Can Cofactor-Binding Sites in Proteins Be Flexible? *Desulfovibrio desulfuricans* Flavodoxin Binds FMN Dimer†

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ABSTRACT: Flavodoxins catalyze redox reactions using the isoalloxazine moiety of the flavin mononucleotide (FMN) cofactor stacked between two aromatic residues located in two peptide loops. At high FMN concentrations that favor stacked FMN dimers in solution, isothermal titration calorimetric studies show that these dimers bind strongly to apo-flavodoxin from *Desulfovibrio desulfuricans* (30 °C, 20 mM Hepes, pH 7, $K_D = 5.8\ \mu M$). Upon increasing the temperature so the FMN dimers dissociate (as shown by ^1H NMR), only one-to-one (FMN-to-protein) binding is observed. Calorimetric titrations result in one-to-one binding also in the presence of phosphate or sulfate (30 °C, 13 mM anion, pH 7, $K_D = 0.4\ \mu M$). FMN remains dimeric in the presence of phosphate and sulfate, suggesting that specific binding of a divalent anion to the phosphate-binding site triggers ordering of the peptide loops so only one isoalloxazine can fit. Although the physiological relevance of FMN and other nucleotides as dimers has not been explored, our study shows that high-affinity binding to proteins of such dimers can occur in vitro. This emphasizes that the cofactor-binding site in flavodoxin is more flexible than previously expected.

Specific binding is fundamental to the molecular organization of living organisms. Virtually all biological phenomena depend on molecular recognition, which is either intermolecular, as in ligand binding to a macromolecule and in the formation of macromolecular complexes, or intramolecular, as in protein folding. The flavin−polypeptide complexes that constitute the large family of flavoproteins represent remarkably versatile examples where function requires noncovalent interaction between a small ligand and a protein. Numerous enzymological and mechanistic studies have established a good understanding of the catalytic and functional steps involving the cofactor in many different flavoproteins (*1*). Structural studies have in addition yielded detailed information about the many interactions between the flavin and the protein as well as the general environment provided by the protein (*1*).

Flavodoxins are small, bacterial flavoproteins that participate in low-potential electron-transfer pathways (*1*). Their small size, well-established physical and structural properties, and availability of high-expressing recombinant constructs make the flavodoxins good model systems for studying the properties and interactions of flavin cofactors and flavoproteins. All known flavodoxins contain a single, noncovalently bound FMN[1] cofactor. Cofactor binding occurs primarily through a combination of hydrogen bonds and aromatic interactions with the apo-protein (*2*). The FMN interacts with three loops of the protein: two aromatic residues (Tyr60 and Trp98 in *Desulfovibrio desulfuricans* flavodoxin) located on opposite loops flank either side of the FMN isoalloxazine ring. The 5′-phosphate moiety of FMN is bound in an atypical phosphate-binding site, anchored by several hydrogen bonds in a loop near the N-terminus, but with no ion-pairing interactions (*2*). The ribityl side chain hydroxyls make some hydrogen bonds but only a minor contribution to the stability of the complex (*3*).

The mechanistic details of how FMN binds to the apo-flavodoxin are not clearly understood. Inspection of the X-ray crystal structure of apo-flavodoxin from *Anabaena* indicates that the phosphate-binding site is well-formed, while the isoalloxazine pocket is closed by one of the aromatic residues (Trp57) normally interacting with the FMN (*4*). On the basis of this observation, it was suggested that binding of FMN is initiated by association at the phosphate-binding site. Conflicting with this hypothesis, NMR solution studies of the structures of the *Anabaena* (*5*) and *Azotobacter* (*6*) apo-flavodoxins indicate great flexibility of the isoalloxazine-binding site and the absence of a preformed phosphate-binding site. Flexibility of the isoalloxazine-binding site is also evidenced by the different X-ray structures observed for the 60s loop in wild-type and mutant *Anabaena* flavodoxins (*3*). Notably, the X-ray structure of *Anabaena* apo-flavodoxin with the preformed phosphate-binding site (*4*) bears a bound sulfate anion in this site perhaps responsible for the observed local structuring of the region.

Earlier this year, two different kinetic models for FMN binding to apo-flavodoxin (from *Desulfovibrio vulgaris* and *Anabaena*, respectively) were reported (*7, 8*). In the first model, it was shown that binding of the FMN isoalloxazine ring to *D. vulgaris* apo-flavodoxin is dependent on the presence of phosphate in the phosphate-binding site (*7*). When phosphate is in the buffer solution, FMN can bind in

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[1] Abbreviations: FMN, flavin mononucleotide; ITC, isothermal titration calorimetry; NMR, nuclear magnetic resonance; K_D, dissociation constant; FAD, flavin adenine dinucleotide.

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either of two ways: by initial insertion of the phosphate group followed by ring binding, or when inorganic phosphate from solution is bound, by initial insertion of the isoalloxazine ring. These results support that a phosphate-triggered (by FMN's phosphate or a buffer phosphate) conformational change is required for the binding of the isoalloxazine ring. In the other kinetic study, ϕ-value analysis (for a set of cofactor-binding site mutants) was used to probe the transition-state complex for FMN binding to *Anabaena* apoflavodoxin (*8*). On the basis of these results, the authors described FMN binding as a hydrophobic encounter at an open binding site, starting with the isoalloxazine ring interactions; the phosphate-binding site formed last, independent of the presence of inorganic phosphate.

