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Mechanism of FMN Binding to the Apoflavodoxin from *Helicobacter pylori*

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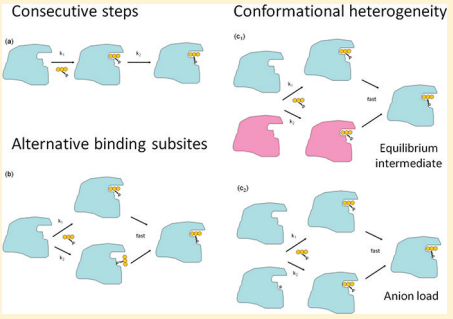
ABSTRACT: Flavodoxins are bacterial electron transport proteins whose redox competence is due to the presence of a tightly but noncovalently bound FMN molecule. While the thermodynamics of the complex are understood, the mechanism of association between the apoflavodoxin and the redox cofactor is not so clear. We investigate here the mechanism of FMN binding to the apoflavodoxin from *Helicobacter pylori*, an essential protein that is being used as a target to develop antimicrobials. This flavodoxin is structurally peculiar as it lacks the typical bulky residue interacting with the FMN *re* face but bears instead a small alanine. FMN binding is biphasic, regardless of the presence of phosphate molecules in solution, while riboflavin binding takes place in a single step, the rate constant of which coincides with the fast phase of FMN binding. A mutational study at the isoalloxazine and phosphate subsites for FMN binding clearly indicates that FMN association is always limited by interaction with the isoalloxazine subsite because mutating residues that interact with the phosphate moiety of FMN in the native complex hardly changes the observed rate constants and amplitudes. In contrast, replacing tyr92, which interacts with the isoalloxazine, greatly lowers the rate constants. Our analysis indicates that the two FMN binding phases observed are related neither with alternative or sequential interaction with the two binding subsites nor with the presence of bound phosphate. It is possible that they reflect the intrinsic conformational heterogeneity of the apoflavodoxin ensemble.



Many proteins contain associated small molecules or ions that perform essential roles either to stabilize the structure of the corresponding apoprotein or to facilitate its function. While a structural and thermodynamic understanding of such apoprotein/cofactor interactions is routinely provided by a combination of techniques such as X-ray, NMR, calorimetry, and simple spectroscopic determinations of equilibrium constants, the actual details of the recognition processes between the molecules involved are not so well understood. Knowledge of binding and dissociation mechanisms of protein ligands at atomic resolution may help to understand and modulate a variety of biological pathways where not only the affinity of the complexes involved but also the frequency at which they form and break are relevant.

Flavodoxins are small protein/ligand complexes[2,3] that constitute simple and convenient models to investigate the association of apoproteins to cofactors. All flavodoxins contain a noncovalently bound molecule of flavin mononucleotide, FMN, which confers electron transfer capabilities to the complex and allows flavodoxins to participate in a variety of essential redox reactions in bacteria. Flavodoxins are typical α/β proteins composed by a central five-stranded β-sheet surrounded by two layers of α-helices, and they have been subdivided in two groups: long-chain flavodoxins and short-chain ones. Long-chain flavodoxins bear an extra 20-residue loop that splits the fifth β-strand and that has been associated with recognition of partner proteins.[4−7] In spite of this structural difference, the FMN binding loops in short and long flavodoxins are similar. Two loops (the 50′ and 90′ loops) provide side chains that interact with the aromatic moiety in FMN: the isoalloxazine fused triple ring. Those side chains are typically aromatic ones. At the 90′ loop, there is almost always a tyrosine interacting with the *si* face of the cofactor. At the 50′ loop, there is often a tryptophan interacting with the cofactor *re* face. The third loop involved in FMN binding is the phosphate binding loop, which is highly conserved.[8] A variety of binding studies, including mutational analysis and the use of FMN analogues, have demonstrated that the interactions of both the phosphate group and the isoalloxazine ring of FMN contribute to the affinity of the FMN−apoflavodoxin complex.[1,9−13] In contrast, the ribityl moiety of FMN, connecting the phosphate

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and the isoalloxazine ring, hardly contributes to the strength of the apoflavodoxin−FMN complex.[10]

The usefulness of flavodoxins to investigate mechanisms of protein ligand recognition is reflected in the abundance of kinetic studies on a variety of flavodoxins from different species. Early studies on the short-chain *Peptostreptococcus elsdenii* protein[14] under pseudo-first-order conditions revealed monoexponential kinetics, compatible with a binding process lacking intermediates. They also revealed a complex dependence of rate constants with ionic strength and buffer composition. Similar kinetics were also described for the binding of FMN to the long-chain apoflavodoxin from *Azotobacter vinelandii*,[9] where faster binding of riboflavin, compared to that of FMN, was noticed. Subsequent temperature-jump relaxation studies, however,[15] proposed a more complex mechanism involving an initial binding step followed by a reorganization leading to the native complex. According to this mechanism, the cofactor phosphate group was key in the initial binding step and induced a conformational change in the apoprotein, which facilitated the rearrangement. Further analysis of short- and long-chain flavodoxins from *Desulfovibrio vulgaris*, *Anabaena variabilis*, and *Azotobacter vinelandii* by stopped-flow kinetics also revealed pseudo-first-order kinetics and confirmed the faster binding of riboflavin and a complex dependence of the binding rate constant on ionic strength and buffer composition.[16]

