BBA 66290

PROPERTIES OF TWO CLOSTRIDIAL FLAVODOXINS

STEPHEN G. MAYHEW
Department of Biological Chemistry, The University of Michigan, Ann Arbor, Mich. 48104 (U.S.A.)

(Received October 12th, 1970)

SUMMARY

1. Flavodoxins have been purified from *Clostridium pasteurianum* and Clostridium MP and their properties examined. Clostridium MP flavodoxin, like flavodoxins from *C. pasteurianum* and *Peptostreptococcus elsdenii*, has a molecular weight of about 14 000, it contains 1 molecule of FMN, and it replaces ferredoxin as an electron carrier in the phosphoroclastic oxidation of pyruvate. The two clostridial proteins have different amino acid compositions.

2. The absorption spectra of flavodoxins from Clostridium MP and *C. pasteurianum* are very similar. Both proteins are reduced by irradiation with visible light in the presence of EDTA, NADPH in the presence of ferredoxin–$NADP^+$ reductase (EC 1.6.99.4), hydrogen in the presence of hydrogenase (EC 1.12.1.1), and $Na_2S_2O_4$. At half reduction, high yields of the blue neutral flavin semiquinone are obtained. Clostridium MP flavodoxin, like *P. elsdenii* flavodoxin, is completely reduced by 1 mole of $Na_2S_2O_4$. *C. pasteurianum* flavodoxin is initially reduced by a small excess of $Na_2S_2O_4$, but the reduced protein slowly reoxidizes to give a mixture of semiquinone and fully reduced flavodoxin. The extent of this reoxidation depends on the $Na_2S_2O_4$ concentration and on pH. Extinction coefficients for the three oxidation–reduction states of the flavodoxins have been determined.

3. The oxidation–reduction potential (E_1) for the couple flavodoxin semiquinone–fully reduced flavodoxin is −0.399 V and −0.419 V at pH 7 and 25° for Clostridium MP and *C. pasteurianum* flavodoxins, respectively. The oxidation–reduction potential (E_2) for the couple oxidized flavodoxin–flavodoxin semiquinone is −0.092 V and −0.132 V at pH 7 and 25° for Clostridium MP and *C. pasteurianum* flavodoxins, respectively. The effects of pH on E_1 suggest that an ionization occurs at about pH 6.7 in the fully reduced forms of these proteins. Potentiometric titrations with *P. elsdenii* flavodoxin have confirmed earlier results which indicated that this flavodoxin also is reduced in two 1-electron oxidation–reduction steps.

4. FMN is displaced from the flavodoxins by low concentrations of phenylmercuric acetate. The rate of this reaction increases with flavodoxins from *C. pasteurianum*, *P. elsdenii* and Clostridium MP, respectively, and a correlation is drawn between the observed rate and the cysteine content of the protein.

INTRODUCTION

Flavodoxin was first discovered in extracts of *Clostridium pasteurianum* grown in iron-poor medium[1–3]. More recently, similar proteins have been isolated from *Desulfovibrio gigas*[4–7], *Desulfovibrio vulgaris*[7] and *Peptostreptococcus elsdenii*[8–10]. A protein which closely resembles flavodoxin in its catalytic and chemical properties has been isolated from *Anacystis nidulans* and termed phytoflavin[11–13]. All of these proteins have a molecular weight of about 15 000, they contain 1 molecule of FMN as prosthetic group, and they function as electron carriers in low potential oxidation–reduction reactions.

This paper reports the isolation of a second clostridial flavodoxin and a comparison of this protein with flavodoxin from *C. pasteurianum*. In some experiments the clostridial flavodoxins are also directly compared with flavodoxin from *P. elsdenii*. The species of the Clostridium from which this new flavodoxin has been isolated is not known; it is referred to in this paper as Clostridium MP. An X-ray crystallographic study on crystals of flavodoxin from Clostridium MP has been reported[14,15].

METHODS AND MATERIALS

Growth of microorganisms

Two clostridial strains were used in this work. One was *C. pasteurianum* strain W5 (ATCC 6013) and was obtained directly from the American Type Culture Collection at the start of this investigation. The other strain was obtained indirectly from the ATCC in 1962 as *C. pasteurianum* strain W5 and had since been maintained through serial transfers by the author and by Dr. J. L. Peel of the A.R.C. Food Research Institute, Norwich, England. In the course of the present study it became clear that the flavodoxin from this strain differed significantly from that prepared from the current ATCC 6013, and that there were also morphological differences between the two strains, the current ATCC 6013 having larger cells. Pending a more detailed bacteriological examination, the strain obtained in 1962 will therefore be referred to in this paper by the trivial name, Clostridium MP.

Clostridium MP was maintained on potato broth medium as described by DAINTY AND PEEL[16]. Large cultures of up to 40 l were grown on the synthetic medium of CARNAHAN AND CASTLE[17], modified to contain 10 g of glucose and 5 g of $CaCO_3$ per l of culture. The iron concentration in the medium was also different from that used by CARNAHAN AND CASTLE[17]. At an early stage in this work, cultures of Clostridium MP were grown in the presence of 120 μg iron per l, but for reasons to be described later, more recent cultures have been grown with 55 μg of iron per l. Iron was added to the medium as an 0.22% solution of $FeSO_4 \cdot 7H_2O$ in 0.05 M H_2SO_4.

The procedure for growth of Clostridium MP was as follows. Tubes of synthetic medium were inoculated with about 5% (v/v) of a vigorously growing culture on potato broth. The tubes were plugged with pyrogallol and incubated for 1–2 days at 30°. Two such tube cultures were used to inoculate 3 l of synthetic medium in a 5-l conical flask which was fitted with an attachment for flushing with gas. The flask was flushed with nitrogen for 10 min before inoculation, and for a further 10 min after inoculation. It was then incubated at 30° for 2 days. This culture was used as

the inoculum for 37 l of synthetic medium in a 12-gal bottle. 40-l cultures were incubated at 30° for 3–4 days, or until gas production ceased.