All studies to date of FMN interactions with apoflavodoxins have utilized the dramatic quenching of the FMN emission upon protein binding as the detection tool (*1*, *3*, *7*, *8*). This allows for studies at very low protein concentrations, which have proven necessary to determine the nanomolar dissociation constants reported for many FMN−flavodoxin complexes. It is well-known that in aqueous solution and poly(vinyl alcohol) films, FMN at high concentrations (mM) is not monomeric (*9*, *10*). At these conditions, FMN preferentially forms stacking-type dimers stabilized by hydrogen bonds but no aggregates of higher order (*11*). Here, we use isothermal titration calorimetry (ITC) to study FMN binding to *D. desulfuricans* apo-flavodoxin at conditions where FMN is dimeric in solution. Remarkably, we find that the FMN dimers bind to apo-flavodoxin with high affinity. However, when the phosphate-binding site is occupied by a phosphate or sulfate from the buffer, only one FMN is inserted per protein, and the binding is 15-fold stronger. Our results show that the FMN-binding site (without an anion in the phosphate-binding site) in flavodoxin is substantially more flexible than that observed in NMR and crystal structures, which is able to accommodate an FMN dimer.

MATERIALS AND METHODS

Protein Preparation. Flavodoxin from *D. desulfuricans* (ATCC strain 29577) was expressed in *Escherichia coli* (*12*, *13*) and purified as described earlier with some modifications. Apo- and holo-flavodoxin were eluted separately on a Q-Sepharose column and further purified individually by gel permeation on a Superdex-75 using FPLC (Amersham-Pharmacia). Purified apo-flavodoxin (>99% pure) and HPLC-purified FMN (Sigma Chemicals) were used in all binding studies (20 mM Hepes, pH 7.0 with sodium phosphate and sodium sulfate added as indicated).

Fluorescence Titration. Quenching of FMN fluorescence upon the addition of small aliquots of apo-flavodoxin was monitored between 500 and 550 nm (excitation at 445 nm) at 30 °C using a fluorometer (Cary Eclipse, Varian). The intensity changes at 525 nm were used for binding analysis according to Scatchard (*14*). FMN concentration was 1 μM, determined using a molar extinction coefficient of $\epsilon_{445} = 12\,500\ M^{-1}\ cm^{-1}$.

Calorimetry. Thermodynamic-binding measurements were made using an isothermal titration calorimeter (VP-ITC, MicroCal) at different temperatures (*15*). Protein and FMN samples were filtered through a 0.22 μm sterile filter membrane (Millipore) and degassed at 30 °C (ThermoVac, MicroCal) before loading into the ITC cell and syringe. Before FMN-protein binding experiments, reference titrations (injecting buffer to buffer and FMN to buffer) were carried out. A typical injection schedule included the addition of 10 μL samples of 1.3 mM FMN to 50 μM apo-flavodoxin with 25−28 injections spaced between 3 min intervals (4 μL injections of apo-flavodoxin samples were used in the case of reverse titrations, Figure 2B). For experiments in the presence of phosphate and sulfate, both FMN and apo-protein solutions contained the specified anion (13 mM sodium phosphate, 135 μM sodium phosphate, or 13 mM sodium sulfate). For each condition studied, at least two independent experiments were performed. The binding isotherms were best fit to a single class of binding sites by Marquardt nonlinear least-squares analysis (Origin 5.0). The resulting ligand-to-protein stoichiometry ranged from 2 to 0.5.

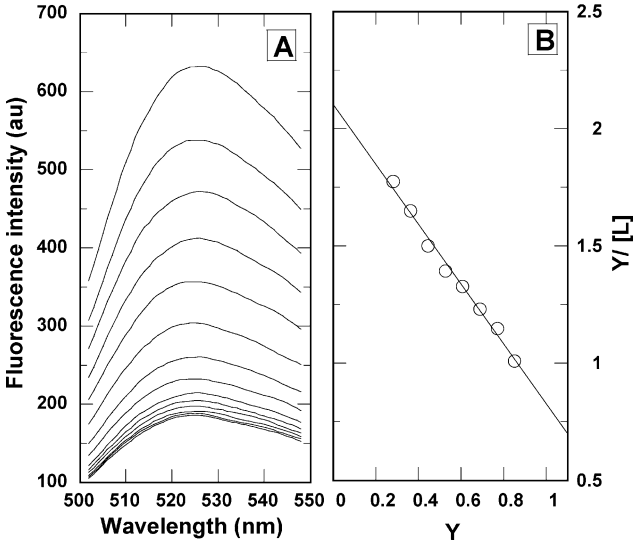

FIGURE 1: FMN binding probed by fluorescence quenching (20 mM Hepes, pH 7, 30 °C). (A) Progressive decrease in FMN fluorescence (1 μM FMN) upon additions of 5 μL aliquots of apo-flavodoxin (final concentration ranging from 0.1 to 1.5 μM). (B) Data analysis according to Scatchard, assuming 1:1 stoichiometry (*14*). *Y* is fractional saturation at different FMN/protein ratios.

NMR. ^{1}H NMR spectral measurements of FMN in D_2O-based solutions were performed on a 400 MHz spectrometer (Varian). The chemical shifts associated with two aromatic and two methyl protons, previously reported as sensitive to stacking (*10*), were measured for samples of FMN (from 1 to 50 mM) in the presence of D_2O (99.9%), 20 mM Hepes, 13 mM sodium phosphate, or 13 mM sodium sulfate (30 °C). Chemical shifts as a function of temperature (25−50 °C) were also measured for 1.3 and 20 mM FMN in D_2O. The chemical shifts were assigned using the center peak of the tetramethylammonium nitrate (1 mM) as the internal reference (*16*). The pD of all the solutions was adjusted to 7.0 using DNO_3 or NaOD (*17*).