Generalization of site-directed mutagenesis together with the availability of the X-ray structure of an apoflavodoxin[17] promoted a renewed interest in the mechanism of apoflavodoxin−FMN complex formation. For *Desulfovibrio vulgaris* flavodoxin, the FMN binding was described as monoexponential in the absence of phosphate but biexponential when phosphate was present in the buffer, while riboflavin kinetics were monoexponential regardless of the presence of phosphate.[18] On the basis of these facts, it was proposed that two populations of apoflavodoxins, differing in whether they carried bound phosphate at the phosphate FMN binding site, could bind FMN following different pathways. Contemporary studies on the *Anabaena PCC 7119* protein indicated monoexponential binding kinetics in absence of phosphate, and a mutational analysis of the binding kinetics led to the proposal that the rate limiting step was the interaction of the FMN isoalloxazine ring with aromatic residues, chiefly Tyr94.[19] A biexponential binding was observed, nevertheless, for variants carrying mutations at the phosphate binding site. On the other hand, the binding of FMN to the apoflavodoxin from *D. desulfuricans*[20] was monoexponential regardless of phosphate presence in the buffer, and the mutational analysis also indicated that the binding might start at the isoalloxazine site. Modulation of binding speed by the presence of phosphate was reported. For *Azotobacter vinelandii* apoflavodoxin, FMN binding was biexponential in pyrophosphate buffer containing free phosphate.[12]

In this work we investigate the mechanism of FMN binding to the long-chain *Helicobacter pylori* (Hp) apoflavodoxin. The X-ray structure of the apoprotein is known,[21] and its conformational stability has been thoroughly characterized,[1,22,23] which has revealed the accumulation of partly unfolded conformations in equilibrium with the native state during the thermal unfolding. When the first X-ray structure of an apoflavodoxin was solved,[17] the presence of a bound phosphate or sulfate anion mimicking the FMN phosphate was noted. When Hp apoflavodoxin was crystallized, care was taken to avoid the presence of phosphate or sulfate anions in the crystallization buffer. Nevertheless, a chloride anion from the buffer was found bound at the phosphate site.[21] The FMN binding site of Hp apoflavodoxin is atypical. The bulky residue at the 50′ loop (often Trp in other flavodoxins) is replaced by an alanine, which lowers the equilibrium binding constant of the complex with FMN compared to that of other long-chain flavodoxins.[1] In addition, it creates a cavity near the redox active site which is being used for drug discovery purposes.[24,25] Despite this cavity, the approximation of the 50′ and 90′ loops that takes place in the apoprotein compared to the holoprotein closes the FMN binding site. Therefore, the X-ray structure of the apoflavodoxin displays a closed isoalloxazine binding subsite and a phosphate binding subsite that carries a bound anion (Figure 1). It is thus not possible to infer a binding mechanism based on the X-ray structure, which calls for a combination of kinetic measurements in a variety of apoflavodoxin variants and in different buffers to try to clarify the order of events leading to formation of the functional complex.

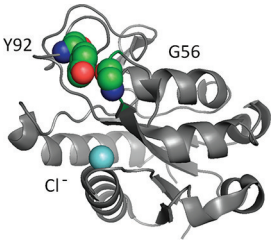

Figure 1. Cartoon of the *Helicobacter pylori* apoflavodoxin structure (PDB code: 2bmw). The FMN isoalloxazine binding cavity of the holoprotein is closed by approximation of the 50′ and 90′ loops, and a chloride ion is bound at the FMN phosphate site. Key residues of the two loops (Y92 and G56) and the bound chloride ion are shown.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis, Protein Expression, and Purification of *Helicobacter pylori* Flavodoxin Variants. The mutant flavodoxins T10A, S12A, N14A, Y92A, Y92F, and D142A were prepared by oligonucleotide-directed mutagenesis (Stratagene) of the *H. pylori* gene cloned in a pET28a vector and transformed into BL21(DE3) gold cells. Mutations were confirmed by DNA sequencing, and the mutants and the wild-type protein were expressed and purified as described.[25] Apoforms were prepared by treatment with 3% trichloroacetic acid.[17]

Stopped-Flow Kinetic Measurements. Rapid binding kinetics were recorded and analyzed using an Applied Photophysics SX17.MV stopped-flow spectrofluorimeter interfaced with an Acorn 5000 computer. Experiments were performed at 25.0 (±0.1) °C in different buffer conditions. Reactions at pH 7.0 were performed in 50 mM MOPS, 8 mM ADA, or 10 mM sodium phosphate. In some experiments, NaCl was added to study the influence of ionic strength on the binding rate. To study the influence of phosphate on the rate constants, a 50 mM MOPS buffer containing sodium phosphate and NaCl at a constant ionic strength of 300 mM was used. The initial concentration of FMN and riboflavin (both 99% purity, supplied by SIGMA) were typically 1 μM. Excess apoflavodoxin concentrations ranging from 15 to 90 μM were used to determine apparent rate constant of flavin binding under pseudo-first-order conditions. In some experiments a 0.1 μM flavin concentration was used. Quenching of flavin

fluorescence was followed to monitor flavin binding to apoflavodoxin.

FMN Binding by Isothermal Titration Calorimetry. Equilibrium binding constants of the complexes between FMN and wild-type or mutant apoflavodoxins were determined at 25.0 °C, in 50 mM MOPS, pH 7.0, using an isothermal titration calorimeter (VP-ITC calorimeter, MicroCal LLC, Northampton, MA). Typically, a 20 μM flavodoxin solution present in the calorimetric cell was titrated with FMN dissolved in the same buffer (300 μM FMN in the injection syringe). Protein and FMN samples were previously degassed, and their concentrations were determined before loading them into the ITC cell and syringe, respectively. Injections into the sample cell were done sequentially every 700 s to allow a complete recovery of the baseline. Data were analyzed with software developed in our laboratory, implemented in the software package Origin 7.0 (MicroCal).

RESULTS

Binding of FMN and Riboflavin to Wild-Type Apoflavodoxin in the Absence of Phosphate. The kinetics of FMN binding to apoflavodoxin at 25.0 ± 0.1 °C, pH 7.0, were monitored in a stopped-flow apparatus under pseudo-first-order conditions, following the quenching of FMN fluorescence after mixing cofactor with excess apoprotein (15-fold protein molar excess or higher). Different buffers were used to better evaluate the potential influence in the mechanism of different anions from the buffer that could be potentially bound at the phosphate binding subsite of the apoprotein. In all cases, the observed kinetic traces of FMN binding began at the fluorescence intensity registered for FMN mixed with buffer in absence of protein, indicating that no FMN quenching were taking place in the dead time of the instrument (Figure 2).