C. pasteurianum (ATCC 6013) was grown in 40-l cultures as described for Clostridium MP. This organism was grown with $(NH_4)_2SO_4$ as nitrogen source on the medium of CARNAHAN AND CASTLE[17], modified to contain only 120 μg of Fe^{2+} per l. *C. pasteurianum* grew more rapidly on this medium than Clostridium MP. The total growth was also higher; the yield of dry cells of *C. pasteurianum* was approximately 1 g/l of culture.

P. elsdenii (strain LC 1) was grown in iron-poor medium when flavodoxin was required, and in iron-rich medium when ferredoxin-free extracts were to be prepared[9].

Cultures of all three organisms were harvested with a Sharples super centrifuge. The harvested cells were dried in a vacuum over H_2SO_4 at room temperature, ground to a fine powder, and stored at −20°.

Flavodoxin assay

Flavodoxins were assayed by using the phosphoroclastic system of *P. elsdenii*[9]. Flavodoxin stimulates the oxidation of pyruvate to acetyl phosphate, H_2 and CO_2 by ferredoxin-free extracts of *P. elsdenii*. In this assay, the acetyl phosphate formed in 10 min at 30° is measured by reaction with neutral hydroxylamine followed by acid $FeCl_3$ reagent[9,18]. A unit of flavodoxin is defined as the amount to cause an absorption change of 1.0 at 540 nm, and is equivalent to the formation of approx. 6 μmoles of acid hydroxamate[19].

Purification of flavodoxins

The procedures used to purify and crystallize flavodoxins from Clostridium MP and *C. pasteurianum* were very similar to those previously described in detail for the purification of *P. elsdenii* flavodoxin[9]. Both organisms provided approx. 0.5 mg of crystalline product per g of dried cells. The shape of the crystals of *C. pasteurianum* flavodoxin was as described by KNIGHT AND HARDY[2]. Flavodoxin from Clostridium MP crystallized as long prisms[14].

Enzyme preparations

Flavodoxin and a crude preparation of hydrogenase (EC 1.12.1.1) were made from dried cells of *P. elsdenii* as described previously[9,10]. Ferredoxin–$NADP^+$ reductase (EC 1.6.99.4) was purified from spinach leaves by a procedure similar to that of SHIN *et al.*[20].

Anaerobic titrations with $Na_2S_2O_4$ or NADPH were performed under a nitrogen atmosphere in an all-glass apparatus as described elsewhere[21]. The experimental procedure of FOUST *et al.*[21] was modified as follows. The side arm on the cuvette in the apparatus contained 0.2 ml of $1 \cdot 10^{-3}$ M indigo disulfonate, and when NADPH was the titrant, 20 μl of $1 \cdot 10^{-5}$ M ferredoxin–$NADP^+$ reductase. After making the apparatus anaerobic, the dye in the side arm was reduced by addition of sufficient titrant to remove the blue color. The apparatus was then gently shaken for approx. 10 min before making the first addition of titrant to the main compartment of the cuvette. This modified procedure gave greater confidence that the gas phase in the apparatus was free of oxygen contamination before a titration was begun, and also that anaerobic conditions were maintained during a titration; even low levels of

oxygen caused a rapid reoxidation of the yellow reduced dye to the blue oxidized form.

For potentiometric titrations, the spectrophotometer cuvette on this apparatus was modified to contain two platinum sheet electrodes sealed into glass tubes, a salt bridge of saturated KCl in 3% agar[22], and a magnetic stirring bar. The platinum electrodes were selected on the basis of their performance during potentiometric titrations of indigo disulfonate with sodium dithionite. The glass tube (5 mm internal diameter) containing the salt bridge was drawn out to give a tip (approx. 1.0 mm internal diameter) which dipped into the solution in the cuvette. This narrow tip prevented the KCl–agar mixture from being drawn into the cuvette by the vacuum applied when the apparatus was made anaerobic. The salt bridge was connected to a saturated calomel electrode (Radiometer Type K 4018) by a flexible tube which contained a saturated solution of KCl. Measurements of potential were made with a pH meter (Radiometer Type TTT 1c). The spectrophotometer cuvette and calomel electrode were thermostated at 25°.

Measurements of oxidation–reduction potential

The methods used to measure the oxidation–reduction potentials of flavodoxin have been described in detail elsewhere in work on the oxidation–reduction potentials of *P. elsdenii* flavodoxin[10]. The oxidation–reduction potential (E_1)* for the couple, flavodoxin semiquinone–fully reduced flavodoxin was determined from equilibrium measurements with either hydrogen in the presence of hydrogenase, or NADPH and a catalytic amount of ferredoxin–$NADP^+$ reductase. For calculations based on the latter type of experiment, the oxidation–reduction potential of $NADP^+$ at pH 9.1 was assumed to be −0.372 V. This value was calculated from the data of RODKEY AND DONOVAN[23] with the assumption that $\Delta E_{m,9.1}/\Delta t$ is −0.0013, a value determined for NAD^+ at pH 7 (ref. 24). The oxidation–reduction potential (E_2) for the couple oxidized flavodoxin–flavodoxin semiquinone was determined from equilibrium reactions with mixtures of flavodoxin and indigo disulfonate. Reducing equivalents were introduced into this system in two ways. First, experiments were performed in the anaerobic titration apparatus described above, adding increments of NADPH in the presence of a catalytic amount of ferredoxin–$NADP^+$ reductase. At equilibrium after each addition of NADPH, the percentage reduction of flavodoxin and dye was determined. Second, experiments were performed on similar mixtures of flavodoxin and indigo disulfonate in 1 ml anaerobic cuvettes in the presence of 0.03 M EDTA[25]. This mixture was exposed to visible light at 25° for short intervals of time (approx. 10 min). After each period of illumination, the system was allowed to equilibrate in darkness at 25°, and at equilibrium, the percentage reduction of flavodoxin and dye was determined. Similar results were obtained by both methods.

