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PHYSICOCHEMICAL PROPERTIES OF FLAVODOXIN FROM *DESULFOVIBRIO VULGARIS*

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SUMMARY

Reductive titration curves of flavodoxin from *Desulfovibrio vulgaris* displayed two one-electron steps. The redox potential E_2 for the couple oxidized flavodoxin/flavodoxin semiquinone was determined by direct titration with dithionite. E_2 was -149 ± 3 mV (pH 7.78, 25 °C). The redox potential E_1 for the couple flavodoxin semiquinone/fully reduced flavodoxin was deduced from the equilibrium concentration of these species in the presence of hydrogenase and H_2. E_1 was -438 ± 8 mV (pH 7.78, 25 °C).

Light-absorption and fluorescence spectra of flavodoxin in its three redox states have been recorded.

Both the rate and extent of reduction of flavodoxin semiquinone with dithionite were found to depend on pH. An equilibrium between the semiquinone and hydroquinone forms occurred at pH values close to the neutrality, even in the presence of a large excess of dithionite, suggesting an ionization in fully reduced flavodoxin with a $pK_a = 6.6$.

The association constants K for the three FMN redox forms with the apoprotein were deduced from the value of K ($K = 8 \cdot 10^7\ M^{-1}$) measured with oxidized FMN at pH 7.0.

Oxidized flavodoxin was found to comproportionate with the fully reduced protein ($k_{comp} = 4.3 \cdot 10^3\ M^{-1} \cdot s^{-1}$, pH 9.0, 22 °C) and with reduced free FMN ($k_{comp} = 44\ M^{-1} \cdot s^{-1}$, pH 8.1, 20 °C).

Fast oxidation of reduced flavodoxin occurred in the presence of O_2. Slower oxidation of semiquinone was dependent on pH in a drastic way.

INTRODUCTION

Flavodoxin is a low molecular weight (about 15 000) electron-transfer flavoprotein. It contains one molecule of flavine mononucleotide. It was originally isolated

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from iron-deficient *Clostridium pasteurianum* [1, 2]. Similar flavoproteins were obtained from the sulfate-reducing bacteria *Desulfovibrio gigas* [3] and *Desulfovibrio vulgaris* [4]. *Desulfovibrio* flavodoxins substitute for the iron-sulfur protein ferredoxin as low-potential electron carriers [3, 5]. Their blue semiquinone state [6] exhibits spectral characteristics comparable with those of dehydrogenases [7]. All these characters – except the molecular weight which changes from 14 000 to 23 000 – are found in flavodoxins and phytoflavins purified from several other microorganisms [8–16].

D. vulgaris flavodoxin is the first flavoprotein for which both the complete amino-acid sequence [17] and X-ray diffraction structure of the oxidized form at 2.5 Å [18] and, more recently, at 2 Å [19] resolution, have been performed. Similar structural investigations on other flavodoxins have been reported [20–23]. The X-ray diffraction structure of the flavodoxin from *Clostridium MP* has been obtained at 3.25 Å resolution [24].

This paper describes the oxidation-reduction properties of the flavodoxin from *D. vulgaris*. Some of these results have already been communicated [25]. Similar determinations have been performed on the flavodoxins from *C. pasteurianum* and *Clostridium MP* [11, 12], from *Escherichia coli* [13], from *Peptostreptococcus elsdenii* [26], and also on the *Azotobacter* (Shethna) flavoprotein [9, 27, 28].

MATERIALS AND METHODS

Proteins

Flavodoxin was prepared from *D. vulgaris* as described previously [3, 4]. Apoflavodoxin was prepared by 3 % trichloroacetic acid precipitation following the method of Hinkson [9] and was used extemporarily. A partly purified preparation of hydrogenase (EC 1.98.1.1) was obtained by the procedure of Hatchikian and Le Gall [29].

Titration with dithionite

Potentiometric titrations with dithionite were performed in an all-glass apparatus built by G. Mazza (Thèse d'Etat; Université de Marseille, December 1971). The solutions of protein (titration vessel) and of reductant (2-ml buret) were bubbled for 5 h before titration with purified argon. The titration vessel was kept in the dark to prevent photoreduction of flavodoxin. The measuring unit was a Metrohm potentiograph, type E 336 A, calibrated with an AOIPW battery. An Ag/AgCl electrode was used as a reference. Stock solutions of dithionite were $5 \cdot 10^{-4}$ M. Solutions of flavodoxin were $3.6 \cdot 10^{-5}$ M. Buffer was 0.05 M phosphate, pH 7.78. The temperature was 25 °C.