In 50 mM MOPS (I = 17 mM), the binding was biexponential, with the two observed rate constants being proportional to apoprotein concentration (Figure 3). The faster process (k_1 = 0.188 ± 0.005 μM^{-1} s^{-1}) accounted for around 15% of the total fluorescence change and the slower one (k_2 = 0.0068 ± 0.0002 μM^{-1} s^{-1}) for around 85%. The data for the faster process of FMN binding (and the data shown below for riboflavin binding) allow to calculate both k_{on} and k_{off} values (see Discussion).

In 8 mM ADA (I = 17 mM), a buffer lacking the sulfate moiety in MOPS and thus less likely to bind at the phosphate binding site in apoflavodoxin, the observed kinetics were similar (k_1 = 0.145 ± 0.014 μM^{-1} s^{-1} with around 5% amplitude, k_2 = 0.0034 ± 0.0001 μM^{-1} s^{-1}, with around 95% amplitude) (see Table 1).

Riboflavin binding to apoflavodoxin was carried out in the same reaction conditions (MOPS and ADA buffers). The fluorescence quenching traces began at the fluorescence intensity corresponding to riboflavin in absence of protein, indicating that no riboflavin quenching were taking place in the dead time of the instrument (Figure 2). In both buffers the binding was monoexponential (k_{on} = 0.191 ± 0.003 μM^{-1} s^{-1} in MOPS and k_{on} = 0.159 ± 0.003 μM^{-1} s^{-1} in ADA) (Table 1 and Figure 3).

Binding of FMN and Riboflavin to Wild-Type Apoflavodoxin in the Presence of Phosphate. Analogous kinetics of FMN binding have been carried out in 10 mM sodium phosphate, pH 7 (I = 18 mM). In this buffer, the quenching of FMN is biexponential (k_1 = 0.0078 ± 0.0002 μM^{-1} s^{-1}, k_2 = 0.0039 ± 0.0001 μM^{-1} s^{-1}) (Table 1). Although the faster rate constant is much slower than in the other buffers tested (Figure 4), the amplitudes of the two phases follow a similar trend (5% and 95%, respectively). The low value of the fast rate constant in 10 mM phosphate buffer together with its relative amplitude being similar to that of the fast phase observed in the other buffers tested has prompted us to examine whether the fast phases observed in the different buffers correspond to the same elemental process. To that end we have determined the value of the fast rate constant in MOPS buffer (at set FMN and apoflavodoxin concentrations of 1 and 16 μM, respectively) in the presence of different concentrations of phosphate (as low as 0.1 nM). As shown in Figure 5, the fast rate constant determined in MOPS buffer is smoothly decreased in presence of increasing concentrations of

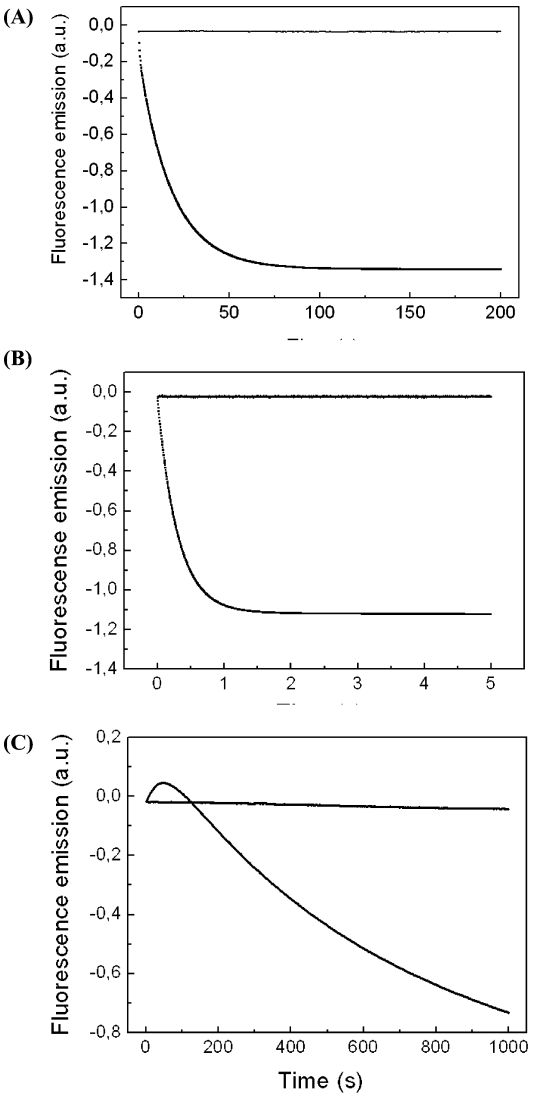

Figure 2. Binding of FMN to wild-type (A) and Y92F (C) *H. pylori* apoflavodoxin. Decrease in emission fluorescence upon FMN complexation with apoflavodoxin after fast mixing in 50 mM MOPS (at a ratio FMN/protein of 30:1). The horizontal line shows the fluorescence of FMN upon mixing with buffer. (B) Binding of riboflavin to wild-type apoflavodoxin in the same conditions. The horizontal line shows the fluorescence of riboflavin upon mixing with buffer. The concentration of FMN or riboflavin was 0.5 μM and the temperature 25.0 °C. The instrument voltage was the same in the three experiments.