In the case of *P. elsdenii* flavodoxin, values for E_1 and E_2 were also determined from potentiometric titrations in the apparatus described above. The cuvette contained in a total volume of 5 ml: $7.9 \cdot 10^{-5}$ M flavodoxin; $3 \cdot 10^{-6}$ M methyl viologen; $3 \cdot 10^{-6}$ M indigo disulfonate and 0.08 M sodium pyrophosphate buffer (pH 8.2).

* The notation used is that recommended by CLARK[33]. E_2, the midpoint potential for the couple oxidized flavodoxin–flavodoxin semiquinone; E_1 the midpoint potential for the couple flavodoxin semiquinone–fully reduced flavodoxin; E_{m7}, the midpoint potential at pH 7; E_h, the potential with reference to the standard hydrogen electrode.

Indigo disulfonate and methyl viologen were added as mediators for the interaction of flavodoxin with the platinum electrodes; in the absence of these dyes, the measured potentials were erratic. Both oxidative and reductive titrations were performed. Oxidized flavodoxin was titrated with $3 \cdot 10^{-3}$ M $Na_2S_2O_4$, and the reduced protein was back-titrated with approx. $3 \cdot 10^{-3}$ M dichlorophenolindophenol. Values for E_1 and E_2 were determined from the measured potentials at 75% and 25% reduction, respectively, and a value of 0.244 V for the saturated calomel electrode.

Materials

NADPH was from P-L Biochemicals; $Na_2S_2O_4$ was from Eastman Kodak; indigo disulfonate was from K and K Laboratories; phenylmercuric acetate was from Hopkins and Williams, Essex, England. FMN was prepared by enzymic hydrolysis of FAD (from Sigma) followed by column chromatography on DEAE-cellulose[26,27]. An extinction coefficient of 12 500 $M^{-1} \cdot cm^{-1}$ at 445 nm was used for FMN at pH 7 (ref. 28). Nitrogen and hydrogen were purified by treatment in a column of copper turnings at 420°.

RESULTS AND DISCUSSION

Effects of iron on Clostridium MP

CARNAHAN AND CASTLE[17] showed that the rate and extent of growth of *C. pasteurianum* are limited by the iron concentration in the growth medium. More recently, KNIGHT AND HARDY[2] found that when this organism is grown in iron-poor medium flavodoxin is synthesized instead of the iron–sulfur protein ferredoxin. Similar effects of iron were found with the rumen organism *P. elsdenii*[9]; cells grown

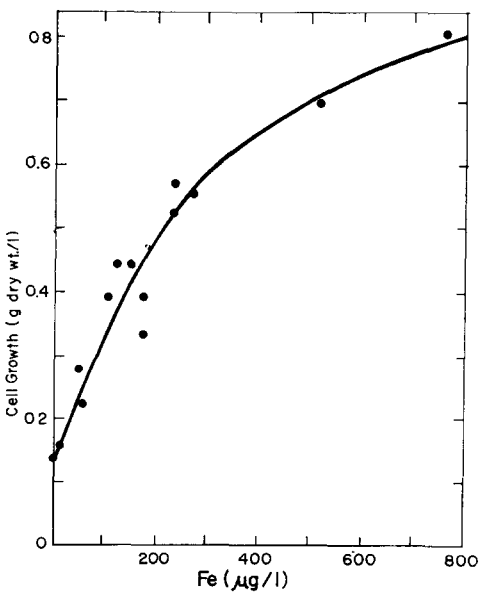


Fig. 1. Effect of iron on growth of Clostridium MP.

in iron-poor medium contained flavodoxin, but not ferredoxin. Iron also affects growth and flavodoxin production by Clostridium MP.

Fig. 1 shows the effect of iron on the total growth of Clostridium MP. Cell growth which occurred in the absence of added iron was probably due to iron contamination in the constituents of the medium. It was found that when the iron added to the medium was greater than 150 μg/l, little if any flavodoxin was obtained; at iron concentrations of 110 μg/l or less, ferredoxin could not be detected. On the basis of these experiments, an iron concentration of 120 μg/l was chosen as the optimum for flavodoxin production with useful cell growth. However, as already mentioned in METHODS AND MATERIALS, the iron concentration in the medium has recently been lowered to 55 μg/l, because more extensive growth with less flavodoxin production occurred at the higher iron concentration. Recent cultures grown at the lower iron concentration provide cells which contain flavodoxin in amounts similar to those previously grown at an iron concentration of 120 μg/l. Flavodoxin from these cultures is identical with flavodoxin obtained from cells grown earlier. The reason for this apparent change in the iron requirement of Clostridium MP is not known; it is possible that there has been an increase of iron contamination in the water supply or the media constituents.

Catalytic activity, molecular weight and chemical composition

The general properties of flavodoxins from Clostridium MP and *C. pasteurianum* are summarized in Table I which also includes data on *P. elsdenii* flavodoxin. The two clostridial flavodoxins replace *P. elsdenii* flavodoxin as electron carriers for the phosphoroclastic oxidation of pyruvate catalysed by extracts of *P. elsdenii*[9]. In this assay the three flavodoxins have the same activity. All three proteins have similar molecular weights and each contains one molecule of FMN as prosthetic group. However, the clostridial flavodoxins differ in their amino acid compositions. Preliminary amino acid analyses of flavodoxin from Clostridium MP (kindly performed by Dr. C. H. Williams, Jr. and Mr. L. D. Arscott) have shown for example that this protein contains approx. 6, 7 and 15 residues of alanine, serine and isoleucine; *C.*

TABLE I

PROPERTIES OF FLAVODOXINS

	Source of flavodoxin		
	Clostridium MP	*C. pasteurianum*	*P. elsdenii (refs. 9, 10)*
Catalytic activity*	1	1	1
Molecular weight	13 800**	14 600 (ref. 2)	14 600–15 600
Prosthetic group	FMN	FMN	FMN
Cysteine residues	3	1	2
Electrons accepted	2	2	2
E_1 (V)***	−0.399	−0.419	−0.372
E_2 (V)***	−0.092	−0.132	−0.115

* Assayed in the pyruvate oxidation system of *P. elsdenii*[9]. Values given are relative values based on a molar activity of 1 for *P. elsdenii* flavodoxin.