The redox potentials (one-electron steps) were calculated by the method of Reed and Berkson [30] from the modified Nernst equation:

$$E_h = E_m + 2.303 \frac{RT}{F} \log C \qquad (1)$$

where E_m is the midpoint potential value and E_h the potential measured after any dithionite addition. Log C is obtained from the equation:

$$E_m = E_r - 2.303 \frac{RT}{F} \log C \quad (2)$$

where E_r is the potential measured after addition of a reference amount of dithionite.

Titration with H_2 and hydrogenase

The redox potential E_1 for the couple flavodoxin semiquinone/flavodoxin hydroquinone was determined with H_2 in the presence of catalytic amounts of hydrogenase, following the equation:

$$E_1 = E_h - 2.303 \frac{RT}{F} \log \frac{[\text{semiquinone}]}{[\text{hydroquinone}]} \quad (3)$$

The potential E_h depends on the pH and on the partial pressure of H_2 P_{H_2}:

$$E_h = -2.303 \frac{RT}{F} \text{pH} - 2.303 \frac{RT}{2F} \log P_{H_2} \quad (4)$$

The concentrations of semiquinone and hydroquinone were determined at equilibrium by spectrophotometry. An optical cuvette fitted with a side arm containing hydrogenase was used. Experiments were performed at pH 7.78 (0.05 M phosphate buffer) and 25 °C.

Spectrophotometric experiments

Sodium dithionite used for the determination of the absorption and fluorescence spectra of fully reduced flavodoxin was recrystallized from half-saturated solutions of Na_2HPO_4. The mixed crystals of dithionite and phosphate were washed with absolute ethanol, then dessicated in vacuo. Their dithionite content was measured spectrophotometrically by anaerobic titration of 10^{-3} M solutions of FMN at pH 9.0. Such dithionite samples exhibited a sharp absorption peak at 315 nm that vanished after air oxidation.

Absorption spectra were recorded either with a Cary 15 or with a Cary 14 instrument. Fluorescence spectra were recorded independently with Fica, Aminco-Bowman or Perkin-Elmer apparatus.

The absorption spectrum of fully-reduced flavodoxin was obtained by reducing the protein in an anaerobic cuvette (1-cm light path) with an excess of dithionite. A variable-light-path cuvette containing dithionite was disposed in the reference compartment of the spectrophotometer. Compensation of the dithionite absorbance was measured from the plot of absorbance variations at 315 and 330 nm versus the length of the variable cuvette.

Photoreduction

Photoreduction of flavodoxin [7] has been performed in the presence of 0.05 M EDTA. A Thunberg cuvette degassed with pure N_2 and a 100-W tungsten lamp were used.

Association equilibria

Microliter quantities of apoflavodoxin were added to $1.2 \cdot 10^{-5}$ M solutions of oxidized FMN (0.1 M Tris buffer, pH 7.0, 25 °C). Binding of FMN to apoprotein

was determined at equilibrium, either by measuring the quenching of flavin fluorescence (at 530 nm) caused by interaction with the apoprotein, or by differential absorption spectrophotometry at 492 nm ($\Delta\varepsilon_{492} = 3350\ M^{-1} \cdot cm^{-1}$ between free and protein-bound FMN). The concentration of apoprotein was determined by titration with FMN as measured by fluorescence quenching.

Nomenclature

The three redox states of flavodoxin have been listed as follows: $Fld_{ox}H$, flavoquinone (neutral), $\varepsilon_{460} = 10\,700\ M^{-1} \cdot cm^{-1}$; $\dot{F}ldH_2$, flavosemiquinone (neutral), $\varepsilon_{580} = 4100\ M^{-1} \cdot cm^{-1}$; $Fld_{red}H_2^-$, flavohydroquinone (anionic), $\varepsilon_{357} = 4100\ M^{-1} \cdot cm^{-1}$; $Fld_{red}H_3$, flavohydroquinone (neutral).

RESULTS

Light-absorption and fluorescence spectroscopy

Anaerobic reduction with dithionite of the yellow flavoquinone form of flavodoxin from *D. vulgaris* at pH 7.8 occurred in two steps. Addition of 0.5 mol of dithionite per mol of protein resulted in the formation of a blue-grey coloured species. The absorption spectrum of this compound is the same as that obtained previously [6] by light irradiation in the presence of EDTA (Fig. 1) and is characteristic of the neutral flavosemiquinone [7, 31]. On further addition of reductant to a total of 1.0 mol per mol flavodoxin, the solution turned pale yellow in colour. The light-absorption spectrum of this fully reduced species (Fig. 1) is characteristic of flavohydroquinone [26, 32, 33].

