CONFORMATIONAL STABILITY OF HELICOBACTER PYLORI FLAVODOXIN. FIT TO FUNCTION AT pH 5.

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Running title: Stability of H. pylori flavodoxin: fit to function at pH 5.0.

Flavodoxin is an essential protein for Helicobacter pylori, a pathogen living in the very acidic environment of the gastric tract and responsible for several diseases. We report the conformational stability of the protein in neutral and acidic pH. The apoprotein remains native between pH 12 and 5, and adopts a monomeric molten globule conformation at more acidic pH values. The equilibrium unfolding in urea appears two-state for either conformation but the native one coexists with a hidden equilibrium intermediate of very similar properties. The stability of H. pylori apoflavodoxin is higher than that of the Anabaena homologue throughout the entire pH interval, which may be related to better charge compensation. H. pylori apoflavodoxin is strongly stabilized by its FMN cofactor. A global analysis of apo and holoflavodoxin equilibrium unfolding, with and without excess FMN, indicates that the cofactor only binds to the native state. Some physical-chemical properties of the protein may represent an adaptation to the acidic environment. Unlike the apoflavodoxin from Anabaena, which becomes highly insoluble at pH 5.0, that from H. pylori remains soluble to at least 40 μM. This fact, together with the high stability of the apoprotein at this low pH that can arise in the bacteria cytoplasm, seem useful to allow newly synthesized apoflavodoxin molecules to fold and remain soluble in order to accomplish cofactor binding, which in turn increases the stability. Also, whenever the cytoplasmic pH drops to 5, preexisting flavodoxin molecules will remain folded and soluble and will retain the FMN cofactor, thus remaining functional.

Keywords: flavodoxin, Helicobacter pylori, protein stability, molten globule, chemical denaturation, ligand binding, protein folding, histidine pKa

Flavodoxins are bacterial electron-transfer proteins that bear a non-covalently bound redox cofactor (flavin mononucleotide: FMN) that allows them to participate in a variety of reactions (1). There is evidence that, in vivo, the apoprotein folds first and then binds the cofactor, and it is clear that when the cofactor is removed, the apoprotein remains folded (2-4). Helicobacter pylori is a Gram-negative bacterium related with digestive diseases such as type B gastritis, stomach and duodenal ulcers (5), adenocarcinomas (6) and stomach lymphomas (7). It is found in the stomach of 50% of the human population (8), and 20% of infected individuals develop pathologies. Recently, it has been reported that the flavodoxin from H. pylori is essential for the survival of the pathogen due to its role as electron acceptor of the pyruvate:flavodoxin oxidoreductase complex (PFOR), which catalyzes pyruvate oxidative decarboxylation (9). Conversely, flavodoxin is the electron donor in the reverse reaction that is used to fix CO₂ (10). The X-ray structure of the protein is available for both the apo (11) and holo (12) forms. H. pylori flavodoxin contains 164 residues, including two tryptophans, and shares 40% sequence identity with Anabaena PCC7119 and Azotobacter vinelandii, and 30 % with Desulfovibrio desulfuricans and Desulfovibrio vulgaris flavodoxins. Interestingly, H pylori flavodoxin lacks a fairly conserved tryptophan residue involved in FMN binding in many flavodoxins and bears instead an alanine residue, which creates an exposed pocket near the redox cofactor (12) that is being used to target small inhibitory molecules (13). In recent years the stability of several (apo)flavodoxins has been investigated in
different laboratories (Anabaena PCC7119 (3,14-16); Azotobacter vinelandii (17,18); Desulfovibrio desulfuricans ATCC27774 (19); Desulfovibrio desulfuricans ATCC29577 (20,21); Desulfovibrio vulgaris (22)). In thermal unfolding, all apoflavodoxins studied seem to populate at moderate temperatures an intermediate conformation (14). In contrast, the chemical unfolding has revealed a less homogeneous behavior. The only apoflavodoxin studied by urea denaturation (Anabaena) follows a two-state model (3) while, using guanidinium hydrochloride, three apoflavodoxins have been investigated and different models have been proposed (15,18,19).

