Kinetics and thermodynamics of the binding of riboflavin, riboflavin 5′-phosphate and riboflavin 3′,5′-bisphosphate by apoflavodoxins

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The reactions of excess apoflavodoxin from *Desulfovibrio vulgaris*, *Anabaena variabilis* and *Azotobacter vinelandii* with riboflavin 5′-phosphate (FMN), riboflavin 3′,5′-bisphosphate and riboflavin are pseudo-first-order. The rates increase with decreasing pH in the range pH 5–8, and, in general, they increase with increasing ionic strength to approach a maximum at an ionic strength greater than 0.4 M. The rate of FMN binding in phosphate at high pH increases to a maximum at an ionic strength of about 0.1 M, and then decreases as the phosphate concentration is increased further. The dissociation constants for the complexes with FMN and riboflavin decrease with an increase of ionic strength. Inorganic phosphate stabilizes the complex with riboflavin. The effects of phosphate on riboflavin binding suggest that phosphate interacts with the apoprotein at the site normally occupied by the phosphate of FMN. Redox potentials determined for the oxidized/semiquinone and semiquinone/hydroquinone couples of the riboflavin and FMN complexes were used with K_d values for the complexes with the oxidized flavins to calculate values for K_d for the semiquinone and hydroquinone complexes. The hydroquinone complexes are all less stable than the complexes with the two other redox forms of the flavin. Destabilization of the hydroquinone is less marked in the complexes with riboflavin, supporting a proposal that the terminal phosphate group of FMN plays a role in decreasing the stability of the hydroquinone complex and in decreasing the redox potential of the semiquinone/hydroquinone couple.

INTRODUCTION

Flavodoxins are small flavoproteins that contain a molecule of non-covalently bound FMN and that function as low-potential electron carriers in a variety of microbial redox reactions (reviewed in [1–4]). The flavin is bound strongly to the apoprotein through numerous non-covalent interactions, but the two components can be separated by acid and other treatments. The apoprotein is usually stable in neutral solution, and it binds FMN to form a complex that is indistinguishable from native flavodoxin. Protein–flavin interactions in flavodoxins stabilize the semiquinone of FMN and destabilize the hydroquinone. These different effects result in a large negative shift of the redox potential of the semiquinone–hydroquinone couple, thus allowing flavodoxins to function as one-electron carriers at a potential close to that of the hydrogen electrode. Much recent work has attempted to elucidate the mechanisms by which apoflavodoxins modulate the redox properties of FMN.

Apoflavodoxins fall into two groups according to their specificity for the terminal phosphate on the side chain of FMN. Proteins in one group, including those from *Azotobacter vinelandii*, *Desulfovibrio vulgaris* and *Anabaena variabilis*, bind riboflavin, whereas proteins in a second group, including those from *Megasphaera elsdenii* and *Clostridium* spp., have a strict requirement for the 5′-phosphate group of FMN. The crystal and solution structures of several flavodoxins have shown that the ribityl phosphate side chain of FMN is buried in the protein, and that the negative charge of the phosphate is not compensated by positive charge on the protein. Instead, hydrogen bonds occur between the phosphate oxygens and amides of the protein backbone and the side chains of serine or threonine residues. Further hydrogen bonding occurs between the protein and the hydroxyl groups of the ribityl side chain. There is much similarity in the sequence of amino acids in the area of the FMN site, but the detailed structure around the flavin varies among the flavodoxins. For example, the isoalloxazine is sandwiched between the side chains of hydrophobic amino acids in all of the structures, and an aromatic amino acid flanks the face of the flavin nearer the solvent, but different amino acids are involved.

The association of flavin and protein almost completely quenches the flavin and protein fluorescence in flavodoxins. Fluorescence measurements therefore provide a sensitive and convenient way to monitor the association and dissociation reactions. The flavodoxins are useful as models for studies on the association of flavin and protein for several reasons: they are small and stable; three-dimensional structures are available in all three oxidation–reduction states; there is natural variation in structure at the flavin site; and cloned genes and mutant proteins have been prepared. The dynamics of the interaction of oxidized flavins with apoflavodoxins have been studied previously with flavodoxins from *M. elsdenii* [5–7] and *Az. vinelandii* [7–10]. We now describe an investigation of the kinetics and thermodynamics of binding of FMN, riboflavin and riboflavin 3′,5′-bisphosphate to apoflavodoxins from *D. vulgaris*, *Anabaena variabilis* and *Az. vinelandii*. In addition, we report redox potentials for the complexes of *A. variabilis* and *Az. vinelandii* apoflavodoxins with riboflavin. A preliminary account of some of these findings has appeared [11].