Solutions of oxidized flavodoxin exhibited a weak fluorescence (maximum emission wavelength: 521 nm). The excitation spectrum closely matched the absorp-

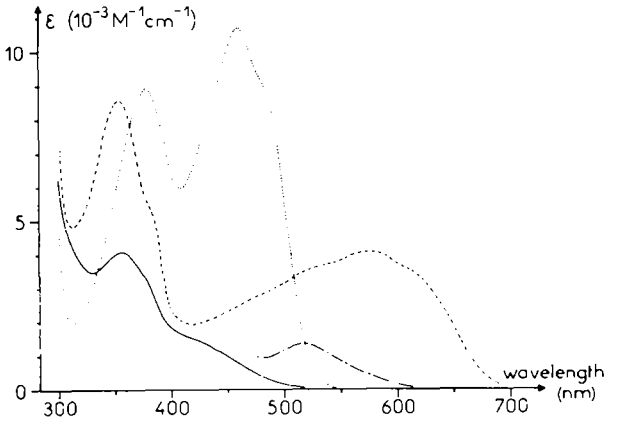


Fig. 1. Light-absorption and fluorescence spectra of flavodoxin from *D. vulgaris* in 0.025 M phosphate buffer (pH 8.1) at room temperature: · · ·, absorption of the oxidized protein (λ_{max} 378 and 460 nm); - - -, absorption of the semiquinone produced by light irradiation in the presence of 0.05 M EDTA (λ_{max} — 352 and 580 nm); , absorption of the protein reduced with an excess of dithionite (see Materials and Methods) (λ_{max} = 357 nm); - · - ·, fluorescence emission (λ_{max} — 519 nm) of the fully reduced form.

tion spectrum of free FMN and the fluorescence yield was only 3 % with respect to oxidized free FMN. Fluorescence of oxidized flavodoxin solutions thus may be due to some denaturation of the protein in the course of lyophylization.

After treatment with a slight excess of sodium dithionite (0.025 M phosphate buffer, pH 8.1), fully reduced flavodoxin exhibited a weak fluorescence (maximum emission wavelength: 519 nm). The excitation spectra shown marked maxima at 292 and 357 nm and a shoulder at 420 nm, i.e. they were characteristic of flavohydroquinone.

Potentiometry

Titration of flavodoxin with dithionite shown two monoelectronic steps (Fig. 2). The slope of the first plateau was 117 mV per electron equivalent, in good agreement with the theoretical slope expected from a one-electron step [30]. Potential values measured at the end of the first plateau diverged from the 117-mV average slope (Fig. 2). Such an effect may come from the way the system is reaching equilibrium in this region of titration [26]. If the non-aligned dots are omitted, a straight line (Fig. 3) can be obtained by using the method of Reed and Berkson [30]; absence of O_2 at any time of the titration was proved by a zero-ordinate intercept. The redox potential E_2 for the couple $Fld_{ox}H/\dot{F}ldH_2$ was obtained from the slope of this line (Fig. 3) and was found to be equal to -149 ± 3 mV (pH 7.78, 25 °C) from an average over three experiments.

The value of the redox potential E_1 for the couple flavodoxin semiquinone/flavodoxin hydroquinone was deduced from equilibrium studies in the presence of hydrogenase and H_2 (see Materials and Methods). E_1 was -438 ± 8 mV (pH 7.78, 25 °C).

At low pH values, the reduction of flavodoxin with a large excess of dithionite

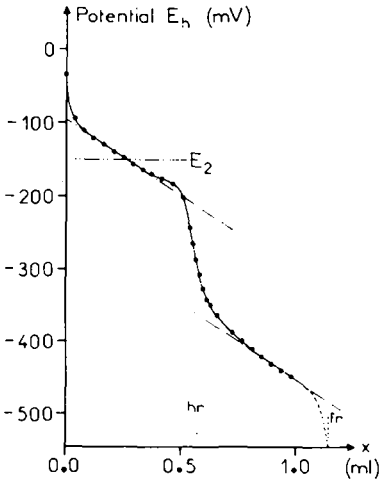


Fig. 2. Potentiometric titration of flavodoxin from *D. vulgaris* with dithionite (see conditions under Materials and Methods). Half-reduction (hr) and full-reduction (fr) were deduced from the value of the redox potential E_1 measured at equilibrium with hydrogenase and H_2. x (abscissa) is the volume of dithionite added. Buffer was 0.05 M phosphate, pH 7.78.