We report here the conformational stability of H. pylori flavodoxin in a wide pH interval to try to understand how the protein can remain functional at moderately acidic pH values where flavodoxin is expected to be either aggregated or highly insoluble (3) and as a contribution to understanding the somewhat controversial equilibrium and binding behavior of the flavodoxin family. According to our analysis, H. pylori apoflavodoxin remains folded in a wide pH-interval and adopts a monomeric molten globule conformation at low pH. Near the isoelectric point, its conformational stability is similar to that of Anabaena apoflavodoxin but its solubility is far higher. A global analysis of the unfolding of the apo and holo forms, with and without excess FMN, shows that only the native state can interact with the cofactor. Overall, it seems that while apoflavodoxins share a fairly common thermal unfolding mechanism (14,17,23,24) they can respond differently to chemical denaturation, probably reflecting a different response of their close to native partly unfolded forms (25,26) to thermal and chemical stresses. Importantly, the apoflavodoxin from Helicobacter pylori remains folded, soluble and in an FMN competent state at the fairly low pH values that can arise in the cytoplasm (27) of the pathogen, and the holoprotein is highly stable.

### Experimental Procedures

**Protein Purification-Recombinant H. pylori** flavodoxin was purified from a culture of E. coli cells (BL21) harbouring plasmid pET28a, which contains the flavodoxin gene (13). The cells were disrupted by sonication in 50 mM Tris-HCl, pH 8, and the supernatant was brought to 60 % saturated (NH₄)₂SO₄, centrifuged and loaded onto a DE-52 DEAE-cellulose column, previously equilibrated with 60 % saturated (NH₄)₂SO₄ in 50 mM Tris-HCl buffer, pH 8. Flavodoxin was then eluted with a linear gradient from 60% to 0% saturated ammonium sulfate in the same buffer. Flavodoxin fractions were poured onto a DE-52 DEAE-cellulose column, previously equilibrated with 50 mM Tris-HCl, pH 8. A purified flavodoxin was eluted with a linear gradient from 0 to 0.5 M NaCl in 50 mM Tris-HCl, pH 8. Finally, the apo and holo forms of flavodoxin were separated and concentrated in a MonoQ10 column (GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 8 from were they were eluted with a linear gradient from 0 to 1 M NaCl. The purity of the protein was monitored by SDS-PAGE and by recording its absorption spectrum. Apoflavodoxin was also obtained by removing the flavin mononucleotide group from the holoprotein by apoprotein precipitation with trichloroacetic acid (28).

**Spectroscopic Characterization**

**Fluorescence emission and far-UV and near-UV circular dichroism (CD) spectra were acquired at 298 ± 0.1 K at several pH values in both native and denaturing conditions (6 M urea or 363 K).** Fluorescence emission spectra (300 to 400 nm) were recorded, exciting at 280 nm, using an Amino-Bowman Series 2 spectrometer. Circular dichroism spectra in the near- and far-UV were recorded in a Jasco 710 or a Chirascan (from Applied-Photophysics) spectropolarimeters. Protein samples at different pH values were prepared by mixing concentrated buffer and protein solutions. Apoflavodoxin concentrations were 2-20 μM for fluorescence, 20 μM for far-UV CD experiments and 20-40 μM for near-UV CD. All final buffer solutions were of a 10 mM ionic strength. For fluorescence and near-UV CD, a 1 cm path-length cuvette was used and, for far-UV CD, a 0.1 cm one. Binding of ANS to apoflavodoxin was measured, as a function of pH, by recording emission fluorescence at 495 nm (excitation at 396 nm). Apoflavodoxin and ANS concentrations were 2 μM and 250 μM, respectively.

**Nuclear Magnetic Resonance spectroscopy-** All NMR experiments were performed on a Bruker DRX-500 spectrometer equipped with a triple resonance probe and z-pulse field gradients. The temperature probe was regularly calibrated by using methanol and ethyleneglycol (29).