RESULTS

FMN Binding to Apo-Flavodoxin at Low FMN Concentrations. When flavin cofactors bind to apo-flavodoxin, the intrinsic fluorescence of the isoalloxazine ring is almost completely quenched, providing a convenient way to probe the binding (*1*). This method has been used to determine the dissociation constant (K_D) for FMN and flavodoxin from

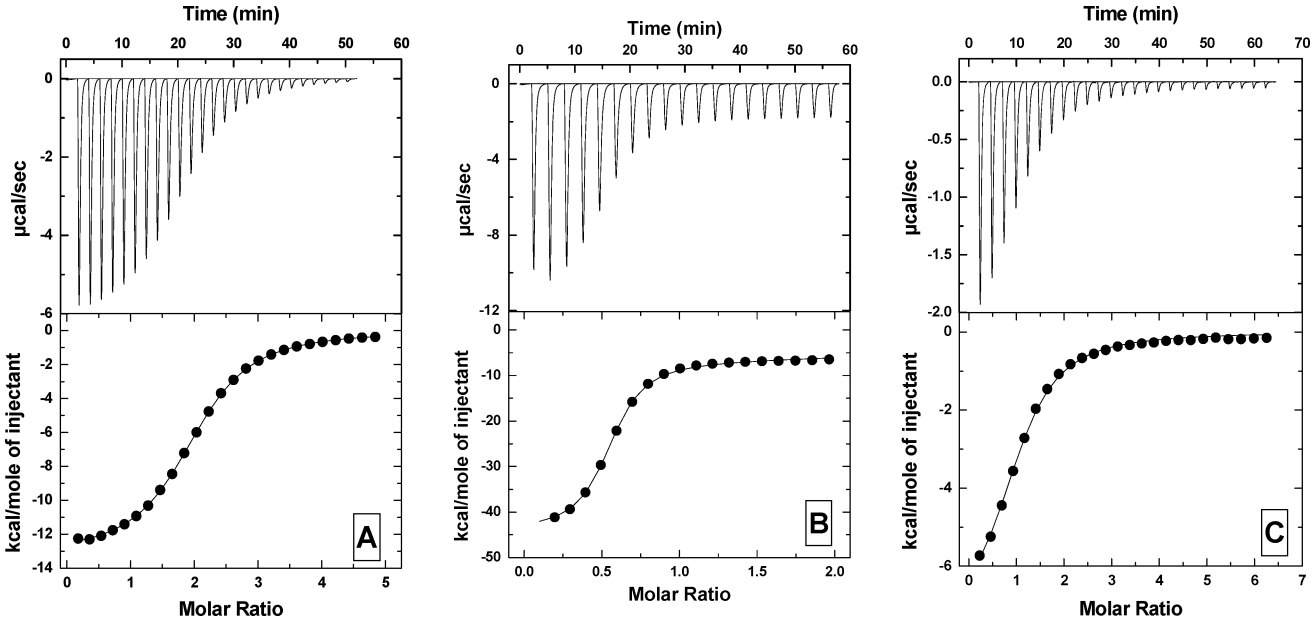

FIGURE 2: FMN binding probed by ITC (20 mM Hepes, pH 7, 30 °C). (A) 1.3 mM FMN (predominantly dimeric) in a syringe titrated against 50 μM apo-protein in the cell. (B) Reverse titration: 4 mM apo-flavodoxin in the syringe titrated against 500 μM FMN (mostly dimeric) in the cell. (C) 60 μM FMN (predominantly monomeric) in the syringe titrated against 2 μM apo-protein in the cell. The upper panels include the heat changes observed upon injections of 10 μL samples (4 μL samples in Figure 2B) into the cell spaced by 3 min intervals. The lower panels show the resulting binding isotherms: solid lines representing fits to single sets of binding sites.

Table 1: Thermodynamic Data, Obtained from ITC Experiments (see Figures 1, 2, 5, and 6), for the Interactions of *D. desulfuricans* Apo-Flavodoxin with FMN Dimers and Monomers at Different Conditions (20 mM Hepes, pH 7)

ligand	T (°C)	FMN oligomeric state[a]	n	K_D (10^{-6} M)	$-\Delta H_{bind}$ (kcal mol^{-1})	$-T\Delta S_{bind}$ (kcal mol^{-1})	$-\Delta G_{bind}$ (kcal mol^{-1})
FMN (1.3 mM)[b]	30	2	2.0 ± 0.01	5.8 ± 0.1	13.2 ± 0.1	5.9 ± 0.5	7.27 ± 0.22
FMN (1.3 mM)	35	1	1.1 ± 0.02	7.5 ± 0.1	19.2 ± 0.3	12.0 ± 1.5	7.23 ± 0.21
FMN (1.3 mM)	45	1	1.1 ± 0.04	50.0 ± 1.0	22.1 ± 0.3	15.8 ± 1.5	6.26 ± 0.14
FMN (50 μM)	30	1	1.0 ± 0.02	0.80 ± 0.02	7.7 ± 0.1	−0.7 ± 0.1	8.43 ± 0.14
FMN (1 μM)[c]	30	1	1.1 ± 0.1	0.78 ± 0.08			8.45 ± 0.21
FMN[d] + 13 mM phosphate	30	2	1.0 ± 0.01	0.40 ± 0.01	13.5 ± 0.3	4.6 ± 0.6	8.88 ± 0.20
FMN[d] + 13 mM sulfate	30	2	1.0 ± 0.09	0.43 ± 0.01	14.3 ± 0.2	5.5 ± 0.8	8.84 ± 0.16
phosphate	30		37 ± 6	5263 ± 50	−2.6 ± 0.7	−5.8 ± 0.9	3.16 ± 0.05
sulfate	30		61 ± 8	862 ± 10	0.27 ± 0.04	−4.0 ± 0.5	4.25 ± 0.05