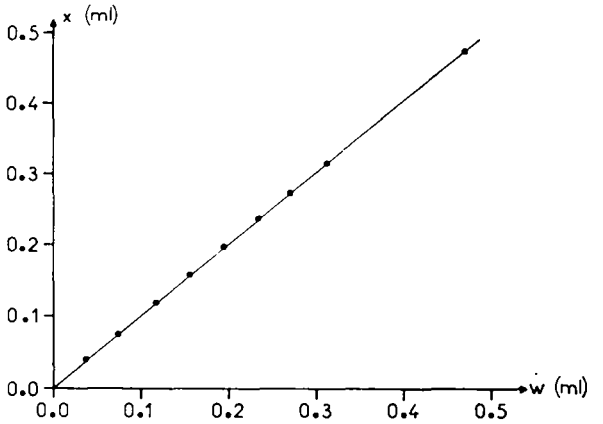


Fig. 3. Determination of E_2 following the method of Reed and Berkson [30]. x (ordinate) is the volume of reducing agent added at any given stage of the titration. w (abscissa) is calculated from a reference amount of dithionite x_r and from the difference $(E_h - E_r)$. $E_2 = E_r - 0.059 \log C$. In this experiment, the reference potential E_r was -0.2405 V and the slope x/w is $(C+1) - 1.031$, then log $C = -1.51$ and $E_2 = -0.151$ V.

did not reach completion, i.e. a pH dependent equilibrium occurred between semiquinone and hydroquinone. Similar effects have already been evidenced in *Azotobacter* flavoprotein [27, 28] and in *P. elsdenii* flavodoxin [34]. The [hydroquinone]/[semiquinone] ratio was dependent on $1/[H^+]$ in an almost linear way and an equivalent point was found at a pH value of about 6.6 (Fig. 4). Following Edmonson and Tollin [27], the pH value where the ratio [hydroquinone]/[semiquinone] = 1 gives the ionization pK_a of flavohydroquinone, agreeing with the entry of one proton in the second reduction step.

The potential E_2 for the couple: $Fld_{ox}H + H^+ + e^- \rightleftharpoons \dot{F}ldH_2$ is given at any pH by the equation:

$$E_2 = E_2^0 - 2.303 \frac{RT}{F} \text{ pH} \tag{5}$$

i.e. E_2 varies with pH like the H_2 electrode since ionization of the semiquinone form is quenched by the protein. From $E_2 = -149 \pm 3$ mV at pH 7.78, we can deduce:

$$E_2^0 = +311 \pm 3 \text{ mV (pH 0.0)} \tag{6}$$

$$E_2^{0'} = -102 \pm 3 \text{ mV (pH 7.0)} \tag{7}$$

The potential E_1 for the couple: $\dot{F}ldH_2 + e^- \rightleftharpoons Fld_{red}H_2^- \rightleftharpoons H^+ \rightleftharpoons Fld_{red}H_3$ is given by the equation:

$$E_1 = E_1^0 + 2.303 \frac{RT}{F} \log (K + [H^+]) \tag{8}$$

where K is the ionization constant of the fully reduced form and $[H^+]$ the H^+ activity. From $E_1 = -438 \pm 8$ mV at pH 7.78, we can deduce:

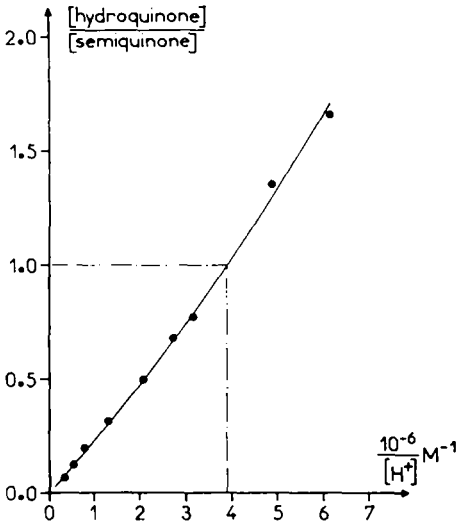


Fig. 4. Determination of the ionization constant of fully reduced flavodoxin. Excess dithionite ($3.5 \cdot 10^{-3}$ M) was added to anaerobic solutions of the protein. 0.2 M phosphate buffers were used. Semiquinone concentration was obtained from $\varepsilon_{580}(\dot{F}ldH_2) = 4100\ M^{-1} \cdot cm^{-1}$. Note that dithionite is stable at all the pH values used [34].

$$E_1^{\,0} = -50 \pm 8 \text{ mV (pH 0.0)} \tag{9}$$

$$E_1^{\,0'} = -431 \pm 8 \text{ mV (pH 7.0)} \tag{10}$$

From Eqn 8, E_1 is shown to be constant ($E_1 = -439 \pm 8$ mV) at alkaline pH values, i.e. at pH $\geqslant$ (pK+2), agreeing with experiments performed on *P. elsdenii* flavodoxin [26]. Hence, we do not agree with the values of the redox potentials given by Barman and Tollin [28].