** Determined with a column of Sephadex G-100 calibrated as described in ref. 9.

*** Values given for pH 7 and 25°.

TABLE II

EXTINCTION COEFFICIENTS AND ISOSBESTIC POINTS OF FLAVODOXINS

Extinction coefficients for oxidized flavodoxins were determined by quantitative extraction of FMN with cold trichloroacetic acid[9], and by treating pure FMN with excess apoprotein[27]. Other values and the isosbestic points were determined from titrations of flavodoxin with sodium dithionite at pH 8.3.

	λ (nm)	Extinction coefficients ($M^{-1} \cdot cm^{-1}$)			Isosbestic points (nm)	
		Ox.	Semiquinone	Red.	Ox. to semiquinone	Semiquinone to red.
Clostridium MP flavodoxin	272	46 800				
	350		8400			
	376	9 100	4850		489; 357	431; 371; 330
	445	10 400	2400	1750		
	575	0	4620	0		
C. pasteurianum flavodoxin	272	45 800				
	350		7660		486; 355	430; 387; 380,
	374	8 470	4650			371; 328
	443	10 400	2080	1600		
	575	0	4550	0		

pasteurianum flavodoxin has 14, 14 and 5 residues, respectively, of each of these amino acids[3]. Furthermore, Clostridium MP flavodoxin contains 3 residues of cysteine, while *C. pasteurianum* flavodoxin has only one residue of this amino acid.

Absorption spectra and extinction coefficients

The visible absorption spectrum of flavodoxin from Clostridium MP has absorption maxima at 445 nm and 376 nm (Fig. 1, Table II). As reported by KNIGHT AND HARDY[2], these maxima are each shifted about 2 nm towards shorter wavelengths in *C. pasteurianum* flavodoxin. The extinction coefficients determined at the absorption maxima were very similar for both proteins (Table II), and are also similar to the extinction coefficients of *P. elsdenii* flavodoxin[9]. The values determined for *C. pasteurianum* flavodoxin are somewhat higher than the extinction coefficients previously reported for this protein by KNIGHT AND HARDY[2], although the relative intensities of the absorption maxima are similar to those determined by KNIGHT AND HARDY[2].

Reduction of flavodoxins

Both of these clostridial flavodoxins are reduced by $Na_2S_2O_4$, irradiation with visible light in the presence of EDTA[25], NADPH in the presence of a catalytic amount of ferredoxin–$NADP^+$ reductase, and hydrogen in the presence of hydrogenase. Under suitable conditions, reduction by these methods gives a blue-colored intermediate which has an absorption spectrum characteristic of the neutral form of flavin semiquinone[25,29]. The absorption spectra of the semiquinone intermediates of Clostridium MP and *C. pasteurianum* flavodoxins differ in detail. In general, the absorption maxima of the semiquinone, and also the isosbestic points between the semiquinone and oxidized forms of the protein, are found at somewhat shorter wavelengths in *C. pasteurianum* flavodoxin (Table II).

Electron paramagnetic resonance (EPR) spectra of solutions of the semiquinone forms of flavodoxins from *C. pasteurianum* and Clostridium MP have not been measured. However, as previously reported[14,15], crystals of Clostridium MP flavodoxin, which form from blue solutions of the semiquinone, do show a free radical which can be detected by EPR. These crystals are red in color while crystals of the semiquinone forms of *C. pasteurianum* and *P. elsdenii* flavodoxins are blue. In contrast to the EPR spectra of solutions of flavoprotein semiquinone which show no fine structure[25], the EPR spectrum of a single crystal of Clostridium MP flavodoxin semiquinone is structured, and the fine structure depends on the orientation of the crystal. It was not possible to determine the line widths of such spectra, and hence the crystal could not be related to either the 'red' or 'blue' class of flavoprotein semiquinone[25]. However, when a slurry of these crystals was made to cancel out any effects due to crystal orientation, the line width of the EPR spectrum was more readily determined. The line width found (21 gauss) showed that although the crystals are red in color, the EPR spectrum is more closely related to the EPR spectrum of the 'blue' class of flavoprotein semiquinone. These experiments were done by Dr. G. Palmer.

Anaerobic titration of flavodoxin from Clostridium MP with $Na_2S_2O_4$ showed that 1 mole of protein is reduced by 1 mole of $Na_2S_2O_4$; approx. 0.5 mole of $Na_2S_2O_4$ generates maximum semiquinone (Fig. 2). Similar results were obtained with *P. elsdenii* flavodoxin[10]. This simple stoichiometry was not observed with flavodoxin from *C. pasteurianum*. In anaerobic $Na_2S_2O_4$ titrations with this flavodoxin, semiquinone was formed linearly with the $Na_2S_2O_4$ added up to 0.5 mole of $Na_2S_2O_4$ per mole of protein. Changes observed upon further additions of $Na_2S_2O_4$ depended upon the pH of the titration. At pH 8.3, a linear reduction of the semiquinone to fully reduced flavodoxin occurred initially, but the titration curve at 575 nm then deviated from a straight line (Fig. 3). When an addition of dithionite was made to this flavodoxin in the non-linear region of a titration, the absorption at 575 nm first decreased,