MATERIALS AND METHODS

Flavodoxin from *Desulfovibrio vulgaris* (Hildenborough) was expressed as the recombinant protein in *Escherichia coli* TG2 and purified as described [12]. *Anabaena variabilis* PCC 7119 flavodoxin was purified from the cyanobacteria grown autotrophically

Abbreviation used: BDQ^{2+}, 1,1′-butylene-2,2′-bipyridylium^{2+}.

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on nitrate at a low concentration of iron (2.6 μM) [13]. *Azotobacter vinelandii* ATCC 478 flavodoxin was purified from cells grown under nitrogen-fixing conditions [14]. The preparation was homogeneous by SDS/PAGE, and we assume that it corresponded to flavodoxin II of Klugkist et al. [15].

Apoflavodoxins were prepared by extraction of the holoprotein with 5% (w/v) trichloroacetic acid [16]. The protein precipitate was dissolved in 50 mM sodium phosphate buffer (pH 7)/1 mM EDTA, and dialysed against the same buffer. The concentration of apoflavodoxin in solution was determined from the end point of a fluorescence titration in which apoflavodoxin was added to a known concentration of FMN; the trichloroacetic acid extract of flavodoxin was used as a source of FMN [16]. Riboflavin was obtained from Sigma Chemical Co.; riboflavin 3′,5′-bisphosphate was purified from commercial FMN as described [17]. All buffers contained 1 mM EDTA.

Values for the dissociation constant (K_d) for complexes of flavins and apoflavodoxins were determined by fluorescence titration at 25 °C, using the quenching of flavin fluorescence to monitor flavin binding [12]. For the relatively weak complexes formed with riboflavin, values for the dissociation constant were also determined from spectrophotometric titrations. In these experiments the flavin–protein complex was determined from the difference spectrum formed in the visible region between free flavin and protein-bound flavin. Values for the absorption coefficients of the complexes were obtained at the end of the titrations by comparison of their spectra with the spectrum of the corresponding protein-free flavin. A value of 12500 $M^{-1}\cdot cm^{-1}$ was used for the absorption coefficient of FMN and riboflavin at 445 nm [18]; it was assumed that the absorption spectrum of riboflavin 3′,5′-bisphosphate is the same as that of FMN. The values for K_d obtained by spectrophotometry were in good agreement with those obtained by fluorimetry. Rate constants for flavin binding were obtained by reaction of flavin with apoflavodoxin under pseudo-first-order conditions (excess apoprotein), by using a stopped-flow spectrofluorimeter that consisted of a Rapid Kinetics Spectrometer Accessory (Applied Photophysics Ltd.; RX-1000) interfaced to the optical system of a Baird Nova fluorimeter, a home-made signal amplifier, an oscilloscope (Hameg Instruments 203-7) and a digital storage adaptor (Thurlby-Thandor DSA524). Values for ionic strength were calculated in the conventional way from the known ionic composition of the solution.

Oxidation–reduction potentials were measured by potentiometry at 25 °C [19], in a spectroelectrochemical cell similar to that described by Stankovich [20]. The dye 1,1′-butylene-2,2′-bipyridylium^{2+} (BDQ^{2+}; $E_{m,7} = -640$ mV [21]) was used to mediate electron transfer from the gold electrode to the protein during electrolysis in the cell. The quaternary salt was synthesized as described [22]. The concentrations of oxidized and reduced protein at each point in the titration were determined from measurements of the absorption spectra [19]. Stepwise oxidation of reduced flavodoxins with air showed that the reductive reactions are fully reversed, and confirmed the potentials determined by electrochemical reduction.

Absorption coefficients for the oxidized flavin–protein complexes were determined as described above, and values for the reduced proteins were calculated from redox data in which the protein was completely converted into the hydroquinone. The following absorption coefficients were used. *A. variabilis* flavodoxin: oxidized, $\epsilon_{464} = 9.4$ $mM^{-1}\cdot cm^{-1}$ [23], semiquinone, $\epsilon_{580} = 5.1$ $mM^{-1}\cdot cm^{-1}$ [19]. *A. variabilis* apoflavodoxin–riboflavin complex: oxidized, $\epsilon_{463} = 9.05$ $mM^{-1}\cdot cm^{-1}$, semiquinone, $\epsilon_{580} = 4.4$ $mM^{-1}\cdot cm^{-1}$. *Az. vinelandii* flavodoxin: oxidized, $\epsilon_{450} = 11.3$ $mM^{-1}\cdot cm^{-1}$ [15], semiquinone, $\epsilon_{580} = 5.7$ $mM^{-1}\cdot cm^{-1}$. *Az. vinelandii* apoflavodoxin–riboflavin complex: oxidized, $\epsilon_{451} = 11.3$ $mM^{-1}\cdot cm^{-1}$, semiquinone, $\epsilon_{580} = 5.7$ $mM^{-1}\cdot cm^{-1}$. The hydroquinone form of flavodoxin does not absorb at 580 nm. Plots of E_h, the potential relative to the standard hydrogen electrode, versus log ([oxidized protein]/[reduced protein]) were used to determine the midpoint potential for the complexes. A straight line was drawn through the points of this Nernst plot to obtain the value of the midpoint potential, E_m, where log ([oxidized protein]/[reduced protein]) is zero.