**Diffusion measurements (DOSY experiments)**-Translational self-diffusion...
measurements were performed using the pulsed-gradient spin-echo (PGSE) NMR method. The theoretical and experimental aspects of PGSE NMR have been extensively reviewed (30,31). Briefly, if during the time between two applied gradient pulses, a molecule moves along the z-axis, when its magnetization is brought back to x-axis, it will not refocus completely and the nuclei resonances will be attenuated. If the molecule diffuses rapidly, the attenuation of its resonances will be large. Conversely, if diffusion is slow, the attenuation will be small. The relationship between the self-diffusion coefficient, \( D \), and the measured NMR parameters is:

\[
\frac{I}{I_0} = \exp\left(D\gamma^2\Delta^2\frac{G^2}{\Delta - \delta/3}\right)
\]

where \( I \) is the measured peak intensity (or volume) of a particular (or a group of) resonance(s); \( I_0 \) is the maximum peak intensity of the same resonance(s) at the smaller gradient strength; \( D \) is the translational self-diffusion constant (in \( \text{cm}^2\text{s}^{-1} \)); \( \delta \) is the duration (in s) of the gradient; \( G \) is the strength of the gradient (in \( \text{T cm}^{-1} \)); \( \Delta \) is the time (in s) between two gradients (i.e., the time when the molecule evolves); and \( \gamma \) is the gyromagnetic ratio of a proton (2.675x10^4 \( \text{G}^{-1}\text{s}^{-1} \)).

The translational diffusion coefficient can be related to the molecular weight and the shape of the macromolecule. The Stokes-Einstein equation relates \( D \) to the molecular shape via the so-called friction coefficient, \( f \):

\[
D = \frac{kT}{f}
\]

where \( T \) is the temperature and \( k \) the Boltzmann constant. The friction coefficient of a protein is determined by its overall dimensions, hydration and surface roughness of the surface exposed to water. Assuming the protein adopts a spherical shape, the friction coefficient follows:

\[
f = 6\pi\eta R
\]

where \( R \) is the hydrodynamic radius of the sphere and \( \eta \) is the viscosity of the solvent. Combining equations (2) and (3), the hydrodynamic radius can be obtained as:

\[
R = \frac{kT}{6\pi\eta D}
\]

The viscosity of a macromolecular solution is very weakly influence by the macromolecule component at low macromolecular concentrations and, therefore, the viscosity of the solution should be that of the solvent. Solvent viscosity is temperature-dependent according to (32): 

\[
\log \eta = a + \left[ \frac{b}{T - c} \right]
\]

The terms \( a \), \( b \) and \( c \) for a 100 % \(^2\text{H}_2\text{O} \) solution are: \( a = -4.2911, \) \( b = -164.97 \) and \( c = 174.24 \), which yields a value of \( \eta = 1.095 \text{ kg/(cm s)} \) at 298 K.

In our experiments, a 5 mm triple-resonance inverse probe with z-gradients was used. The gradient strength was calibrated using the diffusion rate for the residual proton water line in a sample containing 100 % \(^2\text{H}_2\text{O} \) in a 5-mm tube, and back-calculating \( G \). This procedure assumes that the diffusion rate for HDO in a 100 % \(^2\text{H}_2\text{O} \) sample is 1.94 x 10^-5 \( \text{cm}^2\text{s}^{-1} \) at 298 K (32). Experiments were acquired by using the longitudinal eddy current delay (PGF-LED) pulse sequence. Each experiment was averaged over 128 scans and the number of points was 16 K. A post gradient eddy-current relaxation delay of 5 ms was used in all the experiments. The strength of the gradients was varied from 2 % of the total power of the gradient coil to 95 %, and their shape was a sine function. The duration of the gradient was varied between 3 ms and 2.2 ms, and the time between both gradients was changed between 100 and 150 ms. The resonances used to measure the changes of the intensity were those of the most up-field shifted methyl groups (from 0.8 to –1.5 ppm).