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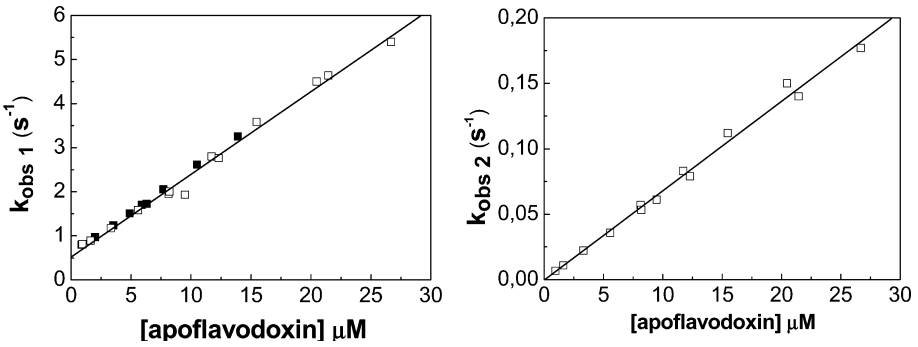

Figure 3. Observed rate constants for FMN (open squares) and riboflavin (close squares) binding to *H. pylori* flavodoxin wild-type in MOPS 50 mM, pH 7, at 25.0 °C.

Table 1. Kinetic Constants for FMN and Riboflavin Binding to Wild-Type Apoflavodoxin at 25 ± 0.1 °C, pH 7.0, in Several Buffers of Same Ionic Strength

	FMN		riboflavin
buffer	k_1 (μM^{-1} s^{-1})	k_2 (μM^{-1} s^{-1})	k_{on} (μM^{-1} s^{-1})
Mops (50 mM)	0.188 ± 0.005	0.0068 ± 0.0002	0.191 ± 0.003
ADA (8 mM)	0.145 ± 0.014	0.0034 ± 0.0001	0.159 ± 0.003
phosphate (10 mM)	0.008 ± 0.001	0.0039 ± 0.0001	0.168 ± 0.003

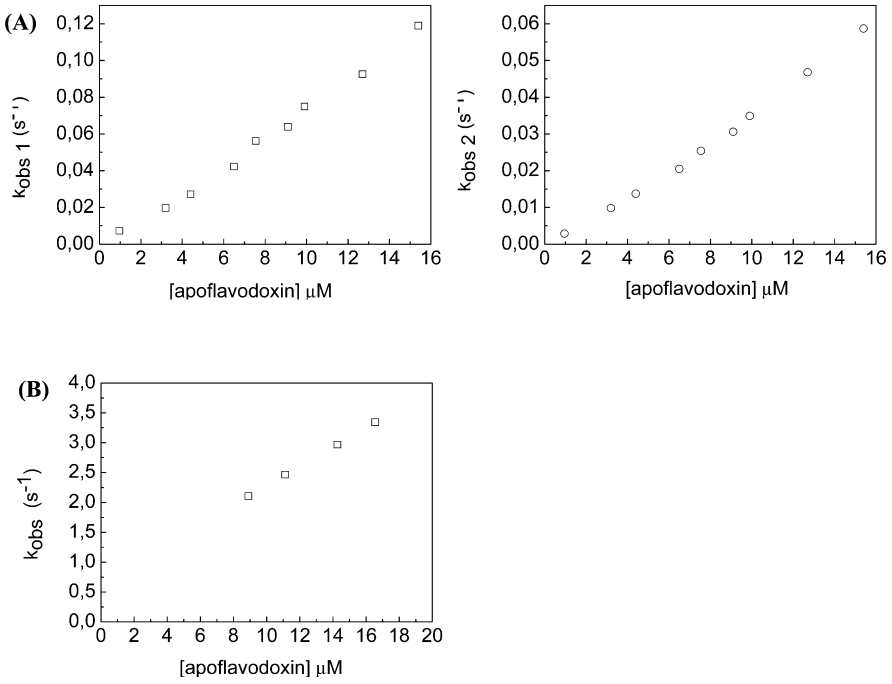

Figure 4. Observed rate constants for FMN (A) and riboflavin (B) binding to *H. pylori* flavodoxin wild-type in sodium phosphate 10 mM, pH 7, at 25.0 °C.

phosphate up to the very low value observed in 10 mM sodium phosphate. In contrast, the slow rate constant is only slightly influenced by the concentration of phosphate.

Riboflavin binding in 10 mM phosphate buffer is monoexponential, as in the other buffers, and the rate constant exhibits a similar value (k_{on} = 0.168 ± 0.003 μM^{-1} s^{-1}) (Table 1). The amplitude change is also similar to the total amplitude change associated with FMN binding.

Influence of Ionic Strength on the Rate Constants of FMN Binding to Wild-Type Apoflavodoxin in the Presence and Absence of Phosphate. The affinity of FMN for Hp apoflavodoxin has been described to increase at high ionic strength.[22] We have tested the influence of ionic strength on the rate constants of FMN binding in absence of phosphate (MOPS buffer) by recording binding kinetics in the presence of different concentrations of NaCl. As shown in Figure 6, increasing the ionic strength from 19 up to 300 mM progressively decreases k_1 and increases k_2. However, in presence of phosphate (10 mM phosphate buffer), both phases become faster (Figure 2): k_2 follows a most similar trend as that observed in MOPS buffer, but k_1 is progressively increased so that it approaches values not very different from those in absence of phosphate. The slowing influence of inorganic phosphate on the fast rate constant (k_1) seems thus to be

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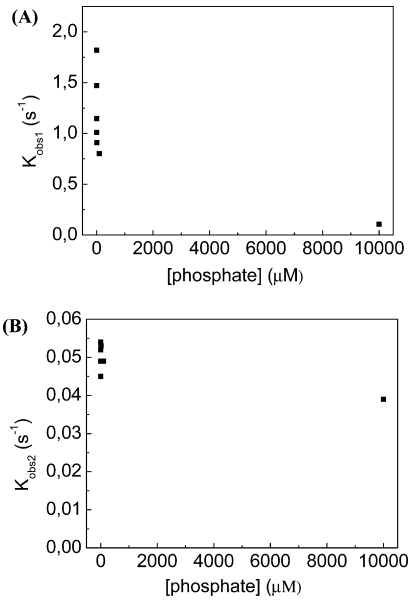

Figure 5. Effects of inorganic phosphate on the observed association constant of the wild-type apoflavodoxin complex with FMN (at constant concentrations of 16 and 1 μM, respectively). The ionic strength was set up to 19 mM, and the temperature was 25.0 °C.