RESULTS AND DISCUSSION

Flavin binding by apoflavodoxins

Since the binding of FMN by apoflavodoxin almost completely quenches the flavin fluorescence, the kinetics of the binding can be measured by fluorescence spectroscopy combined with temperature-jump [7,24,25] or stopped-flow techniques [6]. Stopped-flow fluorimetry was used in the present study to investigate the binding of three different flavins by apoflavodoxins from the three organisms.

The kinetic traces obtained for the reaction of FMN with a large molar excess of *D. vulgaris* apoflavodoxin (Figure 1A) correspond to a single exponential, indicating that the process of fluorescence quenching occurs in a single step. The first-order

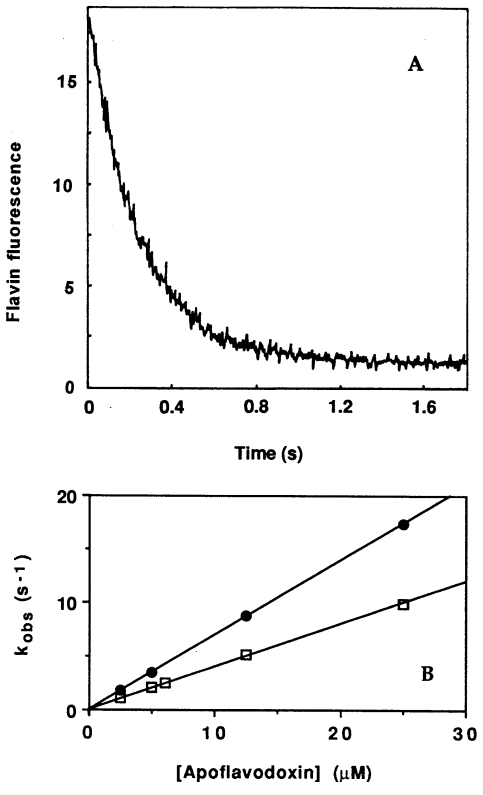

Figure 1 Stopped-flow determination of kinetics of the binding of FMN to *D. vulgaris* apoflavodoxin

(**A**) Stopped-flow kinetic trace monitored at 25 °C in 50 mM sodium phosphate, pH 7.0. Flavin fluorescence, in arbitrary units, is shown plotted against time. Excitation was at 450 nm and emission was recorded at 530 nm. FMN was mixed with apoflavodoxin to give final concentrations of 0.5 μM and 6 μM respectively. (**B**) Plot of the observed pseudo-first-order rate constant (k_{obs}; s^{-1}) of binding of FMN to apoflavodoxin versus apoflavodoxin concentration: ●, 20 mM Tris/HCl buffer, pH 7.5, containing 50 mM NaCl; □, 100 mM sodium phosphate buffer, pH 7.5.

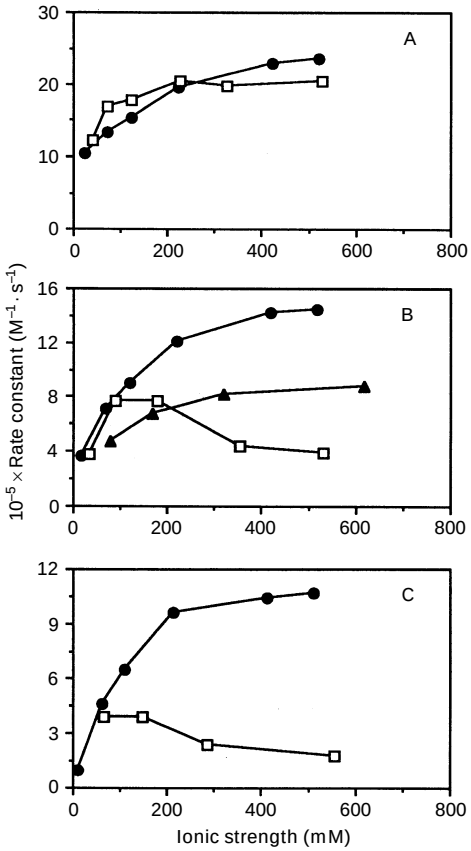

Figure 2 Effect of buffer composition on the second-order constant of binding of FMN to *D. vulgaris* apoflavodoxin

(**A**) 20 mM sodium acetate, pH 5.0; (**B**) sodium phosphate, pH 7.0; (**C**) 20 mM Tris/HCl, pH 8.0. FMN was mixed with apoflavodoxin to give final concentrations of 0.5 μM and 6 μM respectively at 25 °C in 20 mM monoanionic buffers (sodium acetate or Tris/HCl as indicated) containing NaCl (●) or Na_2SO_4 (▲), or in sodium phosphate buffer (□). Second-order rate constants were calculated from the observed pseudo-first-order constant.