[a] In solution, according to ^{1}H NMR chemical shift and visible-absorption data. [b] All experiments performed in duplicate; the FMN titration resulting in $n = 2$ has been repeated four times. [c] Parameters obtained by fluorescence quenching (Figure 1). [d] 1.3 mM.

various species. For example, the K_D for FMN and flavodoxins from *D. vulgaris* and *Anabaena* of 2.4 nM (50 mM phosphate, pH 7, 25 °C) and 0.2 nM (50 mM MOPS, pH 7, 25 °C), respectively, have been reported (*7*, *8*). In Figure 1A, we show the emission quenching that occurs upon additions of apo-flavodoxin from *D. desulfuricans* to 1 μM FMN (20 mM Hepes, pH 7, 30 °C). Saturation is reached at approximately a 1:1 stoichiometry, as expected, and from a Scatchard analysis (Figure 1B), a K_D of 0.56 μM is estimated for the complex. This value is at least 200-fold higher than most K_D values reported for other flavodoxins, although it is in good agreement with a recent recalculation of the K_D to 0.14 μM (20 mM Tris, pH 7.6, 25 °C) for FMN in complex with apo-flavodoxin from another *D. desulfuricans* ATCC strain (*18*).

FMN Binding to Apo-Flavodoxin at High FMN Concentrations. ITC is the most direct method to accurately measure thermodynamics for protein−ligand interactions in solution (*15*). In Figure 2A (top), we show the differential power signal recorded as a function of FMN (L) injections to *D. desulfuricans* apo-flavodoxin (M). After integration with respect to time and normalization per mole of added ligand, the heat can be plotted as a function of the molar L/M ratio as shown in the binding isotherm (Figure 2A, bottom). The only binding model that fit the data in Figure 2A is a single-site model resulting in a stoichiometry (n) of two FMN per apo-protein (thus, two identical binding sites on each protein). The K_D for this complex is 5.8 μM (20 mM Hepes, pH 7, 30 °C; Table 1). To confirm that two FMN molecules are binding per protein, we performed a reverse titration in which M was titrated into a solution of L at similar concentrations (Figure 2B). In this experiment, n (= M/L) becomes 0.5 (i.e., 1 protein per 2 FMN molecules, as expected), and K_D is again ~6 μM.

The reason for this unprecedented observation originates in the properties of FMN in concentrated solutions. In the FMN concentration range used in the ITC experiments above ([FMN] = 1.3 mM in the syringe), FMN has been reported to assemble into stacked dimers, both in aqueous solution and in solid films (*9*, *10*). Considering the flat nature of the isoalloxazine ring, it is not surprising that FMN and related compounds self-stack in solution (*1*). ^{1}H NMR and visible

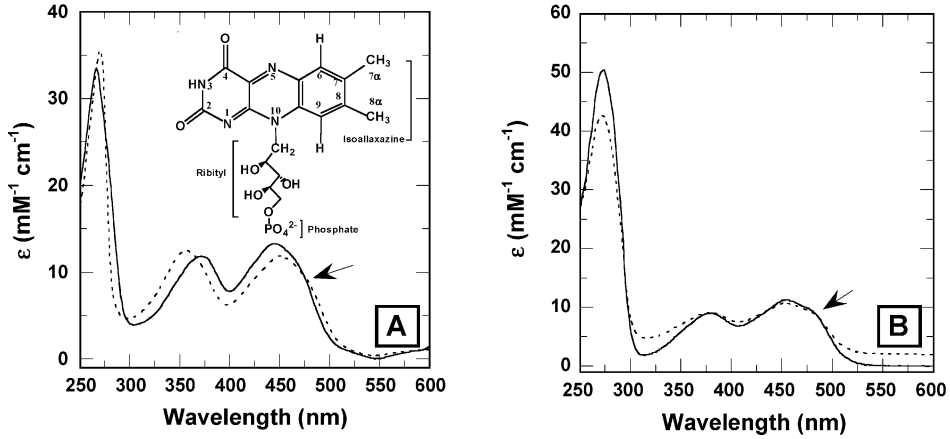

FIGURE 3: Absorption spectra revealing FMN monomers and dimers (20 mM Hepes, pH 7, 30 °C). (A) FMN in solution at high (1.3 mM, broken line) and low (5 μM, solid line) concentrations, showing an isobestic point around 485 nm and a shoulder at higher wavelengths in case of the higher concentration (broken line), in accord with two different species. (B) FMN absorption of purified holo-flavodoxin (solid line) and of the 2:1 FMN-to-protein complex after an ITC experiment and subsequent desalting to eliminate excess FMN (broken line). The same isobestic point and shoulder are observed indicative of FMN dimers bound in one case (broken line), and FMN monomers in the other (solid line) with ϵ_{445} (per FMN) of 10 200 and 10 600 M^{-1} cm^{-1}, respectively. The chemical structure of FMN is shown in the inset of panel A and consists of three parts: the isoallaxazine head, the ribityl trunk, and the phosphate tail. The methyl protons (7α and 8α) and aromatic protons (6 and 9), which are used to probe FMN stacking by NMR in Figures 4 and 7, are indicated.