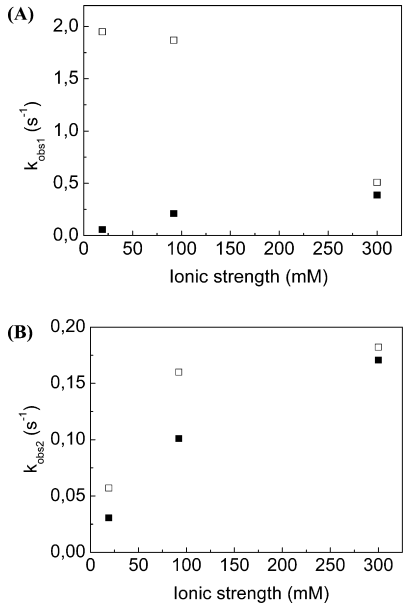

Figure 6. Effect of ionic strength on the first (A) and second (B) observed rates of binding of FMN to wild-type apoflavodoxin in 50 mM MOPS (open squares) and 50 mM sodium phosphate (closed squares), at 25.0 °C. The concentration of apoflavodoxin and FMN was 8 and 0.5 μM, respectively.

progressively reduced by NaCl. This has been further confirmed by determining the influence of phosphate concentration on k_1 and k_2 in the presence of NaCl at a constant ionic strength of 300 mM (Table 2). As the concentration of phosphate increases, at constant ionic strength, the relative amplitudes of the two phases remain rather constant at 5% for the first transition and 95% for the second one, k_1 becomes slower (but less than in the absence of NaCl), and k_2 hardly changes.

Table 2. Effect of Phosphate Concentration on the Apparent Association Constants of FMN to WT Apoflavodoxin at Constant Ionic Strength (300 mM)

[phosphate] (mM)	k_1 (μM^{-1} s^{-1})	k_2 (μM^{-1} s^{-1})
0	0.093 ± 0.007	0.024 ± 0.001
1	0.079 ± 0.007	0.025 ± 0.001
10	0.057 ± 0.004	0.024 ± 0.002
100	0.059 ± 0.002	0.026 ± 0.001

Stability of the Complexes between FMN and Apoflavodoxin Variants. To analyze the contribution of the different residues involved in FMN binding[26] to the stability of the final complex and to the binding kinetics, six apoflavodoxin variants carrying point mutations have been obtained: Y92F, Y92A, T10A, S12A, N14A, and D142A. Y92 is stacked onto the isoalloxazine ring, T10, S12, and N14 appear involved in the binding of the phosphate moiety, and D142 and N14 are involved in binding the ribityl which connects isoalloxazine and phosphate.

The equilibrium binding constant of the wild-type apoflavodoxin/FMN complex was determined in 50 mM MOPS, pH 7, at 25 °C ± 0.1 °C by isothermal titration calorimetry (ITC).[1] In spite of the presence of an alanine residue where most flavodoxins bear a tryptophan or other bulky residues, the complex is strong, albeit less than in other long chain flavodoxins.[1] We have now determined the equilibrium binding constant of the apoflavodoxin/riboflavin complex in the same buffer and temperature, which is significantly lower than that of the FMN complex (Table 3).

Table 3. Thermodynamic Parameters of the Complexes between FMN (or Riboflavin) and Wild-Type and Mutant Apoflavodoxins[a]

protein	ΔH (kcal/mol)	ΔG (kcal/mol)	K_d (nM)
WT	−20.7 ± 1.0	−10.1 ± 0.3	37 ± 6
	−15.4 ± 0.3[b]	−7.2 ± 0.2[b]	5600 ± 840[b]
T10A	−24.9 ± 1.2	−6.2 ± 0.2	27300 ± 400
S12A	−18.2 ± 0.9	−8.9 ± 0.3	296 ± 44
N14A	−16.3 ± 0.8	−9.9 ± 0.3	57 ± 9
D142A	−17.0 ± 0.8	−8.7 ± 0.3	447 ± 67
Y92F	−22.9 ± 1.1	−9.2 ± 0.2	175 ± 26
Y92A	−18.1 ± 0.9	−6.6 ± 0.2	15700 ± 2355

[a]Determined by ITC in 50 mM Mops, pH 7.0, at 25 °C. [b]Data corresponding to the WT complex with riboflavin.

The affinity of the six flavodoxin mutants bearing replacements of key residues has been similarly determined by ITC (Table 3). Replacement of T10 or S12 significantly lowers the affinity of the complex, while replacement of N14 hardly changes the stability. The destabilizations observed in these mutants correlate with the expected relative strengths of their hydrogen bonds with the FMN phosphate as judged by their lengths: 2.43, 3.19, and 3.28 Å for T10, S12, and N14, respectively. Replacement of D142, which is hydrogen bonded to the ribityl, moderately destabilizes the complex. Replacement of Y92, which packs against the *si* face of the FMN isoalloxazine ring, by phenylalanine moderately decreases the affinity, while its replacement by alanine gives rise to a significant destabilization of the complex. Both the wild-type complex and those formed by mutant apoflavodoxins are strongly stabilized by the enthalpy change of binding and severely

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destabilized by entropy changes. In that they differ from the *Anabaena* flavodoxin complex, which is even slightly stabilized by entropy changes.[10] This difference probably reflects a higher contribution of the hydrophobic effect to FMN binding in *Anabaena* apoflavodoxin where a larger burial of hydrophobic surface takes place.