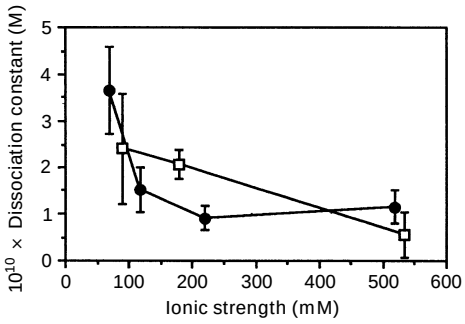

Figure 3 Effect of ionic strength on the dissociation constant of *D. vulgaris* apoflavodoxin–FMN complex, in (●) 20 mM Tris/HCl buffer, pH 7.0, containing NaCl, or (□) sodium phosphate buffer, pH 7.0

Experiments were carried out as described in the Materials and methods section.

nature of the binding is confirmed by a plot of the observed pseudo-first-order rate constant versus the concentration of apoflavodoxin; the plot is linear for experiments done in phosphate buffer and in Tris/HCl buffer, both at pH 7.5, and the plot passes through the origin (Figure 1B).

The effects of pH and ionic strength on the rate of binding of FMN to this apoflavodoxin were studied in the pH range 5–8 using monoanionic buffers [Tris/HCl, sodium cacodylate (results not shown) and sodium acetate, all at 20 mM] and varying the ionic strength with NaCl, and in sodium phosphate buffers (pH 6–7.5) of varying concentration. The values obtained for the second-order rate constant varied with the composition of the buffer and with pH (Figure 2). Greater values were obtained at lower pH, as observed previously for apoflavodoxins from *Az. vinelandii* [10] and *M. elsdenii* [6]. The rate of binding increased with ionic strength when NaCl was used, approaching a maximum at ionic strengths greater than about 400 mM. An increase with ionic strength in the rate constant for FMN binding by *M. elsdenii* apoflavodoxin was observed by Mayhew [5] and Gast et al. [6] at pH values greater than 3.8, whereas at lower pH values an increase of ionic strength decreased the rate of binding. The rate of binding by *D. vulgaris* apoflavodoxin in phosphate buffer increased as the phosphate concentration was increased at low concentrations, but it then decreased as the phosphate concentration was increased further. The decrease in high phosphate was more marked at higher pH (Figures 2B and 2C), suggesting that the effect is caused by dianionic phosphate ($pK_a = 7.2$). When phosphate was used to increase the ionic strength in acetate buffer at pH 5, a pH value at which phosphate is monoanionic, its effect was similar to that of NaCl (Figure 2A). It seems likely that the dianion of inorganic phosphate binds to the protein in the same region as the phosphate group on FMN, thus hindering the binding of the flavin. Sulphate anion at pH 7 gave intermediate values for the rate constants (Figure 2B), an effect that may be due either to the dianionic nature of this ion or to its structural similarity to HPO_4^{2-}. The effects of another similar dianion, SO_3^{2-}, could not be investigated, because of the specific interaction known to occur between sulphite and flavins [26]. An inhibition of FMN binding by phosphate, and reversal of the inhibition by chloride, has previously been noted for apoflavodoxin from *M. elsdenii* [5], a protein that shows no appreciable binding of riboflavin and whose FMN-binding site is different from that of *D. vulgaris* flavodoxin, but similar to that of *Clostridium beijerinckii* flavodoxin [27,28].

Dissociation constants measured at pH 7 in phosphate or Tris/HCl buffer at similar ionic strengths were similar [$(2.08 \pm 0.31) \times 10^{-10}$ M in 100 mM phosphate; $(1.53 \pm 0.48) \times 10^{-10}$ M in 20 mM Tris/HCl with 100 mM NaCl], indicating that, although the complex forms more slowly in the presence of phosphate, it also dissociates more slowly. As shown in Figure 3, an increase in ionic strength causes a decrease of the dissociation constant of the FMN–apoflavodoxin complex, indicating that tighter complexes are obtained at higher ionic strengths.