Association equilibria

The association constant K for oxidized FMN and apoflavodoxin (0.1 M tris buffer, pH 7.0), i.e.:

$$K_{ox} = \frac{[Fld_{ox}H]}{[FMN_{ox}H]\,[\text{apoprotein}]}$$

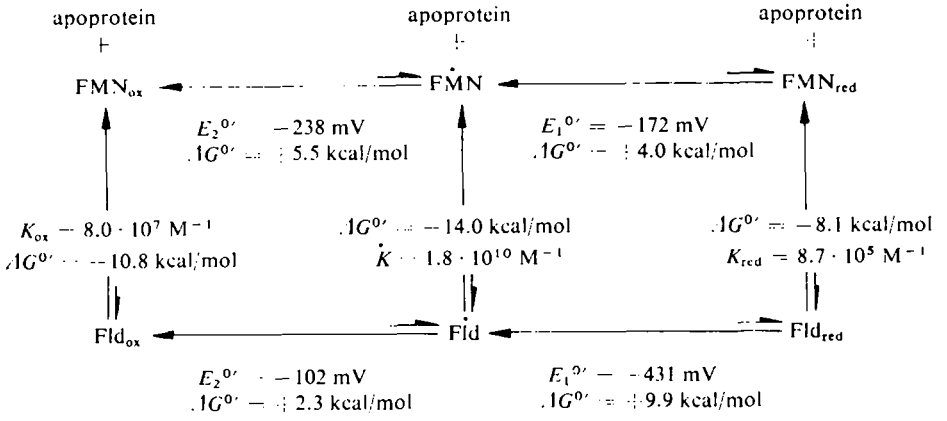
was determined by fluorimetry and by differential absorption spectrophotometry (see Materials and Methods.) The following values were obtained from an average over three experiments:

$K_{ox} = 8.0 \cdot 10^7\ M^{-1}$ at 25 °C

$K'_{ox} = 2.5 \cdot 10^7\ M^{-1}$ at 14 °C

Association is endothermic, i.e. the entropy change for this reaction is large. The association constants for the semiquinone and fully reduced forms of FMN with the apoprotein can be obtained from a free energy diagram (Table I) based on the fact that free energy is path independent. From the values of the redox potentials $E_1^{\,0'}$ and

TABLE I



$E_2^{0'}$ found for *D. vulgaris* flavodoxin (see above), and for free FMN by Draper and Ingraham [41], we found:

$$\dot{K} = 2.0 \cdot 10^{10}\ M^{-1} \quad K_{red} = 8.9 \cdot 10^5\ M^{-1} \quad (pH\ 7.0, 25\ ^\circ C)$$

The free energy change for the binding of FMN to apoflavodoxin is similar for all flavodoxins in the oxidized state [9, 11, 28]. The association constant of fully reduced FMN with apoflavodoxin, however, varies widely with the protein: reduced FMN is less tightly bound to *D. vulgaris* flavodoxin than to other flavodoxins [26, 28].

Dismutation kinetics

Fully reduced flavodoxin reacted with the oxidized form to yield semiquinone, and the semiquinone formation constant K_d was found to obey the equation:

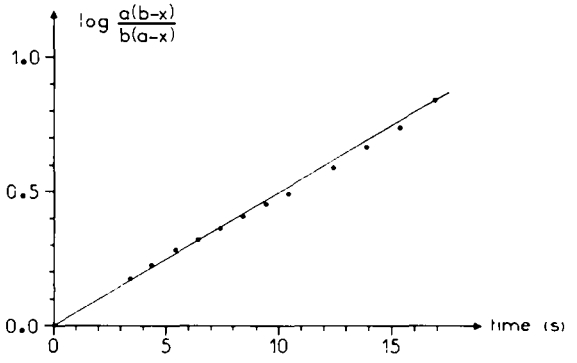


Fig. 5. Second-order analysis of semiquinone formation from a mixture of oxidized and reduced flavodoxin (pH 9.0, 22 °C). At time zero, oxidized flavodoxin was [Fld$_{ox}$H] = a = 2.5 · 10^{-5} M; reduced flavodoxin was [Fld$_{red}$H$_2^-$] = b = 5.1 · 10^{-5} M. Semiquinone was initially present since hydroquinone was obtained from long light irradiation in the presence of 0.05 M EDTA. Observation wavelength: 580 nm.