Kinetics of FMN Binding to Apoflavodoxin Variants. The binding kinetics of the mutant apoflavodoxins to FMN (Figure 7) follow double-exponential kinetics, like those

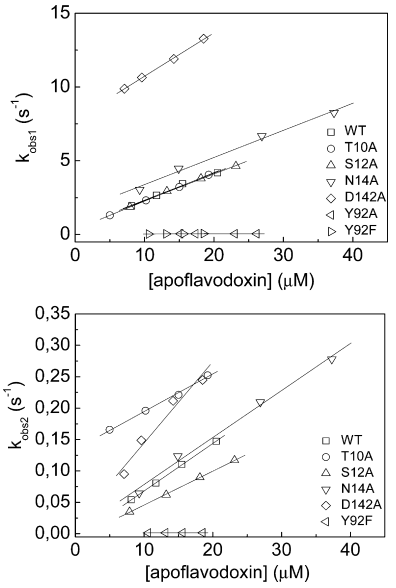

Figure 7. Observed rate constants for FMN binding to wild-type *H. pylori* apoflavodoxin and mutants thereof, in MOPS 50 mM, pH 7, at 25.0 °C.

observed for the wild-type protein. It is remarkable that replacement of the three residues that establish hydrogen bonds with the phosphate moiety of FMN in the functional holoflavodoxin complex (T10, S12, and N14) gives rise to virtually no change in either k_1 or k_2 (Table 4). Besides,

Table 4. Association Rate Constants of FMN to Wild-Type and Mutant Apoflavodoxins at 25 °C ± 0.1 °C, pH 7.0, in 50 mM MOPS

protein	k_1 (μM^{-1} s^{-1})	k_2 (μM^{-1} s^{-1})
WT	0.183 ± 0.010	0.0076 ± 0.0001
T10A	0.193 ± 0.001	0.0060 ± 0.0003
S12A	0.181 ± 0.006	0.0054 ± 0.0001
N14A	0.184 ± 0.012	0.0075 ± 0.0005
D142A	0.294 ± 0.007	0.0129 ± 0.0018
Y92F	0.0029 ± 0.0003	(1.59 ± 0.24) × 10^{-5}
Y92A	0.00038 ± 0.00007	

replacement of the one residue specifically involved in ribityl binding (D142A) only slightly increases the two rate constants, which is probably due to decreased electrostatic repulsions with the FMN phosphate moiety during binding.

In contrast, replacement of the isoalloxazine binding Y92 residue gives rise to slower binding. The very conservative mutation Y92F retains the two kinetic phases observed in wild type, and the binding of FMN also gives rise to strong quenching of FMN fluorescence intensity (Figure 2C). However, k_1 is 63-fold lower than in wild type, and it appears associated with a small increase in intensity rather than to the small decreased seen in wild type and mutants elsewhere in the binding site. As for k_2, it shows an even more pronounced 480-fold decrease. The less conservative replacement of Y92 by alanine gives rise to a further 10-fold decrease in k_1, compared to the Y92F mutant, and k_2 is no longer observed in reasonable stopped-flow times. In Y92A the fast phase is also accompanied by a small fluorescence intensity increase, as observed in Y92F.

DISCUSSION

Rate-Limiting Binding Events Are Interactions between FMN Isoalloxazine and Tyr 92. Hp flavodoxin is a long-chain flavodoxin that lacks the tryptophan residue often involved in cofactor binding in other flavodoxins.[3] Yet, Hp flavodoxin binds FMN tightly, with a dissociation constant in the nanomolar range.[1] The mechanism of FMN−apoflavodoxin complex formation has been studied in several flavodoxins, and a variety of kinetic behaviors have been reported. Often, a monophasic binding has been found, which in some cases becomes biphasic in presence of phosphate.[12,18] In *H. pylori* flavodoxin FMN binding is biphasic (Figure 2) regardless of the presence of inorganic phosphate in solution, as previously found in the W60A variant of *D. desulfuricans* flavodoxin.[18]

In the two apoflavodoxin structures solved by X-ray[17,21] the isoalloxazine site is occluded by approximation of residues at the 50′ and 90′ loops. At the same time, small anions from the buffer solution (phosphate/sulfate or chloride) appear bound at the FMN phosphate subsite. The structures thus can offer little insight on where the primary recognition event between FMN and the apoprotein will take place. On the other hand, solution studies[27,28] suggest that flexibility at both the isoalloxazine and phosphate site is larger than that observed in the X-ray structures, but they do not clarify how the binding takes place. Since the anions bound at the FMN phosphate site could influence the binding mechanism and be related to the observation of biphasic kinetics,[21] we have performed FMN binding kinetics in three buffers of same ionic strength: MOPS (containing chloride), phosphate, and ADA (the negative charges of which are provided by carboxylate groups and deemed less likely to bind strongly to the protein). In the three cases we obtained biphasic kinetics with similar relative amplitudes. In addition, the slow rate constant displayed the same value in the three buffers, and only the fast rate constant was progressively slowed down by increasing phosphate concentration. This suggests that the existence of the two binding processes observed need not be related to the presence of bound anions in apoflavodoxin.

Of all the information gathered in this work, the most revealing one is that derived from comparison of the binding kinetics of wild-type and mutant apoflavodoxins. Small, conservative mutation of the polar residues that establish hydrogen bonds with either the FMN phosphate or ribityl gave rise to apoproteins which still bind FMN, although with lower affinity than the wild type (Table 3). For these mutants, the apoflavodoxin/FMM complexes are 1−1.5 kcal/mol less stable and, in one case (T10A), 4 kcal/mol less stable than the wild-type complex. In spite of these substantial stability decreases, all these mutants display close to identical biphasic binding kinetics with very similar values for the two observed rate constants and associated amplitudes (Table 4). Only D142A binds slightly faster than the other variants, which is probably related to the removal of a negative charge allowing a faster

binding of negatively charged FMN. It is thus very unlikely that any of the two phases observed bear relation with a binding mechanism involving initial contact of FMN at the phosphate binding site of the apoprotein. We have also analyzed the influence of replacing Y92, which stacks onto the isoalloxazine group of FMN, by phenylalanine and alanine. The conservative Y92F mutation gives rise to a complex 1 kcal/mol less stable than wild-type and, in this respect, analogous to most of the complexes formed by mutants of the phosphate and ribityl site (Table 3). However, the binding kinetics of Y92F, while still biphasic, are much slower than those of wild-type (k_1 is 60-fold and k_2 480-fold slower). In the more drastic mutation Y92A, the affinity of the complex is reduced by 3.5 kcal/mol, similar to the debilitation observed in the T10A mutant. However, while the T10A mutant exhibits binding kinetics identical to wild-type, the Y92A mutant exhibits an extremely slow binding. Only the fast phase with its characteristic small amplitude could be observed, which was almost 500-fold slower than in wild-type. These results together clearly indicate that Y92 is the key residue of the binding site where the FMN molecule establishes the rate-limiting contacts of the binding mechanism. They also indicate that the phosphate and ribityl binding residues contribute to the stability of the final functional complex but play no significant role in the binding mechanism.