Some apoflavodoxins bind only FMN and flavin derivatives that carry the terminal phosphate group on the ribityl side chain, whereas others are less specific and also bind riboflavin. The binding of riboflavin by *Az. vinelandii* apoflavodoxin is known to occur much more rapidly than the binding of FMN [8], but the kinetics of the reaction with riboflavin have not previously been studied in detail with any flavodoxin. Figure 4(A) shows a kinetic trace obtained for the binding of riboflavin to *D. vulgaris* apoflavodoxin. The process is more rapid than the corresponding reaction with FMN. The pseudo-first-order rate constant for riboflavin binding shows a linear dependence on the concentration of apoprotein, and the line passes through zero (Figure 4B), indicating that, as with FMN binding, the reaction occurs in a single step. The rate depends on the buffer composition (Figure

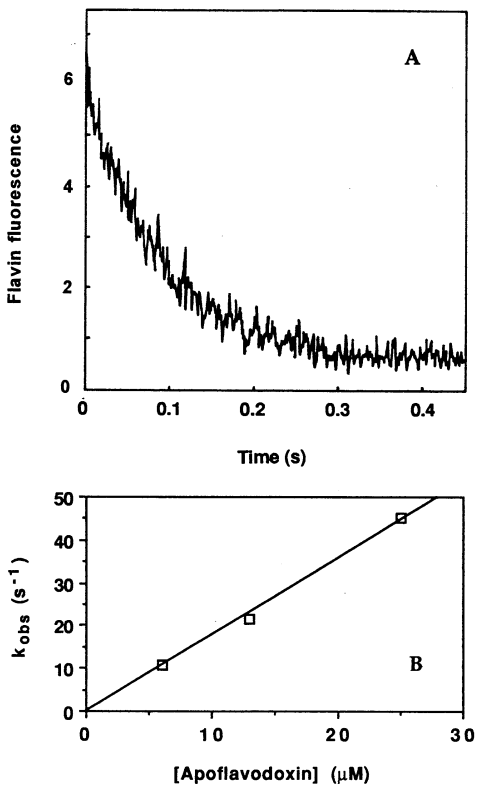

Figure 4 Stopped-flow determination of the kinetics of binding of riboflavin to *D. vulgaris* apoflavodoxin

(**A**) Stopped-flow kinetic trace monitored at 25 °C in 50 mM sodium phosphate, pH 7.0. Flavin fluorescence, in arbitrary units, is shown plotted against time. Excitation was at 450 nm and emission was recorded at 530 nm. Riboflavin was mixed with apoflavodoxin to give final concentrations of 0.5 μM and 6 μM respectively. (**B**) Plot of the observed pseudo-first-order rate constant of binding versus apoflavodoxin concentration in 50 mM sodium phosphate buffer, pH 7.0.

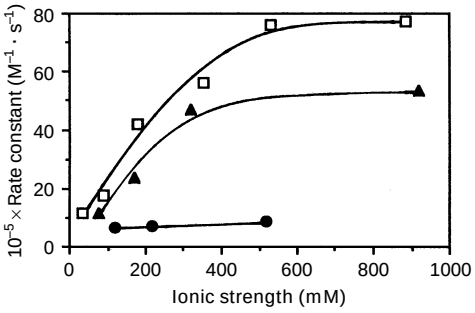

Figure 5 Effect of buffer composition on the second-order constant of binding of riboflavin to *D. vulgaris* apoflavodoxin

Riboflavin was mixed with apoflavodoxin to give final concentrations of 0.5 μM and 6 μM respectively at 25 °C in 20 mM Tris/HCl buffer, pH 7.0, containing NaCl (●) or Na_2SO_4 (▲), or in sodium phosphate buffer, pH 7 (□). Second-order rate constants were calculated from the observed pseudo-first-order constant.

5). In Tris/HCl buffer there was a slight increase of the calculated second-order rate constant with ionic strength when NaCl was added, but the presence of phosphate increased the rate by a factor of 10. Sulphate at high concentrations had a similar effect.

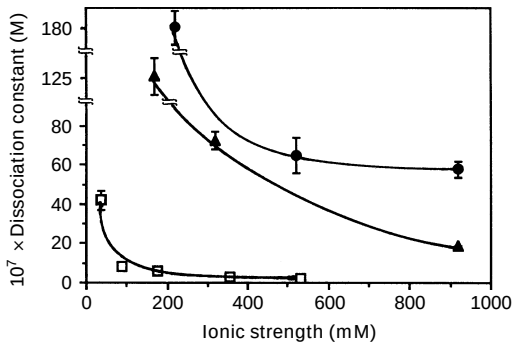

Figure 6 Effect of buffer composition on the dissociation constant of *D. vulgaris* apoflavodoxin–riboflavin complex: (●) 20 mM Tris/HCl buffer, pH 7.0, containing NaCl; (▲) 20 mM Tris/HCl buffer, pH 7.0, containing Na_2SO_4; (□) sodium phosphate buffer, pH 7.0

Experiments were carried out as described in the Materials and methods section.