$$2.303\frac{RT}{F}\log K_d = E_2^0 - E_1^0 - 2.303\frac{RT}{F}\log(1+K/[H^+]) \quad (11)$$

where K is the ionization constant of the fully reduced form. The dismutation kinetics were second order (Fig. 5). Under alkaline conditions, reduced flavodoxin is anionic, while semiquinone is not ionizable. The dismutation reaction then may be described by the following scheme:

$$Fld_{ox}H + Fld_{red}H_2^- \rightleftharpoons (H^+) \underset{k_-}{\overset{k_+}{\rightleftharpoons}} 2\ \dot{F}ldH_2$$

The rate constant $2k_+$ was found $4.3 \cdot 10^3\ M^{-1} \cdot s^{-1}$ (pH 9.0, 22 °C). From $K_d = 5000$ at pH 9.0, we can deduce $2k_- = 0.86\ M^{-1} \cdot s^{-1}$.

Oxidized flavodoxin could also comproportionate with reduced free FMN. The reaction kinetics were recorded with an anaerobic stopped-flow apparatus built by one of us. One drive syringe contained oxidized flavodoxin degassed by argon, the other contained a 10-fold excess of FMN reduced with H_2 platinum black. The total yield of flavosemiquinone at equilibrium was less than in the previous experiment, agreeing with the instability of the FMN radical under the conditions used [35]. The rate constant $2k_+$ was deduced from an initial rate analysis and was found to be equal to $44\ M^{-1} \cdot s^{-1}$ (pH 8.1, 20 °C).

Dithionite reduction

In the presence of a large excess of dithionite, reduction of flavodoxin semiquinone to the hydroquinone form followed pseudo-first order kinetics (Fig. 6). The reducing agent is probably the monomeric species SO_2^- [34, 36]. The rate of reduction at constant dithionite concentration was found to be faster at low pH: the initial rates were $V_i = 1.2 \cdot 10^{-6}\ M \cdot s^{-1}$ at pH 10 and $V_i = 3.6 \cdot 10^{-6}\ M \cdot s^{-1}$ at pH 6.

Reduction of the oxidized form with the same excess of dithionite followed more complex kinetics. Semiquinone, possibly due to ionic strength-promoted com-

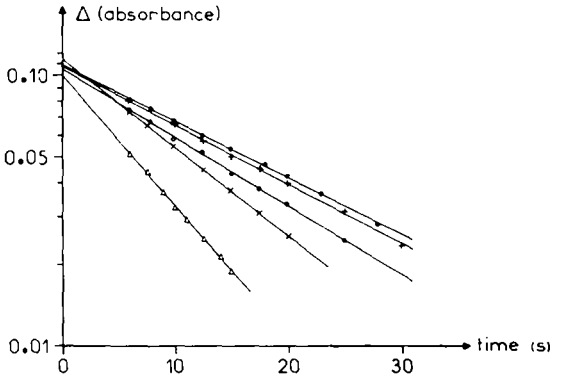


Fig. 6. First order analysis of the reduction of semiquinone with dithionite (22 °C). At time zero, semiquinone was about $2.5 \cdot 10^{-5}$ M over the five experiments; dithionite was $3.5 \cdot 10^{-3}$ M. Buffers were 0.2 M phosphate or carbonate. △: pH 6.0; ×: pH 7.0; ○: pH 8.1; +: pH 9.0; ●: pH 10.0. Observation wavelength: 580 nm.

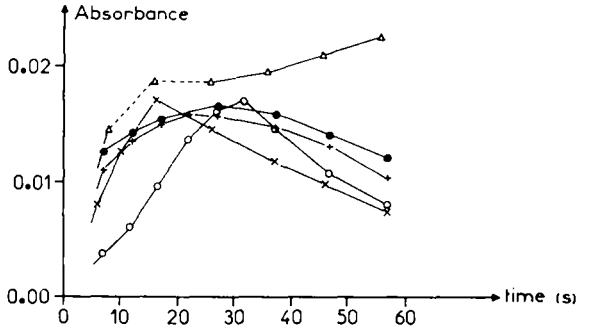


Fig. 7. Semiquinone formation during the reduction of oxidized flavodoxin with dithionite (22 °C). Flavodoxin was $1.1 \cdot 10^{-5}$ M. Dithionite was $3.5 \cdot 10^{-3}$ M. Buffers were 0.2 M phosphate or carbonate. △: pH 6.0; ×: pH 7.0; ○: pH 8.1; +: pH 9.0; ●: pH 10.0. Observation wavelength: 580 nm.

proportionation [34], was found to accumulate at pH 6.0; at higher pH values, its formation was only transitory (Fig. 7). Initial rate analysis shown that flavoquinone is much less reactive with dithionite than semiquinone and that the reaction is slightly acid-inhibited: $V_i = 3.0 \cdot 10^{-7}\ M \cdot s^{-1}$ at pH 10 and $V_i = 1.5 \cdot 10^{-7}\ M \cdot s^{-1}$ at pH 6.