The possibility that the biphasic kinetics reflect a two-step binding of FMN, first contacting at the isoalloxazine site and then docking at the phosphate site can be ruled out because of the nonzero-slope linear dependence of the slow binding phase on protein concentration (Figure 3). If the initial ligand binding step were followed by a rearrangement step leading to optimized protein–ligand interactions, k_2 would be concentration independent.[29,30] It thus appears that the two phases observed for the binding of FMN are related to two different binding mechanisms both beginning at the isoalloxazine site.

The Fast Minor Phase of FMN Binding Corresponds to a Simple Binding Event Identical to That of Riboflavin Binding. In contrast to FMN binding kinetics, that of riboflavin is monoexponential (Figure 2). It is highly unlikely that a very fast phase takes place in the dead time of the instrument because the observed binding trace begins with exactly the same emission fluorescence intensity as that of free riboflavin (Figure 2B). This indicates that all fluorescence quenching processes associated with complex formation are observed in the recorded time window, exactly the same as in the case of the biexponential FMN binding (Figure 2A,C). This simple binding behavior of riboflavin allows to determine[31] the k_{on}, from the slope of the linear concentration dependence observed for the rate constant, at 1.9×10^5 M^{-1} s^{-1}, and the k_{off}, from the intercept, at 0.58 s^{-1}. The equilibrium dissociation constant calculated from these values is K_d = 3.1 μM, which compares well with that directly determined from ITC (5.6 μM) (see Table 3), confirming a simple riboflavin binding mechanism without intermediates. Comparison of the observed rate constants of FMN binding (in the absence of phosphate) with that of riboflavin strongly suggests that the fast FMN binding phase reflects binding via the same mechanism as that of riboflavin. Indeed, the values of the k_{on}, k_{off}, and K_d calculated assuming the fast phase of FMN binding corresponds to a two-state binding equilibrium (1.9×10^5 M^{-1} s^{-1}, 0.51 s^{-1}, and 2.7 μM) are essentially identical to those calculated for the binding of riboflavin (see above).

This leaves open the question of whether the slower phase reflects (see Figure 8): (a) a reorganization of the initial complex formed in the fast phase; (b) an alternative FMN binding mode beginning at a different protein subsite; or (c1) FMN binding to a fraction of protein molecules in a different conformational state or (c2) carrying a different anion load. Possibility (a) will not be further considered because a reorganization of a pre-existing apoflavodoxin/FMN complex would not give rise to the observed linear dependence of the slow rate constant on protein concentration. Possibility (b) can also be ruled out based on the lack of effect of mutations at the phosphate and ribityl subsites in the binding kinetics of FMN.

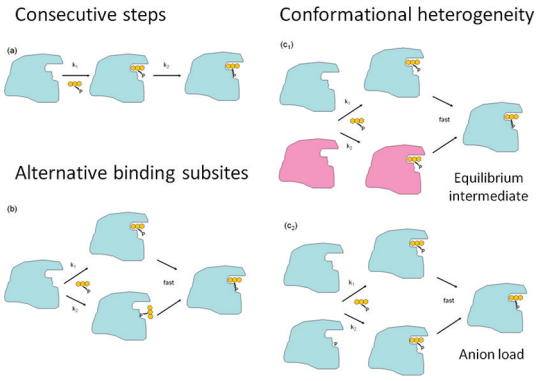

Figure 8. Schemes showing different possible binding mechanisms of FMN to *H. pylori* apoflavodoxin: (a) sequential binding, first at the isoalloxazine site, then at the phosphate site; (b) alternative initial binding to either isoalloxazine or phosphate site, followed by fast reorganization; (c1) alternative binding to either native or partly unfolded apoflavodoxin followed by fast reorganization; (c2) alternative binding to either phosphate-loaded or phosphate free apoflavodoxin followed by fast reorganization.

The Slow Major Phase May Represent FMN Binding to the Native State and the Fast One to an Equilibrium Intermediate. A variety of effects of phosphate anions on the binding of FMN to different apoflavodoxins have been reported. In presence of phosphate, the binding becomes faster in *D. desulfuricans* flavodoxin,[20] while in *Anabaena* it becomes slower.[19] In *D. vulgaris*, phosphate anions change the kinetics from monoexponential to biexponential.[18] For Hp apoflavodoxin, comparison of the FMN binding rate constants in wild-type as recorded in the same ionic strength but in MOPS or in phosphate buffer reveals a strong influence of phosphate on the fast rate constant, which is markedly slowed down. That the two phases observed in the presence of phosphate correspond to the same two phases observed in MOPS is suggested by the fact that their relative amplitudes are very similar and by the similar value of the slow rate constant in either buffer. We have nevertheless confirmed this correspondence by recording binding kinetics at constant ionic strength in the presence of different concentrations of phosphate. The slowing down effect of phosphate on the fast rate constant is observed at low phosphate concentrations. As the phosphate concentration is raised, the value of the rate constant is reduced from the value observed in MOPS buffer to that observed in 10 mM phosphate buffer (Figure 6). This confirms that the two phases observed in phosphate buffer correspond to those observed in MOPS. Although the strong effect of phosphate concentration in the value of fast rate constant could be interpreted as indicating that the fast binding process begins at the phosphate subsite, the fact that the amplitude of this fast phase is independent of phosphate concentration makes possibility (c2) unlikely and clearly calls for a different explanation. It seems

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that, as the phosphate concentration increases, the fast binding of FMN starting at the isoalloxazine subsite is slowed down, possibly as a consequence of electrostatic repulsions between approaching FMN molecules and bound phosphate. This is consistent with the lack of effect of increasing phosphate concentrations on riboflavin binding kinetics (Table 1).