**Histidine pKa determination-** 1D proton NMR spectra of apoflavodoxin solutions at different pH values were recorded to determine the chemical shifts of the resonances corresponding to the two histidine residues in the protein. To allow for complete H-D exchange of amide protons, protein samples were first incubated at pH 11.5 for fifteen minutes in 90 % \(^2\text{D}_2\text{O} \). The required pH of each sample was then set by adding 50 \( \mu \)l of buffer in \(^2\text{D}_2\text{O} \). The buffers used were, at a final 50 mM concentration: pH 2.0-3.0, deuterated glycine; pH 3.0-5.5, deuterated acetic acid; pH 6.0-7.0, NaH\(_2\)PO\(_4\); pH 7.5-9.0, deuterated Tris acid; pH 9.5-12. The actual pH of the samples was measured with a thin Aldrich electrode in a Radiometer (Copenhagen) pH-meter before and after recording the spectrum. No corrections were done for the isotope effect.

The 1D spectra were acquired with 32 K data points, averaged over 2 K scans with 6000 Hz of spectral width (12 ppm), with water suppression achieved by WATERGATE (33). Baseline correction and zero-filling were applied for all pH values with the same parameters. Spectra were referenced to an external frequency. Histidine resonances were analyzed (34,35) assuming that the protonated and deprotonated species contributed to the observed chemical shifts according to:
\[ \delta = \delta_\nu + \delta_c \cdot 10^{(pH-pK_a)} \]  
(5)

where \( \delta \) is the chemical shift observed at a particular pH, \( \delta_\nu \) is the chemical shift being observed for the acidic species, \( \delta_c \) is the chemical shift observed at high pH values and pK\(_a\) is the apparent pK of the corresponding histidine. C\(_3\)H protons of the imidazole rings were followed. Detection of C\(_3\)H resonances in the 1D spectra was precluded by the many aromatic residues present in the protein.

**Urea-induced unfolding**—The chemical stability of the apoprotein has been characterized in a wide pH interval (between 1 to 12 every 0.5 pH units) using urea as denaturing agent. Samples were prepared by mixing 900-\(\mu\)l urea solutions with 100-\(\mu\)l aliquots of buffered apoflavodoxin in order to obtain protein samples of 2 \(\mu\)M (for fluorescence experiments) or 20 \(\mu\)M (for far- and near-UV dichroism experiments) at the desired pH and urea concentrations. The different buffers used were prepared so that the ionic strength was always 10 mM. The protein samples were equilibrated at 298 K for at least 30 min before recording the spectroscopic signal. Unfolding was followed throughout the entire pH interval, by emission fluorescence (ratio of 320/360 nm emission with excitation at 280 nm). A ratio of emission signals at two wavelengths was used to minimize protein concentration errors. In some cases, this can give rise to inaccuracies in the \(T_m\)'s. However, when the reference wavelength is chosen so that the signal does not change with the unfolding transition (as is the case of 360 nm for \(H.\) pylori apoflavodoxin), the ratio of intensities quantitatively follows the advance of the reaction (36). In addition, the unfolding equilibrium was also followed by CD at 222 nm (far-UV) and 291 nm (near-UV) at pH 2, 5, 7 and 9.

Unfolding data were analyzed as described (37,38), assuming a two-state equilibrium where the free energy of unfolding, \(\Delta G\), is considered to be a linear function of denaturant concentration, [D]:

\[ \Delta G = \Delta G_w - m[D] \]  
(6)

where \(\Delta G_w\) is the free energy of unfolding in buffered water and \(m\) is a proportionality constant. The spectroscopic signals of the folded and unfolded states (\(S_F\) and \(S_U\) at 0 M urea) are assumed to vary linearly with urea concentration, \(m_F\) and \(m_U\) being the corresponding slopes. Thus, the observed spectroscopic signal was fitted to the following equation:

\[ S = \frac{S_F + m_F[D] + (S_U + m_U[D])e^{-(\Delta G_w-m[D])/RT}}{1+e^{-(\Delta G_w-m[D])/RT}} \]  
(7)

where \(R\) is the gas constant and \(T\) the absolute temperature.