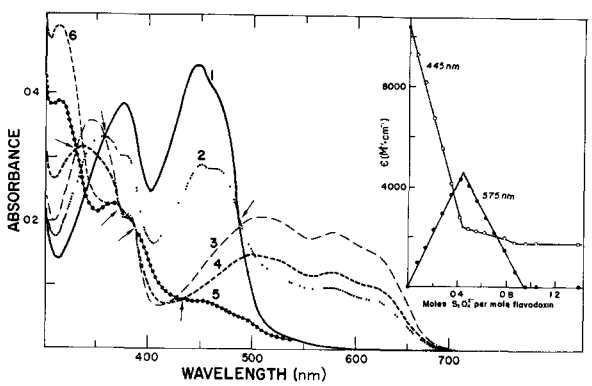
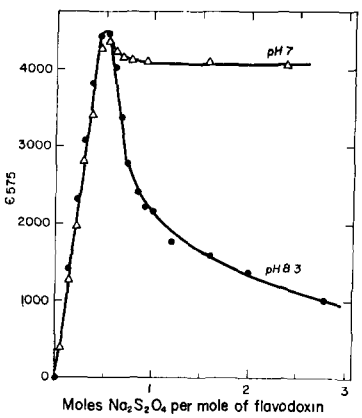


Fig. 2. Anaerobic titration of flavodoxin from Clostridium MP with $Na_2S_2O_4$. Flavodoxin, $4.28 \cdot 10^{-5}$ M, in 2.34 ml of 0.07 M sodium pyrophosphate buffer (pH 8.3) was titrated at 25° with $1.73 \cdot 10^{-3}$ M $Na_2S_2O_4$ dissolved in 0.01 M sodium pyrophosphate buffer (pH 8.3). Absorption spectra are not corrected for dilution. Arrows indicate isosbestic points. Curve 1, untreated flavodoxin; Curves 2–6, after addition of 0.20, 0.42, 0.63, 0.96 and 1.4 moles of $Na_2S_2O_4$ per mole of flavodoxin. The inset shows a plot of the observed extinction coefficients at 445 nm and 575 nm *versus* the $Na_2S_2O_4$ added. Experimental points in this plot are corrected for dilution.

showing that semiquinone had been rapidly reduced. The absorption then slowly increased to a value which was lower than the absorption before this addition of $Na_2S_2O_4$ had been made. At pH 7, similar effects were observed, but at this pH much larger amounts of semiquinone were formed during the slow reoxidation (Fig. 3). A titration done in 0.1 M glycine–NaOH buffer at pH 9.1 gave a titration curve very similar to that at pH 8.3. The explanation for these effects is not known. It appears that this flavodoxin either is reacting chemically with an excess of $Na_2S_2O_4$ or it is catalysing the decomposition of $Na_2S_2O_4$ to a compound with a more positive redox potential. One of the breakdown products of $Na_2S_2O_4$ is SO_3^{2-} (ref. 30). An attempt was made to shift the equilibrium in the experiment of Fig. 3 by performing a $Na_2S_2O_4$ titration of *C. pasteurainum* flavodoxin in the presence of 0.09 M Na_2SO_3 at pH 8.3. However, the titration curve obtained was the same as that in Fig. 3.

When solutions of fully reduced flavodoxin from either Clostridium MP or *C. pasteurianum* are aerated, the semiquinone forms very rapidly. This slowly



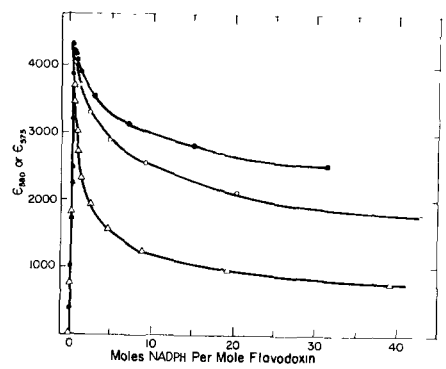


Fig. 3. Anaerobic titration of flavodoxin from *C. pasteurianum* with $Na_2S_2O_4$. The observed extinction coefficient at 575 nm is plotted *versus* the $Na_2S_2O_4$ added. Conditions for the titration at pH 8.3 were as described in Fig. 2. For the titration at pH 7, the flavodoxin was dissolved in 0.1 M potassium phosphate buffer (pH 7). Experimental points are final points obtained after the slow reoxidation described in the text.

Fig. 4. Anaerobic titration of flavodoxins with NADPH in the presence of ferredoxin–$NADP^+$ reductase at pH 9.1. Approx. 0.11 μmole of flavodoxin, $7 \cdot 10^{-7}$ M ferredoxin–$NADP^+$ reductase and 220 μmoles of glycine–NaOH buffer (pH 9.1) in 2.5 ml was titrated with $9 \cdot 10^{-3}$ M NADPH at 25°. The observed extinction coefficient at 575 nm for Clostridium MP (○—○) and *C. pasteurianum* (●—●) flavodoxins and at 580 nm for *P. elsdenii* flavodoxin (△—△) is plotted *versus* the NADPH added.

reoxidizes in a pseudo first order reaction, similar to that observed wtih *P. elsdenii* flavodoxin[10]. The pseudo first order rate constants for the decay of semiquinone in air saturated solution at pH 8.3 and 20° were 0.034 min^{-1} and 0.092 min^{-1} for flavodoxin from Clostridium MP and *C. pasteurianum*, respectively. Under similar conditions the rate constant for the decay of the semiquinone of *P. elsdenii* flavodoxin was approximately 0.1 min^{-1}. In the case of this flavodoxin, it was found that the rate of oxidation increases as the pH is increased above pH 7 (see ref. 10).