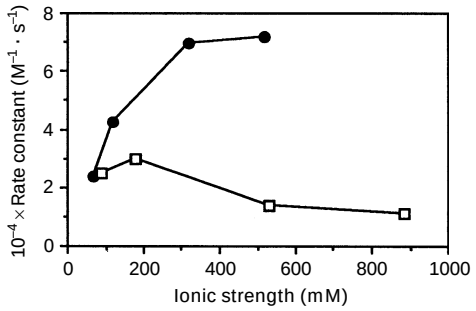

Figure 7 Effect of buffer composition on the second-order constant of binding of riboflavin 3′,5′-bisphosphate to *D. vulgaris* apoflavodoxin

Riboflavin 3′,5′-bisphosphate was mixed with apoflavodoxin to give final concentrations of 0.5 μM and 6 μM respectively at 25 °C in 20 mM Tris/HCl buffer, pH 7.0, containing NaCl (●), or in sodium phosphate buffer (□). Second-order rate constants were calculated from the observed pseudo-first-order constant.

These results suggest that phosphate, or a similar dianion, is required for rapid and tight binding of riboflavin. It is possible that phosphate ion binds at the same site as the phosphate group of FMN and induces a protein conformational change that allows riboflavin to bind more rapidly. A similar buffer effect was observed on the dissociation constant (Figure 6), with a much tighter complex being formed in the presence of phosphate. The riboflavin–apoflavodoxin complex is about 10 times weaker in Tris/HCl buffer and NaCl, even at high ionic strengths. Intermediate values were obtained when sulphate replaced chloride.

The binding of riboflavin 3′,5′-bisphosphate to *D. vulgaris* apoflavodoxin was also investigated. The kinetics of the process were much slower than the reaction with FMN. The progress curve is monoexponential (results not shown), indicative of a single-step process. A similar dependence to that observed for FMN binding was found with regard to the ionic strength and phosphate concentration of the buffer (Figure 7). The dissociation constant for the complex (3.08 nM, at pH 7) indicates that, although the flavin is bound very tightly, its complex is nearly 13 times weaker than the complex with riboflavin 5′-phosphate (FMN). The values determined for the second-order rate constant for binding are in reasonable agreement with a value reported

Table 1 Rate constants for binding and dissociation constants for complexes of FMN, riboflavin and riboflavin 3′,5′-bisphosphate with apoflavodoxins from *D. vulgaris*, *A. variabilis* and *Az. vinelandii*

Measurements were made at 25 °C in 50 mM sodium phosphate buffer, pH 7.0. Values given for k_{on} are the averages of at least four measurements; the experimental error of the values given is estimated to be less than 5%.

		Flavin		
Constant	Flavodoxin	FMN	Riboflavin	Riboflavin 3′,5′-bisphosphate
k_{on} ($M^{-1} \cdot s^{-1}$)	*D. vulgaris*	7.1×10^5	1.8×10^6	2.5×10^4
	A. variabilis	2.6×10^5	2.5×10^6	8.1×10^3
	Az. vinelandii	1.1×10^5	1.2×10^6	1.7×10^4
K_d (M)	*D. vulgaris*	$(2.4 \pm 1.2) \times 10^{-10}$	$(7.5 \pm 1.9) \times 10^{-7}$	$(3.1 \pm 0.3) \times 10^{-9}$
	A. variabilis	$(1.9 \pm 0.6) \times 10^{-10}$	$(1.1 \pm 0.1) \times 10^{-6}$	$(1.5 \pm 0.3) \times 10^{-9}$
	Az. vinelandii	$(4.4 \pm 0.9) \times 10^{-10}$	$(7.7 \pm 0.7) \times 10^{-7}$	$(3.7 \pm 0.4) \times 10^{-9}$

previously (8.6×10^4 $M^{-1} \cdot s^{-1}$ [29]) under different conditions (10 mM acetate, pH 6, and 200 mM NaCl at 20 °C); the K_d value reported in the earlier work (1 nM) was measured at 20 °C in 50 mM potassium phosphate, pH 7, and again it is in reasonable agreement with the value in Table 1, determined in the same buffer but at 25 °C.

In a similar way, values for the rate constants for the binding of FMN, riboflavin and riboflavin 3′,5′-bisphosphate to apoflavodoxins from *A. variabilis* and *Az. vinelandii* were determined. Both of these proteins have been shown previously to bind riboflavin [30,31]. The values, listed in Table 1, were obtained at 25 °C in 50 mM sodium phosphate, pH 7.0. The constants were similar to those with *D. vulgaris* apoflavodoxin under similar conditions. The rate constants for the binding of riboflavin to all three apoflavodoxins were higher than those for FMN binding, whereas the binding of riboflavin 3′,5′-bisphosphate was at least 10 times slower, suggesting that the side chain of FMN sterically hinders access to the binding site. This effect is increased by the presence of the second phosphate group in riboflavin 3′,5′-bisphosphate. The dissociation constants for the complexes with riboflavin were three to four orders of magnitude greater than those with FMN, suggesting that the phosphate of FMN stabilizes the complex. The dissociation constants for the complexes with riboflavin 3′,5′-bisphosphate are also larger, but only by about one order of magnitude, indicating that, although one phosphate group stabilizes the complex, the second one destabilizes it.