absorption have been used to probe this tendency of FMN to self-stack (*9*, *10*). Various K_D values for the FMN (monomer-to-dimer) equilibrium, ranging between 1 and 50 mM, have been reported (*19*, *20*). In Figure 3A, we show the absorption spectra of FMN in solution at the high concentration (1.3 mM) used in the ITC experiments and at a lower (5 μM) concentration (normalized to the same concentration). On the basis of published reports (*9*), the isobestic point around 485 nm (marked in Figure 3) and the shift of the 370 nm absorption peak to 355 nm indicate that the two traces represent two kinds of FMN molecules: dimers and monomers. The absorption shoulder above 490 nm (found in the spectrum for high FMN concentration in Figure 3A) reports on the dimer, according to theoretical deconvolution of the absorption into component bands (*21*). The absorption spectra of the 2:1 flavodoxin complex after a completed ITC experiment and of purified holo-flavodoxin also exhibit these spectral differences, although there is no shift in the 370 nm peak (Figure 3B). Thus, FMN is predominantly dimeric at the ITC conditions (and K_D for the dimer-to-monomer equilibrium in solution pH 7, 30 °C is below 1.3 mM; see also ^{1}H NMR data next), and these dimers bind with micromolar affinity to apo-flavodoxin from *D. desulfuricans* (Hepes, pH 7, 30 °C). It is reasonable that any FMN−dimer dissociation that may occur due to dilution in the ITC experiments is slower than hydrophobic association with the apo-protein (*8*) since the kinetics for the latter process is fast (B.K.M. and P.W.-S., unpublished results).

The ITC titration was repeated at 10-fold lower concentrations of FMN and apo-protein, which should be closer to or below the FMN (dimer-to-monomer) K_D. As expected, we now observe 1:1 binding and a K_D of 0.8 μM (Figure 2C), which is in good agreement with the result obtained in the fluorescence-quenching experiment (Figure 1).

FMN Binding to Apo-Flavodoxin as a Function of Temperature. ^{1}H NMR chemical shifts have been used to characterize self-association of nucleosides and their derivatives: upfield shifts of the resonances of nucleobase protons observed with increasing concentration report on the presence of stacking (*16*, *22*). In the case of FMN, the chemical shifts of the isoalloxazine protons H6, H9, H7α, and H8α (inset, Figure 3A), which were shown to be sensitive to stacking and assigned previously, shift upfield (i.e., to lower ppm) in a curved dependence on the FMN concentration demonstrating that self-stacking occurs (*19*, *20*). In Figure 4, we show the chemical shifts for the FMN (1.3 and 20 mM) protons H6, H9, H7α, and H8α as a function of temperature. It is clear that the resonances shift downfield at temperatures above 30 °C in agreement with dimer dissociation as the temperature is increased. The chemical shifts for the aromatic protons H9 and H6 (1.3 mM FMN) become practically identical at higher temperatures (Figure 4A), which has also been reported previously at high temperature or at infinite dilution (*23*−*25*). Therefore, at 30 °C (20 mM Hepes, pH 7), 1.3 and 20 mM concentrations of FMN exist mostly in the dimeric form.

If two FMN molecules bind to apo-flavodoxin at 30 °C because they exist as dimers in solution, then increasing the temperature of the ITC experiment should result in 1:1 FMN to protein binding. This was tested by ITC experiments at 35 and 45 °C (Table 1). Importantly, thermal unfolding of apo-flavodoxin, as monitored by far-UV CD and fluorescence, shows that the folded structure is unperturbed up to 50 °C (data not shown). At the higher temperatures, FMN binding is somewhat weaker based on the increased K_D values (Table 1); however, the most important difference is that at both 35 and 45 °C, the stoichiometry of the reaction (*n*) reduces to one FMN per protein.

FMN Binding to Apo-Flavodoxin in the Presence of Divalent Anions. All experiments presented previously were performed in the absence of phosphate or sulfate anions in the buffer. To address the effect of phosphate and sulfate anions on FMN dimer interactions with *D. desulfuricans* apo-flavodoxin, we performed ITC experiments (pH 7, 30 °C) in their presence (Figure 5, Table 1). It is clear from the data in the presence of phosphate (Figure 5A) and sulfate (Figure 5B) that only one FMN binds per apo-protein. Moreover, the K_D is decreased about 15-fold as compared to the dimer complex: to 0.4 μM (13 mM phosphate or sulfate, pH 7, 30 °C). Notably, the K_D values obtained by