**Analysis of proton linkage**—Conformational stability values obtained by chemical denaturation at different pH values were fitted to equation 7, which links stability and pH:

\[ \Delta G = \Delta G_0 - \sum_i n_iRT \ln \left( \frac{1 + 10^{[pK_a^U_i-pH]} \times 10^{[pK_a^N_i-pH]}}{1 + 10^{[pK_a^U_i-pH]} \times 10^{[pK_a^N_i-pH]}} \right) \]  
(8)

where different \(i\) groups of ionizable residues have been considered. pK\(_a^U\) and pK\(_a^N\) are the mean pK\(_a\) values for a specific group of residues \(i\) in the unfolded and folded protein, respectively; \(n_i\) is the number of residues with a change in pK\(_a\) upon unfolding large enough to promote variations in the proton fraction between states and \(\Delta G_0\) is the hypothetical stability value when the protein is completely unprotonated. From the experimental curve shape (see Figure 4b), at least three transitions are expected, i.e. \(i=3\).

The number of excess bound protons in the native state relative to the unfolded one was estimated to be, as a function of pH:

\[ <\Delta N_i> = \sum_i \Delta N_i = \sum_i n_i \left( \chi_{U,i}^H - \chi_{N,i}^H \right) = \sum_i n_i \left[ \frac{10^{[pK_a^U_i-pH]}}{1 + 10^{[pK_a^U_i-pH]}} \times \frac{10^{[pK_a^N_i-pH]}}{1 + 10^{[pK_a^N_i-pH]}} \right] \]  
(9)

where \(N_i\) is the number of bound protons of each titratable group of residues (for \(H.\) pylori apoflavodoxin \(i=3\), as has been mentioned); \(n_i\) is the number of titratable residues in each group; \(\chi_{U,i}^H\) and \(\chi_{N,i}^H\) are the proton fraction of each residue bound to the unfolded and native state, respectively; and pK\(_a^{U,i}\) and pK\(_a^{N,i}\) the mean pK values for each group of residues.

**Guanidinium-induced unfolding**—The influence of FMN cofactor binding in protein stability was studied using guanidinium hydrochloride as denaturant. The unfolding of apoflavodoxin, holoflavodoxin and holoflavodoxin with 93 \(\mu\)M excess FMN in MOPS 50 mM, pH 7 was monitored. The unfolding of apoflavodoxin was observed by far-UV CD at 225 nm and fluorescence emission at 320 nm (exciting at 280 nm). For holoflavodoxin, the CD signals at 225, 291 and 373 nm as well as fluorescence emission at 350
nm (exciting at 280 nm) and at 525 nm (exciting at 453 nm), were used to follow the reaction. In the experiment with excess FMN, the unfolding was recorded by far-UV (225 nm), near-UV (291 nm) and visible CD (373 nm). In all cases, the protein concentration used was 20 μM. In CD measurements, the buffer ellipticity was subtracted from that of the samples. A global analysis of all the guanidinium-induced unfolding curves of apo and holoflavodoxin, with and without excess FMN, was performed according to a two-state unfolding model (equation 7) coupled with ligand binding to the native state for holoflavodoxin curves. Both the binding and folding equilibria are assumed to display free energy differences that are linearly dependent on denaturant concentration:

\[ K(D) = K_D^n \cdot e^{-\Delta G^D_{\beta}/RT} \]  

(10)

FMN is assumed to bind only to the native state, so that protein stability corresponds to the stability in the absence of ligand plus an additional term, \( \Delta G^b_{\beta} \):

\[ \Delta G^b_{\beta} = R T \ln(1 + [L]/K_D) \]  

(11)

where \( K_D \) is the dissociation constant of the protein-ligand complex that varies with denaturation agent concentration according to equation (10), and \([L]\) the concentration of free ligand, is given by the following equation:

\[ [L] = \frac{[L]_T}{1 + [N]/K_D} \]  

(12)

where \([L]_T\) is total ligand concentration and \([N]\) is native state concentration, which varies according to:

\[ [N] = \frac{\{K_D(1 + K_{NU}) + [L]_T - P_T\}}{2(1 + K_{NU})} + \frac{\sqrt{(K_D(1 + K_{NU}) + [L]_T - P_T)^2 + 4(1 + K_{NU})(-K_D P_T)}}{2(1 + K_{RU})} \]  