Oxidation–reduction potentials

When flavodoxins are titrated anaerobically with NADPH in the presence of a catalytic amount of ferrodoxin–NADP$^+$ reductase, a linear production of semiquinone occurs during the addition of 0.5 mole of NADPH per mole of flavodoxin (Fig. 4). Further additions of NADPH, to about 40 moles/mole of flavodoxin, cause a non-linear reduction of semiquinone to fully reduced flavodoxin. The extent of reduction in the second part of the titration is different for each flavodoxin. Values for E_1, the oxidation–reduction potential for the couple flavodoxin semiquinone–fully reduced flavodoxin, were calculated at each experimental point in the second parts of the titration curves of Fig. 4 (see ref. 10). The determined values for E_1 at pH 9.1 were -0.410 ± 0.002 V for Clostridium MP flavodoxin, -0.433 ± 0.002 V for *C. pasteurianum* flavodoxin and -0.375 ± 0.002 V for *P. elsdenii* flavodoxin.

The effects of pH on E_1, determined with hydrogen and hydrogenase, are shown in Fig. 5. The change in slope of the curves in Fig. 5 indicates an ionization in fully reduced flavodoxin. Values for the ionization constants describing these curves

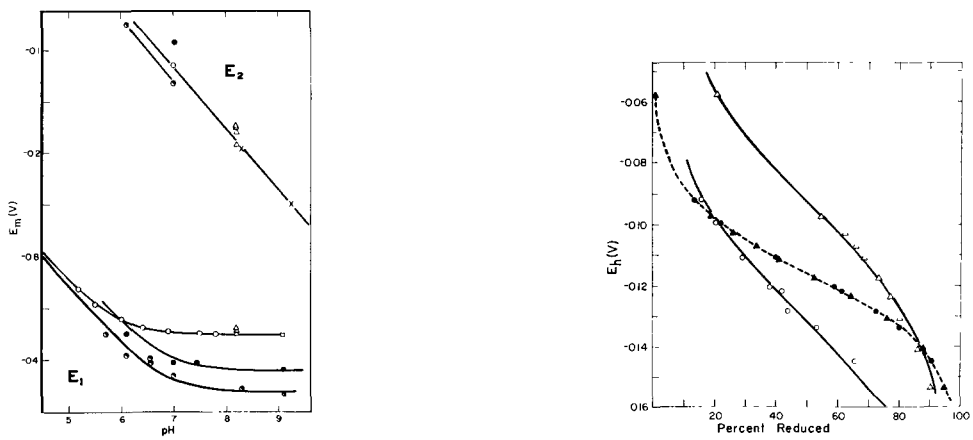


Fig. 5. Effect of pH on the oxidation reduction potentials of flavodoxins. Values for E_1 and E_2 were obtained at 25° as described in the text. ■, ●—●, Clostridium MP flavodoxin; ◪, ◑—◑, *C. pasteurianum* flavodoxin; □, ○—○, *P. elsdenii* flavodoxin, ■, ◪, □, E_1, determined with NADP$^+$ and ferredoxin–NADP$^+$ reductase (Fig. 4); △, values for *P. elsdenii* flavodoxin obtained by potentiometric titration (Fig. 7); ×, theoretical values for E_2 for *P. elsdenii* flavodoxin[10]. The solid lines for E_1 show theoretical curves relating E_1 to pH for 1-electron oxidation–reduction reactions in which the reduced species has a $pK' = 5.8$ (*P. elsdenii* flavodoxin) or a $pK' = 6.7$ (Clostridium MP and *C. pasteurianum* flavodoxins), and the unprotonated form has as apparent oxidation–reduction potential at pH 0 of -0.375 V (*P. elsdenii* flavodoxin), -0.409 V (Clostridium MP flavodoxin) and -0.430 V (*C. pasteurianum* flavodoxin).

Fig. 6. Oxidation–reduction potential (E_2) for the couple oxidized flavodoxin–flavodoxin semiquinone. Approx. $4 \cdot 10^{-5}$ M flavodoxin, $2 \cdot 10^{-5}$ M indigo disulfonate, $2 \cdot 10^{-6}$ M ferredoxin–NADP$^+$ reductase and 0.06 M sodium phosphate pH 7 in a final volume of 2.5 ml was titrated anaerobically with $1 \cdot 10^{-3}$ M NADPH at 25°. — — —, curve relating calculated values of E_h to the experimentally determined percentage reduction of indigo disulfonate after each addition of NADPH to mixtures containing either Clostridium MP flavodoxin (▲), or *C. pasteurianum* flavodoxin (●). △, plot relating E_h to the percentage reduction of Clostridium MP flavodoxin; ○, plot relating E_h to the percentage reduction of *C. pasteurianum* flavodoxin (for the flavodoxins 100% reduction in this plot is maximum semiquinone). Solid lines drawn through the experimental points are theoretical curves for 1-electron oxidation–reduction reactions for which $E_m = -0.092$ V and -0.132 V.

were determined at each of the experimental points by assuming that the apparent oxidation–reduction potential at pH 0 of the unprotonated flavin is −0.430 V and −0.409 V for *C. pasteurianum* and Clostridium MP flavodoxins, respectively[10]. The average values for the ionization constants were $1.77 \cdot 10^{-7} \pm 0.7 \cdot 10^{-7}$ for Clostridium MP flavodoxin and $1.85 \cdot 10^{-7} \pm 0.7 \cdot 10^{-7}$ for *C. pasteurianum* flavodoxin. These values indicate an apparent pK of about 6.7 for both flavodoxins. Fig. 5 shows theoretical curves for 1-electron oxidation–reduction reactions in which the reduced forms ionize with a pK' of 6.7. The midpoint potentials at pH 7 from these theoretical curves are −0.419 V and −0.399 V for *C. pasteurianum* and Clostridium MP flavodoxins, respectively.