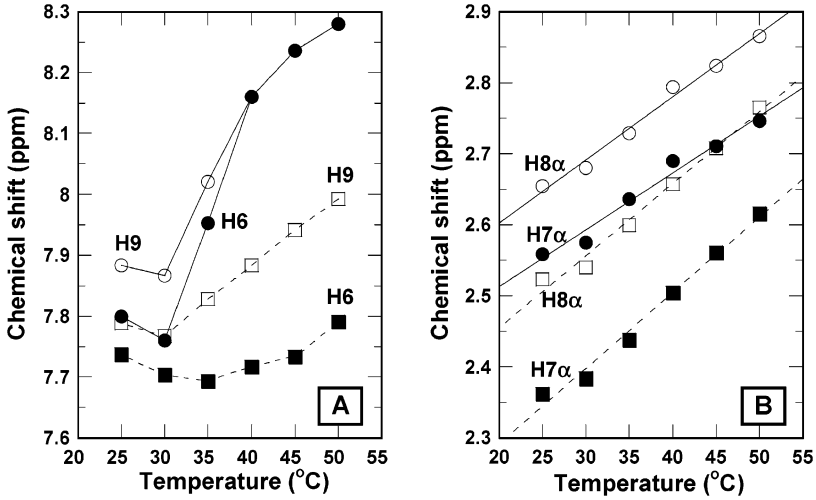

FIGURE 4: Variable temperature ^{1}H NMR studies probing FMN−dimer dissociation (20 mM Hepes, pD 7, 25−50 °C). The chemical shifts for (A) aromatic protons H9 and H6 and (B) methyl protons H8α and H7α are shown as a function of temperature for 1.3 mM FMN (circles) and 20 mM FMN (squares). Downfield shifts of both aromatic and methyl protons indicate dissociation of stacked FMN oligomers upon increasing the temperature.

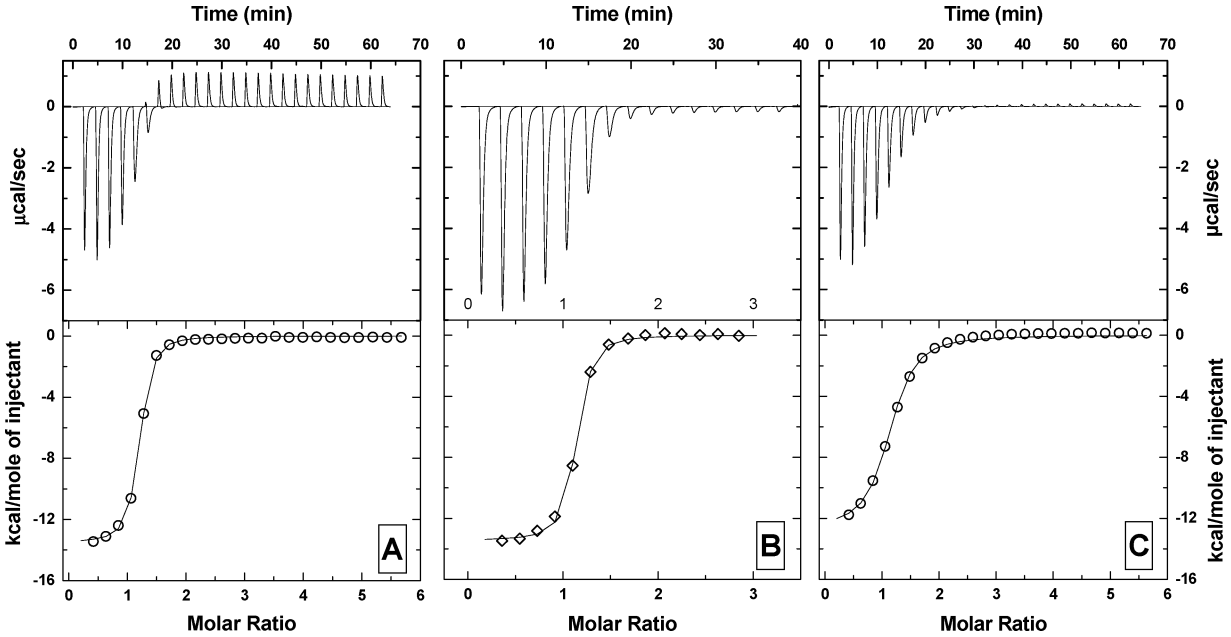

FIGURE 5: FMN binding probed by ITC in the presence of phosphate and sulfate (20 mM Hepes, pH 7, 30 °C). Calorimetric titrations were identical to Figure 2A, with the apo-flavodoxin and FMN samples preincubated at 30 °C for 30 min with (A) 13 mM phosphate, (B) 13 mM sulfate, and (C) 135 μM phosphate, respectively, before being loaded in the calorimeter.

ITC (Figures 5 and 2C) and fluorescence (Figure 1) experiments (all for the 1:1 complex) are similar.

^{1}H NMR chemical shifts reveal that FMN in the millimolar concentration range is dimeric regardless of the presence or absence of phosphate or sulfate. In Figure 6A,B, we compare the chemical shifts for the aromatic protons (H6 and H9) and the methyl protons (H7α and H8α) of the isoalloxazine ring as a function of FMN concentration in the presence and absence of phosphate (pD 7, 30 °C). Curved upfield shifts are observed at all conditions as functions of increased FMN concentration and are in excellent agreement with published chemical-shift data (*10*). The chemical shifts of FMN's ribityl protons are not sensitive to the concentration-dependent stacking of FMN (Figure 6B, inset), in accord with the proposed FMN dimer structure in which the ribityl chains point in opposite directions (*9*). The NMR results suggest that specific binding of a divalent anion to the apo-protein restricts cofactor binding to one isoalloxazine ring. although FMN is dimeric in solution.