(13)

where \( P_T \) is the total protein concentration and \( K_{NU} \) is the protein unfolding equilibrium constant. Therefore, the partition function of the system (Q) and the population of each state (\( F_N, F_{NL}, F_U \), corresponding to apo, holo and unfolded flavodoxin, respectively) are given by:

\[ Q = 1 + [L]/K_D + K_{NU} \]  

(14)

\[ F_N = 1/Q; F_{NL} = [L]/(K_D \cdot Q); F_U = K_{NU} / Q \]  

(15)

Results

Spectroscopic characterization of apoflavodoxin-Fluorescence emission, far-UV and near-UV circular dichroism spectra of apoflavodoxin were recorded at pH 2, 5, 7, 9 and 12 under “native” (298 K and no denaturant) and denaturing conditions (Figure 1). The fluorescence emission spectra of apoflavodoxin (Figure 1a) at pH 5, 7 and 9 under native conditions are similar in emission maximum (323-325 nm) and quantum yield. At pH 2, however, the quantum yield is reduced and the emission maximum is slightly shifted to 329 nm. Heating to 363 K shifts the maximum of the samples to around 346 nm (except at pH 5, where the protein aggregates at high temperatures). At pH 12, under “native” conditions, the emission maximum is at 341 nm and shifts to 349 nm when heated to 363 K. Addition of 6 M urea, at any pH, shifts the maximum to 351 nm.

Circular dichroism spectra in the far-UV (Figure 1b) indicate that, under “native” conditions apoflavodoxin contains a substantial amount of secondary structure at all pH values tested, except at pH 12. The shape of the spectrum is, nevertheless, different at pH 2, which could be due to a combination of a reduced contribution of aromatic residues to the spectrum (4,39) and of a slightly lower helical content (75% of that at neutral pH). Both heating and addition of 6M urea strongly unfolds the protein at all pH values tested. Near-UV CD spectra are very similar at pH 5, 7 and 9. Compared to the spectrum of the four-tryptophan containing Anabaena apoflavodoxin (3), they show a small signal consisting of three distinct peaks at 265 nm, 287 nm and 295 nm. At pH 2 the signal is totally lost and the protein shows a flat near-UV CD spectrum.

The 1H NMR spectrum at pH 7 shows a well-dispersed amide region and several high-field methyl resonances (Figure 2), which is indicative of a well-defined tertiary structure, and the spectrum at pH 5.0 is very similar. At pH 12, the high-field resonances have disappeared and the dispersion of the aromatic and amide protons is reduced. At pH 2, the spectrum is considerably similar to that at pH 12, indicating the loss of the vast majority of the tertiary contacts, although some shoulders can be observed in the high field region, specifically at 0.4, 0.65 and 0.75 ppm; furthermore, the signals are broader than those observed at pH 12. Upon
addition of 6 M urea, the broad lines observed at pH 2 in the methyl region sharpen (Figure 2), and all the aromatic protons cluster between 7.0 and 7.5 ppm (not shown).

The affinity of ANS for proteins increases when hydrophobic clusters are exposed to solvent, as typical of molten globule intermediates (40). From pH 12 to 5.5, ANS fluorescence is not modified by apoflavodoxin (Figure 3). In contrast, there is a sharp increase from pH 5 to 2.5 when the apoprotein is present in the solution. The binding of ANS by apoflavodoxin at acidic pH suggests a greater exposure of hydrophobic area compared to the native state at neutral pH. The fact that the increase in ANS fluorescence follows a sigmoidal curve suggests there could be equilibrium between the native state and a “molten globule” state at acidic pH, with a transition midpoint at around pH 3.7. A similar but sharper transition has been observed in Anabaena apoflavodoxin (3). The difference may be related to either the higher number of acidic residues in Anabaena (31 versus 26 in H. pylori) and/or to the fact that, unlike the molten globule of H. pylori apoflavodoxin (see below) the Anabaena molten globule is aggregated.