Possibility (c1): that the slow and fast phases correspond to FMN binding to fractions of apoflavodoxin molecules in different conformations thus remains the more likely explanation for the occurrence of biexponential kinetics in the binding of FMN to Hp apoflavodoxin. The nature of this conformational heterogeneity affecting FMN binding is uncertain at present. One obvious possibility is the occurrence of partly unfolded conformations of apoflavodoxin in equilibrium with the native state. In fact, most apoflavodoxins whose equilibrium thermal unfolding has been analyzed populate an intermediate conformation.[3,32] Hp apoflavodoxin is even less cooperative than other apoflavodoxins as it unfolds via two sequential intermediates, as the temperature is increased.[1] The intermediate that accumulates at lower temperature is analogous to the only intermediate observed in *Anabaena* apoflavodoxin thermal unfolding. The structure of the *Anabaena* intermediate has been determined by NMR and SAXS[33,34] and combines a large native-like region with a non-native one that comprises the FMN binding loops and the long-loop characteristic of long-chain flavodoxins. On the basis of the correspondence between the disordered regions of the flavodoxin intermediate and the binding sites of native flavodoxin (for FMN and for partner proteins), we have proposed in previous work that the intermediate conformation might play functional roles, such as assisting in ligand binding. It is thus tempting to speculate that native apoflavodoxin may bind FMN at a certain rate and the intermediate conformation at a different one.

For this (c1) binding scenario one would expect that the molar fractions of native and intermediate roughly corresponded to the amplitude fractions of the two observed phases. Quantification of the molar fraction of Hp apoflavodoxin intermediate that is present in the solution conditions of the binding kinetics reported here is in principle possible by extrapolating the thermal unfolding data previously obtained for this protein (1) using the integrated Gibbs–Helmholtz equation. However, the presence of two consecutive intermediates appearing in the thermal unfolding (1) together with the errors associated with the determined enthalpy and heat capacity changes, and midtemperature of the N-to-I_1 equilibrium makes the calculated molar fraction of intermediate (0.20–0.25) only an approximation. It is nevertheless clear that this intermediate accumulates substantially in MOPS buffer. For the *Anabaena* apoflavodoxin, where only one intermediate accumulates and a more precise extrapolation of the thermodynamic data is possible, the molar fraction of intermediate in MOPS buffer is of 0.10–0.15.[34]

On the basis of the large molar fraction of the native conformation, compared to that of the intermediate, at the solution conditions used to monitor FMN binding,[134] we propose that the fast phase should reflect binding to the intermediate while the slow phase could represent binding to the native conformation. Given the uncertainties in the extrapolated molar fractions, the correspondence between those calculated for the intermediate (0.20–0.25 for Hp and 0.10–0.15 for *Anabaena* apoflavodoxin) and the fractional amplitude of 0.15 observed for the fast phase in the same buffer (50 mM MOPS, pH 7.0) is reasonable. On the other hand, the conformational stability of apoflavodoxin is quite sensitive to changes in buffer composition.[35] Changes in the relative stability of native and intermediate apoflavodoxin in the different buffers used (MOPS, ADA, and phosphate) could explain the presence of different molar fractions of intermediate in the different buffers tested here and, hence, the different relative amplitudes of the fast phase in those buffers.

Although the apoflavodoxin intermediate does not form a stable complex with FMN,[1] it could provide a faster pathway during the binding reaction. Thus, the fast binding step would take place at open, partially unfolded FMN binding site, readily accessible to cofactor binding, whereas the slow binding step would occur at the closed, folded native state, requiring larger activation energy for cofactor binding. In this scenario, which is plausible but by no means certain given the available evidence, the influence of NaCl on the apparent rates constants (Figure 6) would be explained as follows: a higher NaCl concentration allows replacing phosphate bound at the phosphate subsite by chloride, which lowers k_1 less than phosphate because it gives rise to less electrostatic repulsion with FMN. The moderate increase of k_2 at high ionic strength may be due to a variety of reasons including a reduced repulsion between approaching FMN and apoflavodoxin or to the fact that ionic strength enhances the hydrophobic interactions. On the other hand, the fact that riboflavin does not discriminate between the two apoflavodoxin conformations, and therefore only one fast binding step takes place would imply that the phosphate group in FMN slows down the binding to the native conformation of apoflavodoxin but not to the intermediate conformation.

CONCLUSIONS

Taking into account all the available evidence, of which the mutational one is most revealing, we propose that the binding of FMN to Hp apoflavodoxin starts by interaction of the isoalloxazine ring and tyrosine 92. The two binding processes observed do not appear to relate to FMN binding to apoflavodoxin carrying or lacking bound anions at the phosphate subsite, nor do they appear to reflect a two-step binding at the isoalloxazine subsite followed by rearrangement. Rather, they appear to reflect conformational heterogeneity of the apoflavodoxin molecule, possibly relating to the equilibrium between the dominant native conformation and an intermediate exhibiting partial unfolding at the FMN binding site. Previous work highlighted the need to consider bound anions as part of the binding scenario.[21] This work adds complexity to the binding scenario. It seems now that conformational heterogeneity of the apoflavodoxin molecule will need also to be considered in future work.

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■ ABBREVIATIONS

Hp, *Helicobacter pylori*; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; ADA, *N*-(2-acetamido)iminodiacetic acid.

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