Photoreduction

At constant quanta per time unit, the rate of semiquinone production by light irradiation in the presence of EDTA was found to increase from neutrality to pH 10 [6] and to decrease at more alkaline pH values. The isosbestic point observed at 507 nm during conversion of the oxidized form into semiquinone at pH 7 was shifted to 511 nm at pH 10. Sharpening of the 490-nm shoulder of oxidized flavodoxin was also observed upon basification; this may be ascribed to a change in the vibronic structure of electronic transitions of the flavin, due to a modification of the coenzyme environment [35], since no radical anion was obtained at pH 11.

On the other hand, formation of fully reduced flavodoxin occurred slowly upon prolonged photoreduction at slightly alkaline pH values (e.g. 24 h at pH 9 gave 80 % hydroquinone). The absorption of oxidized flavodoxin was fully restored on air oxidation. Reduction to the hydroquinone state can be explained in terms of disproportionation (since the semiquinone formation constant K_d is decreased on the basic side, see Eqn 11) assuming that the first photoreduction step ($Fld_{ox}H \rightarrow \dot{F}ldH_2$) is much faster than the second ($\dot{F}ldH_2 \rightarrow Fld_{red}H_2^-$). Note that the dismutation rates are fast under the conditions of photoreduction because of ionic strength effects from the dissociation products of EDTA [34].

Oxidation by O_2

Fully reduced flavodoxin rapidly reacted with O_2 to yield almost quantitative semiquinone. Semiquinone then decayed at a lower rate. Oxidation of the radicalar form with a large excess of O_2 followed pseudo-first order kinetics; the logarithm of the first order rate constant varied linearly with the pH (Fig. 8). Stabilisation of the superoxide radical under basic conditions [37] might explain such an effect:

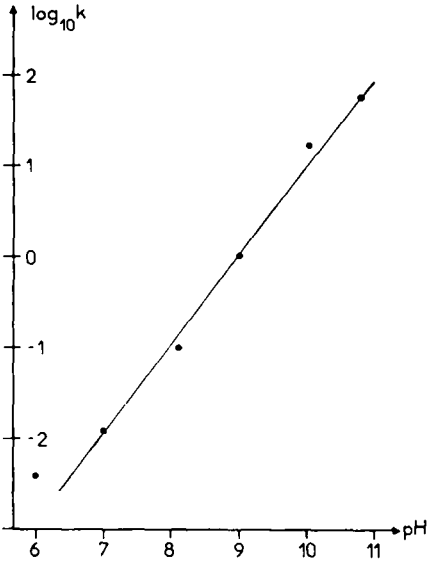


Fig. 8. pH dependence of the first-order rate constant for oxidation of semiquinone with excess O_2 (22 °C). Semiquinone was $3 \cdot 10^{-5}$ M; oxygen was $1.4 \cdot 10^{-3}$ or $1.4 \cdot 10^{-4}$ M. Buffers were 0.2 M in phosphate or carbonate.

pH	6.0	7.0	8.1	9.0	10.0	10.8
k (min^{-1})	0.004	0.012	0.101	1.05	17.2	60.4

$$\dot{F}ldH_2 + O_2 \rightarrow Fld_{ox}H + H^+ + \dot{O}_2^- \quad \dot{O}_2^- + H^+ \rightleftharpoons H\dot{O}_2 \quad H^+ + OH^- \rightleftharpoons H_2O$$

However, no lag time due to accumulation of the superoxide radical in the solvent [38] was observed at any pH. It can be concluded that flavodoxin semiquinone reacts readily with O_2 as well as with $\dot{O}_2^-$ and that the first reduction step of O_2 may be promoted by OH^- present at the flavin site. Semiquinone from *P. elsdenii* flavodoxin was found to react with O_2 in a way similar to the semiquinone from *D. vulgaris* flavodoxin, although with a much smaller rate constant [26].

DISCUSSION

The redox potentials $E_1^{0'}$ and $E_2^{0'}$ for the couples flavohydroquinone/flavosemiquinone and flavosemiquinone/flavoquinone, respectively, vary among flavodoxins and phytoflavins from various sources [12, 13, 26, 39] at a slight extent only, with a marked exception for the *Azotobacter* flavo-protein [28]. $E_1^{0'}$ is shifted to very negative values with respect to free FAD [40] or free FMN [40, 41] in such a way that flavodoxins may substitute for ferredoxin [16]. Flavodoxins work essentially as one-electron carriers, but bielectronic reactions are allowed [42].

