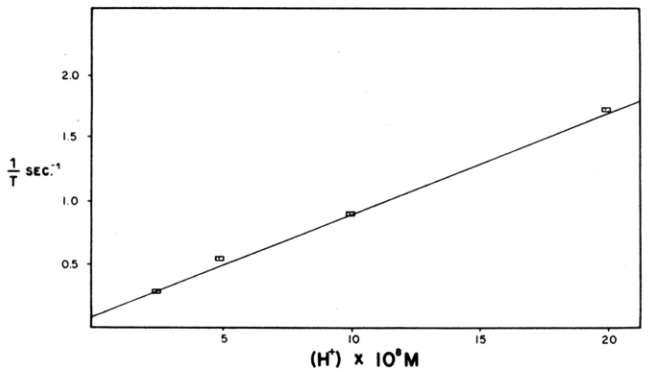


FIGURE 4: Plot of $1/\tau$ *vs.* [H$^+$] for the *Azotobacter* flavodoxin semiquinone–hydroquinone equilibrium. $k' = 3.5 \times 10^4$ M^{-1} sec^{-1}; $k'' = 6.5 \times 10^6$ M^{-1} sec^{-1}.

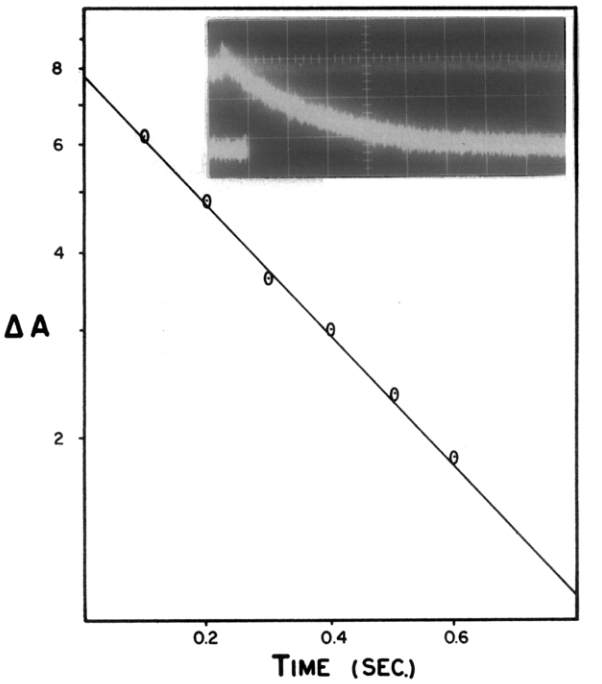


FIGURE 5: Relaxation trace (0.1 sec/division) and semilog plot for the *P. elsdenii* flavodoxin semiquinone–hydroquinone equilibrium: pH 5.8, 0.1 M phosphate buffer; 10 mol % methylviologen; 3×10^{-5} M protein.

However, this equilibrium could not be maintained as above with a simple manipulation of pH using excess dithionite. Attempts were made to achieve an equilibrium by using a less than stoichiometric amount of dithionite. However, in order to bring the kinetics into an accessible time range, a twofold molar excess of methylviologen over protein had to be used. In the presence of this excess viologen and low dithionite concentration, the sample became extremely sensitive to oxidation by the slow leakage of oxygen into the temperature-jump cell. Therefore it proved impossible to maintain the oxidized–semiquinone flavoprotein equilibrium and the kinetics could not be determined. It should be noted that the kinetics of the semiquinone–hydroquinone equilibrium were not affected by a slow leakage of oxygen, due to the presence of the large excess of dithionite.

Summary

Analysis of the temperature-jump relaxation behavior of the *Azotobacter* flavodoxin semiquinone–hydroquinone equilibrium revealed only a single relaxation suggesting the absence of conformational changes accompanying interconversion. This is consistent with earlier studies (Edmondson and Tollin, 1971) in which it was found that the far-uv CD spectra of the oxidized, semiquinone, and hydroquinone forms of the flavoprotein were essentially identical. Therefore, the large enhancement in the binding energy of semiquinone flavine over that of hydroquinone flavine must not result from a conformational change in the protein, or if there is a conformational change, it must be relatively small and highly localized,

It is also interesting that the *P. elsdenii* flavodoxin showed similar relaxation behavior to that of the *Azotobacter* protein. This indicates that the mechanism of reduction is probably the same for both proteins. Additional experiments involving other flavodoxins would be of interest.

The reduction potentials for *Azotobacter* flavodoxin are consistent with the previously determined redox behavior of this enzyme [*e.g.*, (a) semiquinone stability to oxygen (Hinkson and Bulen, 1967; Edmondson and Tollin, 1971); (b) ability to catalyze nitrogen reduction (Benemann *et al.*, 1969); (c) ability to replace ferredoxin in $NADP^+$–photoreduction (Van Lin and Bothe, 1972); (d) oxygen reactivity of the hydroquinone (Hinkson and Bulen, 1967; Edmondson and Tollin, 1971)]. The stability to oxygen is probably a consequence of the very large binding constant of the semiquinone flavine to the apoprotein. Previous work (Vaish and Tollin, 1971) has shown that the neutral form of the flavine radical (which is the form occurring in the flavodoxins) is quite unreactive toward oxygen compared to the anion radical. Thus, it is not unreasonable to suppose that the oxidation of the flavoprotein radicals occurs *via* the prior dissociation of the radical from the protein followed by oxidation through the anionic species. This would account for the correlation between redox potential and oxygen reactivity which we have observed (both Clostridial and *P. elsdenii* flavodoxin semiquinones react with oxygen at approximately the same rate, which is about two orders of magnitude faster than the *Azotobacter* flavodoxin species (Edmondson and Tollin, 1971)).

The fact that the hydroquinone form of this protein is such a powerful reducing agent may aid in the elucidation of its metabolic role. Because the *Azotobacter* flavodoxin is naturally occurring (*i.e.*, not induced through iron deprivation), the second reduction potential cannot be compared to a ferredoxin that it replaces (inasmuch as *Azotobacter* simultaneously produces a ferredoxin), as with most of the other flavodoxins. Therefore, a search for a metabolic substrate is necessary in order to understand the biologial significance of such a negative reduction potential.

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