Oxidation–reduction potentials

The redox potentials of the FMN complex with *Az. vinelandii* apoflavodoxin and the riboflavin complexes with *Az. vinelandii* and *D. vulgaris* apoflavodoxins were determined by potentiometry (Table 2). Equilibration of the proteins with the mediators occurred readily throughout the titrations, and plots of E_h versus log ([oxidized]/[semiquinone]) or log ([semiquinone]/[reduced]) were linear, with slopes of 0.059 ± 0.003 V (Figure 8), indicating that with all of the complexes two successive one-electron reduction reactions occurred. As summarized by Mayhew and Tollin [3], published values for the redox potentials of the two one-electron steps of reduction of flavodoxin preparations from *Az. vinelandii* have varied over a wide range, although it should be noted that the measurements were not all made at the same pH. Three flavodoxins have been isolated from the species of *Az. vinelandii* (ATCC 478) used in the present study [15]. The synthesis of only one of these responded to growth under

Table 2 Midpoint redox potentials (E_m) of complexes of *D. vulgaris*, *A. variabilis*, *Az. vinelandii* and *M. elsdenii* apoflavodoxins with FMN, riboflavin and riboflavin 3′,5′-bisphosphate

Measurements were made at 25 °C in 50 mM sodium phosphate buffer, pH 7.0, unless otherwise indicated. Abbreviations used: ox., oxidized; sq, semiquinone; red., reduced; n.d., not determined.

		Flavin		
E_m(mV)	Flavodoxin	FMN	Riboflavin	Riboflavin 3′,5′-bisphosphate
ox./sq (E_2)	*D. vulgaris*	−110[a]	−193[a]	n.d.
	A. variabilis	−212[b]	-213 ± 5	n.d.
	Az. vinelandii	-168 ± 2	-181 ± 5	n.d.
	M. elsdenii	−115[c]	[d]	−118[c]
sq/red. (E_1)	*D. vulgaris*	−420[a]	−262[a]	n.d.
	A. variabilis	−436[b]	-258 ± 6	n.d.
	Az. vinelandii	-477 ± 6	-332 ± 6	n.d.
	M. elsdenii	−390[c]	[d]	−410[c]

[a] Ref. [12], determined at pH 6.5.
[b] Ref. [19].
[c] Ref. [29], determined at 20 °C.
[d] No detectable binding [5].

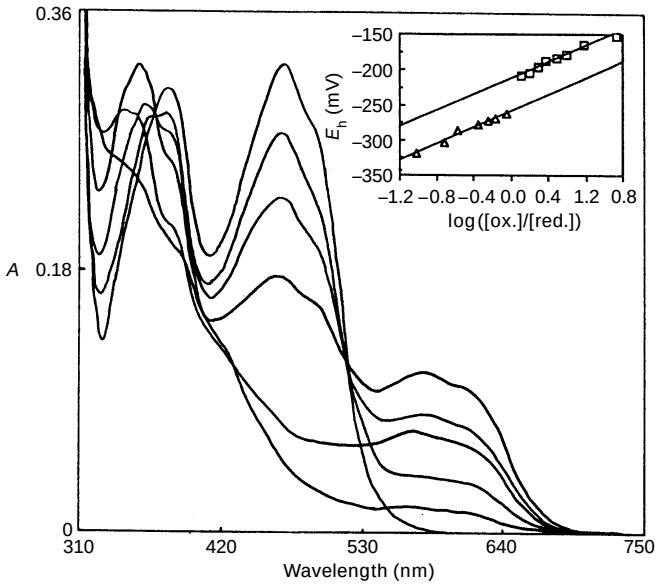

Figure 8 Potentiometric titration of *A. variabilis* apoflavodoxin–riboflavin complex at pH 7.0

Absorption spectra and redox potentials were recorded during electrochemical reduction as described in the Materials and methods section. Inset: Nernst plot of the data: □, oxidized (ox.)/semiquinone couple; △, semiquinone/reduced (red.) couple. The reaction mixture contained, at 25 °C: 36 μM apoflavodoxin–riboflavin complex, 50 mM sodium phosphate buffer, pH 7.0, 15% (v/v) glycerol, 100 μM BDQ^{2+}, 1 μM anthraquinone-2-sulphonate and 1 μM anthraquinone-2,6-disulphonate.

Table 3 Dissociation constants calculated for complexes of the semiquinone (sq) and reduced (red.) forms of FMN and riboflavin with *D. vulgaris, A. variabilis* and *Az. vinelandii* apoflavodoxins

The values given were calculated from measured values for K_d for the complexes with oxidized flavin (Table 1) and for the redox potentials (Table 2), and using published values for the redox potentials of protein-free FMN [32], as described in the text.