Phosphate and sulfate bind weakly to apo-flavodoxin according to separate ITC experiments in which these ions were added into solutions of apo-protein (Table 1). Approximately 40 phosphate and 60 sulfate ions bind to apo-flavodoxin with K_D values of 5.0 and 0.9 mM (20 mM Hepes, pH 7, 30 °C), respectively (see Table 1). A similar binding affinity and stoichiometry of inorganic phosphate to *D. vulgaris* apo-flavodoxin has been reported (*7*). Although the binding of phosphate ions to apo-flavodoxin appears weak overall, binding of a particular phosphate in the phosphate-binding site is probably stronger (*7*) (but not resolved by ITC). This is evident from ITC titrations of FMN dimers (1.3 mM FMN, 20 mM Hepes, pH 7, 30 °C) to apo-flavodoxin in the presence of 135 μM phosphate, which also results in a 1:1 FMN-to-protein stoichiometry (Figure 5C).

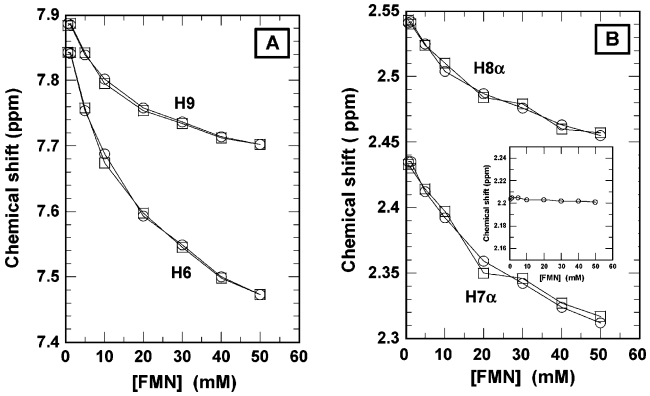

FIGURE 6: ^{1}H NMR data showing that anions do not affect the FMN monomer−dimer equilibrium (20 mM Hepes with and without 13 mM phosphate, pD 7, 30 °C). Chemical shifts for (A) aromatic protons H9 and H6 and (B) methyl protons H8α and H7α as a function of FMN concentration in 20 mM Hepes (circles) and 20 mM Hepes with 13 mM phosphate (squares). The chemical shifts in the presence of 13 mM sulfate (in 20 mM Hepes) were identical to the phosphate data (not shown). The curved upfield chemical shifts of the aromatic and methyl protons as a function of FMN concentration reports on the presence of dimers in this concentration range. The value of K_D for the FMN dimer-to-monomer equilibrium at our conditions (pH 7, 30 °C, ±anions) lies above 50 μM but below 1.3 mM. (B, inset) Chemical shifts of FMN ribityl protons as a function of FMN concentration. The lack of chemical shifts for these protons as a function of FMN concentration supports the reported model of the dimer structure in which the ribityl side chains point in opposite directions (see Discussion) (*9*).

DISCUSSION

Two major conclusions that have far-reaching implications can be derived from our observations. First, at some conditions, FMN dimers bind to *D. desulfuricans* apo-flavodoxin. Second, in the presence of sulfate or phosphate, only one FMN binds, although FMN is dimeric in solution.

How can two FMN molecules bind to flavodoxin when only one binding site exists? Since FMN is dimeric in solution when the 2:1 complex forms, it is reasonable to assume that the FMN dimers stay dimeric upon protein interaction: one FMN stacks with Trp60 and the other with Tyr98, creating a stack of four (instead of three) aromatic rings. Support for FMN being dimeric in the 2:1 complex comes from its absorption features, which differ from those of the 1:1 complex (Figure 3B). Since the two loops with Trp60 and Tyr98 are flexible in the absence of FMN according to NMR data (*5*, *6*), and they are situated on the protein's surface, there can easily be sufficient space to accommodate an additional FMN molecule in the cofactor-binding site. Nonetheless, our observation of the 2:1 complex emphasizes that the FMN-binding site in flavodoxin is substantially more flexible than previously predicted based on spectroscopic data or estimated from NMR and crystal structures.

It has been suggested by molecular modeling that the FMN monomers are oriented face-to-face in the dimer in a water-based solution, with the ribityl chains situated on opposite sides (*9*). This structural model is supported by our NMR results: the isoalloxazine protons are sensitive to stacking but not the ribityl protons (Figure 6). The estimated distance between monomer units in this dimer is 3.5 Å (*9*), in agreement with the smallest distance allowed by the van der Waals' interactions. If this model is true also for the dimer−flavodoxin complex, it implies that one phosphate group is binding in the phosphate-binding site, and the other is pointing toward the solution. This binding geometry (and the fact that the 1:1 complex has ~15-fold higher affinity; Table 1) explains why, upon extensive dialysis of the 2:1 complex, one FMN (presumably the molecule with the phosphate group facing the solvent) dissociates resulting in a 1:1 complex (data not shown).

Why has the flavodoxin complex with FMN dimers not been observed before? First, most flavodoxins have nanomolar affinities for FMN (*1*, *7*, *8*); thus, titrations to determine K_D are performed at very low FMN and protein concentrations (conditions where FMN is monomeric in solution). Second, crystallization efforts of holo-flavodoxin have mainly been successful in the presence of phosphate or sulfate (*2*, *26*), which according to our findings, results in 1:1 complexes although the FMN concentration may be high. Third, flavodoxin purification (after overexpression in *E. coli* or isolated from the natural host) involves many steps in the presence of high salt concentrations and various buffer exchanges; therefore, the purified protein will have only one FMN per protein regardless of the starting stoichiometry in the growing cells.