For comparison with these results, Fig. 5 also includes data obtained with *P. elsdenii* flavodoxin. Potentiometric titrations at pH 8.2 and a titration at pH 9.1 with NADPH and ferredoxin–$NADP^+$ reductase (Fig. 4) have extended and confirmed the measurements made previously on this protein with the hydrogen–hydrogenase system[10]. The pK' in fully reduced *P. elsdenii* flavodoxin is about one pH unit lower than the pK' in the clostridial flavodoxins. The pK' in the clostridial flavodoxins is the same as that reported for free FMN[31].

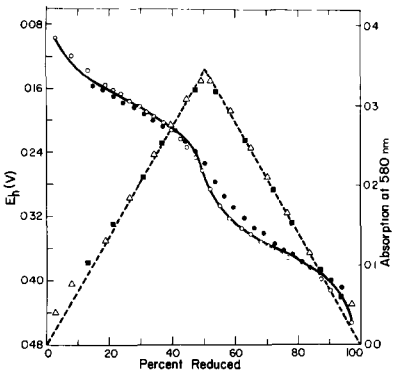
Values for E_2, the oxidation–reduction potential for the couple oxidized flavodoxin–flavodoxin semiquinone, were obtained from equilibrium and spectrophotometric experiments with the dye indigo disulfonate (Fig. 6). The determined values for E_2 at pH 7 and 25° were −0.092 V and −0.132 V for Clostridium MP and *C. pasteurianum*, respectively.

Values for E_2 above pH 7 previously reported for *P. elsdenii* flavodoxin were theoretical values based on the determined semiquinone formation constants for this protein and the assumption that E_1 is pH independent above pH 8 (ref. 10). Fig. 7 shows the results of a direct determination of E_1 and E_2 for *P. elsdenii* flavodoxin by potentiometric titrations at pH 8.2. The potential referred to the standard hydrogen electrode and the absorption at 580 nm are plotted *versus* the percentage reduction of flavodoxin to the fully reduced form. The plot of absorption at 580 nm deviates from the straight lines near 0% and 100% reduction. These deviations were due to absorption by the dyes added as mediators; oxidized indigo disulfonate and reduced methyl viologen absorb at 580 nm.

The data of Fig. 7 show clearly that reduction of *P. elsdenii* flavodoxin occurs in two 1-electron steps. The midpoint potentials for these steps were determined at 25% and 75% reduction from a number of reductive and oxidative titrations. The determined values for E_1 and E_2 from the experiment of Fig. 7 were −0.365 V and −0.175 V, respectively. These values are in reasonable agreement with theoretical values of −0.375 V and −0.187 V for E_1 and E_2, respectively at pH 8.2[10]. The potentiometric data indicate a semiquinone formation constant[10] of 1660. The theoretical line for an oxidation–reduction system with this value for the semiquinone formation constant is shown by the solid line in Fig. 7.

Effect of sulfhhydryl reagents

KNIGHT AND HARDY[3] reported that FMN is slowly dissociated from *C. pasteurianum* flavodoxin by an excess of the mercurial reagent sodium mersalyl. Phenylmercuric acetate causes a similar displacement of flavin from flavodoxin. Fig. 8 shows the effect of a 3–4-fold molar excess of phenylmercuric acetate on the flavin



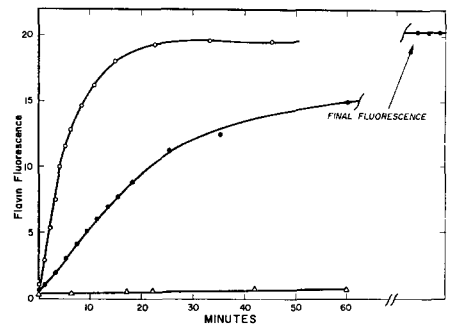


Fig. 7. Potentiometric titration of flavodoxin from *P. elsdenii* at pH 8.2. Experimental conditions were as described in MATERIALS AND METHODS. Values for E_h and the absorption at 580 nm are shown plotted *versus* the percentage reduction of flavodoxin to the fully reduced protein. ●, ■, reductive titration with $Na_2S_2O_4$; ○, △, back titration of the same solution of reduced flavodoxin with dichlorophenolindophenol. For clarity, some of the experimental points have been omitted from the plot of absorption. The solid line is a theoretical curve relating E_h to percent reduction for a 2-electron oxidation–reduction reaction in which semiquinone is formed with a semiquinone formation constant of 1660 and a midpoint potential of −0.270 V.

Fig. 8. Effect of phenylmercuric acetate on the flavin fluorescence of flavodoxins. Phenylmercuric acetate was mixed with flavodoxin in 2 ml 0.1 M potassium phosphate buffer (pH 6.8) containing $3 \cdot 10^{-4}$ M EDTA and 0.14 M KCl at 21°. The changes in fluorescence were followed with an Aminco Bowman fluorimeter. Fluorescence excitation was at 450 nm, and fluorescence emission was at 530 nm. ○, $3.53 \cdot 10^{-6}$ M Clostridium MP flavodoxin *plus* $1.13 \cdot 10^{-5}$ M phenylmercuric acetate. ●, $3.88 \cdot 10^{-6}$ M *P. elsdenii* flavodoxin *plus* $1.49 \cdot 10^{-5}$ M phenylmercuric acetate. △, $4.83 \cdot 10^{-6}$ M *C. pasteurianum* flavodoxin *plus* $1.96 \cdot 10^{-5}$ M phenylmercuric acetate.

fluorescence of flavodoxins from Clostridium MP, *C. pasteurianum* and *P. elsdenii*. The fluorescence of Clostridium MP and *P. elsdenii* flavodoxins increased to a value corresponding to the release of all of the FMN; in the case of *C. pasteurianum* flavodoxin, the changes were very slow and they were not followed to completion. The reactivity of these flavodoxins with phenylmercuric acetate increases with the number of cysteine residues in the protein. Thus the reactivity increased from *C. pasteurianum* to Clostridium MP flavodoxin (flavodoxins from *C. pasteurianum*, *P. elsdenii* and Clostridium MP contain 1, 2 and 3 residues of cysteine, respectively).