		Flavin	
Constant (M)	Flavodoxin	FMN	Riboflavin
K_d (sq)	*D. vulgaris*	2.6×10^{-13}[a]	6.7×10^{-9}
	A. variabilis	3.5×10^{-12}	2.1×10^{-8}
	Az. vinelandii	1.5×10^{-12}	4.3×10^{-9}
K_d (red.)	*D. vulgaris*	6.6×10^{-8}[a]	1.4×10^{-6}
	A. variabilis	6.8×10^{-7}	4.0×10^{-6}
	Az. vinelandii	1.4×10^{-6}	1.4×10^{-5}

[a] Values of $E_1 = -0.440$ V and $E_2 = -0.139$ V were used to calculate these constants [12].

nitrogen-fixing conditions (flavodoxin II), and the reported potential for E_1 for this protein was -500 ± 10 mV at pH 7, and therefore not too different from the value in Table 2 (-477 mV).

The yield of semiquinone at half-reduction for all of the riboflavin–apoflavodoxin complexes is much less than for the FMN complexes, and is a consequence of the smaller difference between the potentials for the two one-electron steps of reduction. For this reason, data were obtained near the start and the end of the titration, when more than 70% of the protein was either fully oxidized or fully reduced, and in this way errors that result from having all three redox species present at the same time were minimized.

Dissociation constants for the complexes of flavin semiquinone and hydroquinone were calculated by using the following equation [32], with values for the dissociation constants measured for the complexes with the fully oxidized flavin, the redox potentials measured for the complexes, and published values for the redox potentials of protein-free FMN and riboflavin (ox., oxidized; red., reduced):

$$E_m(\text{bound flavin}) = E_m(\text{free flavin}) + 0.059\left(\frac{K_d(\text{ox.})}{K_d(\text{red.})}\right)$$

The values listed in Table 3 were obtained with the redox data of Anderson [33] for free FMN and riboflavin. Use of the earlier data of Draper and Ingraham [34] leads to larger K_d values for the semiquinone and hydroquinone forms of all of the complexes, although, as pointed out by Ludwig and Luschinsky [4], the ratio of the dissociation constant for the semiquinone complex to that for the hydroquinone complex is independent of the values used for the redox potential of free FMN. In all cases there is an increase in binding affinity when one electron is added to the flavin to form the semiquinone, and a decrease in binding affinity when a second electron is added to form the hydroquinone. Similar changes in K_d with the redox state of FMN have been observed for several flavodoxins [1,3]. FMN semiquinone binding becomes up to three orders of magnitude stronger than that of the oxidized form, whereas binding of the hydroquinone can be up to three orders of magnitude weaker. The K_d values for the complexes of riboflavin follow the same pattern, but the differences with redox state are less marked.

It has been suggested that the weak binding of flavin hydroquinone by apoflavodoxins, and the very negative potential for the semiquinone/hydroquinone couple of flavodoxins that occurs as a consequence, may result from charge–charge interactions between the hydroquinone anion, which carries a negative charge in the N(1)–C(2)0 region of the flavin, and negative charge elsewhere in the protein–flavin complex [35–37]. Moonen et al. [35] calculated theoretical values for the redox potential of flavodoxins that might result from interaction between the negative charge on N(1) of the isoalloxazine ring and the two negative charges on the phosphate group, the negative charges on the protein and the protein dipole. It was observed experimentally that the addition of a second phosphate group in the complex between riboflavin 3′,5′-bisphosphate and *M. elsdenii* apoflavodoxin caused the redox potential to become even more negative (Table 2) [29]. The absence of the side-chain phosphate in the riboflavin complexes eliminates two of the charges and is perhaps responsible for the less negative redox potential with this complex, as proposed previously for the riboflavin complex of *D. vulgaris* apoflavodoxin [12] and now observed also for the complexes with *A. variabilis* and *Az. vinelandii* apoflavodoxins. Inorganic phosphate was present in the redox titrations of the riboflavin complexes, but the theoretical calculations by Moonen et al. [35] were based on the assumption that the phosphate group is highly immobilized and that the charge contribution is inversely proportional to the distance between the negative charge on N(1) and the phosphate group. It is possible that the redox potential of the riboflavin complexes could be even less negative in a monoanionic buffer. This measurement was not made because of the low stability of the complex in the absence of phosphate (see Figure 6). Mutants of *D. vulgaris* apoflavodoxin in which one or more of the negatively charged amino acid side chains have been removed by site-directed mutagenesis also cause less destabilization of the flavin hydroquinone [38,39].

Replacement of surface aspartate and glutamate residues by asparagine and glutamine, respectively, causes on average a change of about 15 mV in the redox potential per substitution [39]. It is clear that replacement of FMN by riboflavin causes a much larger change in redox potential, supporting the proposal that the charge on the phosphate of FMN in the native complex contributes to destabilization of the hydroquinone complex and to the large negative shift in the redox potential of the semiquinone–hydroquinone couple.

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