Is there any biological relevance for FMN dimers? The (self- and nonself) stacking of flat aromatic compounds is well-established (*19*, *20*, *22*, *28*) in vitro, and these type of complexes have been suggested to act as excitation-energy traps in biological systems (*29*). There are many examples where the stacking of nucleotides and related compounds are important in vivo, although there has been no function assigned for FMN dimers in flavodoxins. In many plants, flavins play the role of blue-light photoreceptors and probably exist in the chloroplasts' membranes as stacked dimers or higher-order oligomers (*30*, *31*). It has also been suggested that ATP occurs in a self-associated form in certain cell organelles where its concentration is high (*16*). Flavoenzymes use FMN or FAD cofactors to catalyze various reactions, such as deamidation, hydroxylation, mono-oxygenation, and reduction. In many such cases, the aromatic substrates bind to the active site by stacking with the FMN or FAD (*1*). Moreover, synthetic indole-based enzyme inhibitors can block the activity of flavoenzymes upon complex formation with flavin cofactors (*32*). Several proteins (such as pyridoxine 5′-phosphate oxidase and NADH:ubiquinone oxidoreductase) contain two FMN molecules per protein, although they have different binding sites (*32*, *33*). Most comparable to the dimer−flavodoxin complex described here is DNA photolyase. In this protein, the FAD cofactor is in a hairpin conformation with the adenine ring stacked over the isoalloxazine ring (*34*). This is the only flavoprotein studied to date that has a stacked cofactor arrangement.

Does the observation of the dimer−flavodoxin complex give insight into the (monomer) FMN-binding mechanism? First, it is clear that the FMN-binding loops, including Trp60 and Tyr98, in *D. desulfuricans* apo-flavodoxin must be very flexible in the absence of phosphate/sulfate since a FMN dimer can fit. Thus, the earlier speculation (*4*) that in the absence of FMN the loops are collapsed and the binding pocket closed appears unreasonable, at least for *D. desulfuricans* apo-flavodoxin. Our finding instead supports that the FMN−protein interactions start as a hydrophobic encounter at an open binding site (*8*). Second, binding of a phosphate/

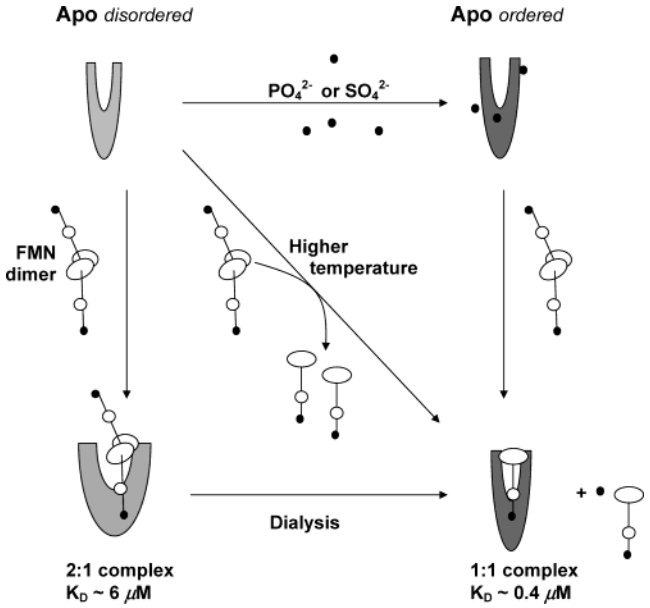

FIGURE 7: Scheme of pathways for the FMN dimer (FMN cartoon: isoalloxazine ring (oval), ribityl (open circle), and phosphate (filled circle); see also Figure 3A inset) interactions with apo-flavodoxin (gray) from *D. desulfuricans*. The apo-protein can be in two forms, indicated as disordered without bound anion and ordered with bound anions (filled circles), of which one is occupying FMN's phosphate-binding site. In the absence of sulfate or phosphate in the buffer, FMN can bind to apo-flavodoxin as an intact dimer; in the presence of phosphate or sulfate, the FMN-binding site is structurally different, which restricts binding to only one FMN (although FMN remains dimeric in solution). Upon heating (35−45 °C), the FMN dimer dissociates into monomers in solution, and accordingly, only 1:1 FMN-to-protein binding is observed. Finally, the 2:1 FMN-to-protein complex decomposes to the (15-fold) tighter 1:1 complex upon extensive dialysis.

sulfate to the phosphate-binding site must induce a substantial conformational change in these loops since, in the presence of these ions, only one FMN can be accommodated. That the phosphate/sulfate ion promotes a transition from a more disordered to a more ordered binding site is evident upon comparing the entropy changes of dimer and monomer binding (Table 1). In the absence of phosphate, the entropy change accompanying FMN binding is higher than in the presence of phosphate, implying (if the final states have the same entropy) that the apo-protein starts from a more disordered state in the absence of phosphate. In Figure 7, we present a mechanistic model that summarizes the conditions for FMN−dimer interactions with *D. desulfuricans* apo-flavodoxin.

CONCLUSION

We here report the remarkable and unprecedented observation that FMN dimers bind to *D. desulfuricans* apo-flavodoxin with micromolar affinity. This binding is modulated by divalent anions at the protein level. Our discovery emphasizes that cofactor-binding sites in proteins may be more flexible and promiscuous than those predicted based on crystal and NMR structures. Although FMN dimers have not been explored as biologically relevant cofactors in flavodoxins, there are many examples in living systems where the stacking of nucleotides and related compounds are important.

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