ELLMAN'S[32] reagent (5,5'-dithiobis-(2-nitrobenzoic acid)) causes similar increases in the flavin fluorescence of *P. elsdenii* flavodoxin. However, *N*-ethylmaleimide and iodoacetate have no effect on this flavodoxin, even when these reagents are present in a large molar excess. In addition, when either *P. elsdenii* or Clostridium MP flavodoxins were reacted with isotopically labelled iodoacetate at pH 7 for 3 h at 0° and then separated from small molecules on a column of Sephadex G-25, none of the radioactivity was associated with the protein band (M. L. LUDWIG AND M. LEQUESNE, personal communication). These results indicate that the sulfhydryl groups in the native forms of these flavodoxins are not available for reaction with iodoacetate. Experiments with the apoproteins from flavodoxins suggest that at least one sulfhydryl group is important for flavin binding. These experiments are described in the accompanying paper[27].

ACKNOWLEDGEMENTS

This work was supported by a grant from the U.S. Public Health Service (GM 11106) to Dr. V. Massey.

REFERENCES

1 E. KNIGHT, JR., A. J. D'EUSTACHIO AND R. W. F. HARDY, *Biochim. Biophys. Acta*, 113 (1966) 626.
2 E. KNIGHT, Jr. AND R. W. F. HARDY, *J. Biol. Chem.*, 241 (1966) 2652.
3 E. KNIGHT, Jr. AND R. W. F. HARDY, *J. Biol. Chem.*, 242 (1967) 1370.
4 J. LEGALL AND E. C. HATCHIKIAN, *Compt. Rend., Ser. D*, 264 (1967) 2580.
5 M. DUBOURDIEU, J. LEGALL AND F. LETERRIER, *Compt. Rend., Ser. D*, 267 (1968) 1653.
6 L. J. GUARRAIA, E. J. LAISHLEY, N. FORGET AND H. D. PECK, *Bacteriol. Proc.*, 129 (1968).
7 M. DUBOURDIEU AND J. LEGALL, *Biochem. Biophys. Res. Commun.*, 38 (1970) 965.
8 S. G. MAYHEW, *Federation Proc.*, 27 (1968) 789.
9 S. G. MAYHEW AND V. MASSEY, *J. Biol. Chem.*, 244 (1969) 794.
10 S. G. MAYHEW, G. P. FOUST AND V. MASSEY, *J. Biol. Chem.*, 244 (1969) 803.
11 R. M. SMILLIE, *Plant Physiol.*, 38 (1965) 28.
12 R. M. SMILLIE, *Biochem. Biophys. Res. Commun.*, 20 (1965) 621.
13 H. BOTHE, in H. KAMIN, *3rd Intern. Symp. on Flavins and Flavoproteins, Durham, N.C., 1969*, University Park Press, in the press.
14 M. L. LUDWIG, R. D. ANDERSEN, S. G. MAYHEW AND V. MASSEY, *J. Biol. Chem.*, 244 (1969) 6047.
15 M. L. LUDWIG, R. D. ANDERSEN, P. A. APGAR AND M. LEQUESNE, in H. KAMIN, *3rd Intern. Symp. of Flavins and Flavoproteins, Durham, N.C., 1969*, University Park Press, in the press.
16 R. H. DAINTY AND J. L. PEEL, *Biochem. J.*, 117 (1970) 573.
17 J. E. CARNAHAN AND J. E. CASTLE, *J. Bacteriol.*, 75 (1958) 121.
18 F. LIPMANN AND L. C. TUTTLE, *J. Biol Chem.*, 159 (1945) 21.
19 W. LOVENBERG, B. B. BUCHANAN AND J. C. RABINOWITZ, *J. Biol. Chem.*, 238 (1963) 3899.
20 M. SHIN, K. TAGAWA AND D. I. ARNON, *Biochem. Z.*, 338 (1963) 84.
21 G. P. FOUST, B. D. BURLEIGH, Jr., S. G. MAYHEW, C. H. WILLIAMS, JR., AND V. MASSEY, *Anal. Biochem.*, 27 (1969) 530.
22 H. BORSOOK AND H. SCHOTT, *J. Biol. Chem.*, 92 (1931) 535.
23 F. L. RODKEY AND J. A. DONOVAN, Jr., *J. Biol. Chem.*, 234 (1959) 677.
24 F. L. RODKEY, *J. Biol. Chem.*, 234 (1959) 188.
25 V. MASSEY AND G. PALMER, *Biochemistry*, 5 (1966) 3181.
26 V. MASSEY AND B. E. P. SWOBODA, *Biochem. Z.*, 338 (1963) 474.
27 S. G. MAYHEW, *Biochem. Biophys. Acta*, 235 (1971) 289.
28 L. G. WHITBY, *Biochem. J.*, 54 (1963) 437.
29 F. MÜLLER, P. HEMMERICH, A. EHRENBERG, G. PALMER AND V. MASSEY, *Eur. J. Biochem.*, 14 (1970) 185.
30 M. WAYMAN AND W. J. LEM, *Can. J. Chem.*, 48 (1970) 782.
31 H. J. LOWE AND W. M. CLARK, *J. Biol. Chem*, 221 (1956) 983.
32 G. L. ELLMAN, *Arch. Biochem. Biophys.*, 82 (1959) 70.
33 W. M. CLARK, *Oxidation–reduction potentials of organic systems*, The Williams and Wilkins Co., Baltimore, 1960.