The free energy change for the formation of semiquinone from *D. vulgaris* flavodoxin is $\Delta G^{0'} = -7.60 \pm 0.25$ kcal/mol (pH 7.0, 25 °C). Partial hindrance of the ring inversion of flavohydroquinone could provide this energy since the activation barrier for ring inversion is about 10 kcal/mol [43]. Sequence determination [17]

and X-ray structures of oxidized flavodoxin from *D. vulgaris* at atomic resolution [18, 19] shown that the FMN moiety is "sandwiched" between two aromatic amino acid residues, viz. tyrosine at Position 98 and tryptophan at Position 60 of the polypeptide chain. Ring inversion of flavohydroquinone thus could be partly hindered if tight enough stacking exists in the fully reduced state. Locking the ring inversion of anionic-reduced free flavin, as obtained in a rigid ethanolic glass at liquid N_2 temperature, results in total resolution of an absorption band at 415 nm which reveals the first electronic transition of flavohydroquinone, and in fluorescence of reduced flavin [44]. Loss of both fluorescence and resolution of the 415-nm band in a fluid solvent is due to conformational instability of free flavohydroquinone. Decrease of the ring inversion rate of reduced FMN inside *D. vulgaris* flavodoxin is supported by the weak fluorescence emission from the coenzyme moiety at room temperature, but complete hindrance of the reduced flavin vibration is unlikely since the 400–500-nm shoulder of the reduced holoprotein (Fig. 1) is uncompletely resolved as compared with free flavohydroquinone in ethanol at 77 °K [44]. On the other hand, quenching the fluorescence of oxidized flavin may be due to hydrogen bridges between protein and coenzyme or to some complexation, possibly of charge-transfer type, between the isoalloxazine ring and the (aromatic) amino acid residues, in agreement with Ghisla et al. [44].

The light-absorption and fluorescence-emission maxima of fully reduced *D. vulgaris* flavodoxin are blue-shifted of about 10 nm with respect to flavodoxin from *P. elsdenii* [44]. Determination of the ionic state of fully reduced flavodoxin from the position of these maxima appears unreliable, but ionization of reduced coenzyme has been evidenced in flavodoxins from the study of oxidation-reduction equilibria [11, 26, 27]. We obtained the ionization constant of *D. vulgaris* flavodoxin-hydroquinone from the pH dependence of reduction with a large excess of dithionite. The pK value obtained (pK = 6.6) is very close to the pK of reduced free FMN [41]. The N(1) position of *D. vulgaris* flavodoxin-bound flavohydroquinone thus is thought to be widely accessible to the solvent.

Electron-transfer reactions between flavodoxin molecules from *D. vulgaris* may proceed through the benzene ring since only this part of the oxidized flavin moieties is sufficiently exposed at the surface of the protein [18, 19]. Owing to electron delocalization over the aromatic part [47], side contact can be quite effective for electron transfer. This idea has received support from kinetic studies of the dismutation reactions of free flavins in non-aqueous media (Favaudon, V. and Lhoste, J.-M., unpublished). Most flavodoxins appear to comproportionate at a rather fast rate, and the biological importance of such a comproportionation has been proposed [42]. At this day, however, comparing the values of the comproportionation rate constants for various flavodoxins (whether in direct experiments or in cross-reactions with free FMN) is unwarranted in view of the drastic influence from ionic strength [34].

The blue semiquinone of flavodoxin from *D. vulgaris* is not ionizable, even at pH 11 as far as the protein does not undergo denaturation, while the pK value of free FMN semiquinone is 8.5 [35, 41].Such a stabilization may be due to interaction between the N(5) position of the semi-reduced isoalloxazine ring and a proton-donor amino acid residue [45] or through water present [19] at the flavin site, without preventing contact between semiquinone and water molecules from the solvent [31,46]. A conformation change of the protein may be required during the first reduction step

of oxidized *D. vulgaris* flavodoxin to allow the formation of a strong hydrogen bridge at N(5). Hence, stabilization of the radicalar form of flavodoxin is due to entropy factors connected with protonation at N(5) as well as with planarization of flavohydroquinone. In fact, kinetic studies of the dismutation reactions of free flavins in non-aqueous media (i.e. under conditions allowing an absolute control of the acid-base reactions) have shown that disproportionation of the neutral radical proceeds through a labile intermediate complex, the heterolytic splitting of which requires previous release of a proton [47].

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