Hydrodynamic characterization of apoflavodoxin—The translational diffusion coefficient, \( D \), at infinite dilution, of \( H. pylori \) apoflavodoxin at pH 7 is \( 1.06 (\pm 0.02) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1} \) at 298 K, from which an experimental hydrodynamic radius of \( 18.8 \pm 0.4 \text{ Å} \) can be calculated for a spherical apoflavodoxin molecule. Conversely, the \( D \) of the protein at pH 2 is \( 0.97 (\pm 0.01) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1} \) at the same temperature, which yields a slightly higher hydrodynamic radius of \( 20.6 \pm 0.2 \text{ Å} \). It is thus clear that \( H. pylori \) apoflavodoxin is also monomeric at pH 2.0 and exhibits a moderate increase in volume as is characteristic of partly unfolded conformations (41-43).

We can compare these values with those obtained for an ideal random coil of apoflavodoxin. The radius of gyration, \( R_g \), for a random coil conformation, with no disulfide bonds, can be estimated from (44):

\[
6^{1/2} \left( R_g^2 \right)^{1/2} = \left( 130n \right)^{1/2}
\]  

where \( n \) is the number of residues. The corresponding hydrodynamic radius, \( R \), is then (41):

\[
R = 0.655 \left( R_g^2 \right)^{1/2}
\]

In \( H. pylori \) apoflavodoxin yields a hydrodynamic radius of 39.0 Å, which is larger than that determined for the folded and partially folded protein. If, as it is usually assumed (41), a monolayer of hydration (2.8 Å) is included, the observed hydrodynamic radius should be 41.8 Å. It is also interesting to compare the obtained measurements of \( R \) for the fully folded protein with those predicted theoretically. The hydrodynamic radius for an ideal unsolvated spherical molecule can also be calculated considering that the anhydrous molecular volume, \((M\bar{V}/N)\), equals the volume of a sphere, which yields (44,45):

\[
R = \sqrt\left( \frac{3M\bar{V}}{4N\pi} \right)
\]

where \( M \) is the molecular weight of the protein, \( \bar{V} \) is the partial specific volume of the protein, and \( N \) is the Avogadro’s number. The molecular weight of \( H. pylori \) apoflavodoxin is 17495.3 Da, and \( \bar{V} = 0.704 \text{ cm}^3/\text{g} \), as calculated from amino acid composition (44). Thus, the hydrodynamic radius of a spherical non-hydrated conformation would be 17.0 Å, which agrees fairly well with that determined by diffusion measurements for the fully folded apoflavodoxin at pH 7. Usually, the values of hydration for proteins are in the range 0.2 to 0.6 (gram of water per gram of protein) (45). Under these conditions of hydration, the \( R \) of a solvated sphere is:

\[
R = \sqrt\left( \frac{3M\bar{V} + \delta V}{4N\pi} \right)
\]

where \( \delta \) is the hydration and \( V \) is the partial specific volume of water (1 cm\(^3\)/g). If it is assumed that the molecule of apoflavodoxin has a hydration of 0.2, the corresponding hydrodynamic radius is 18.4 Å, in good agreement with our experimental value of 18.8 ± 0.4 Å. A similar reasoning can be applied to the apoflavodoxin at pH 2, leading to the conclusion that the high experimental \( R \) can be only due to a high hydration (0.5 gram of water per gram of protein).

Urea-induced unfolding of apoflavodoxin—Apoflavodoxin can be unfolded by moderate urea concentrations and the conformational changes can be followed from the decrease in emission fluorescence as well as from the change in ellipticity in both the far- and near-UV. The spectroscopic unfolding curves of apoflavodoxin at pH 2, 5, 7 and 9 are shown in Figure 4a. All the curves are concentration independent in the 2-40 µM protein concentration range (not shown). At each pH, the spectroscopic curves are
superimposable and, accordingly, they were fitted to a two-state model (see Table 1). Only fluorescence and far-UV CD data were considered for these global fits because the near-UV CD curves are noisy due to the small intensity of the near-UV CD spectrum (see spectroscopic characterization section). The \( m \) value is very similar at \( \text{pH} \) 5, 7 and 9, which suggests that the same states are involved in the unfolding transition in this pH interval. In contrast, the \( m \) value at \( \text{pH} \) 2 is much lower, which is consistent with the fact that at this pH the folded conformation of the protein displays a greater exposure to solvent than at neutral pH. To better characterize the conformational stability of \( H. \text{pylori} \) apoflavodoxin as a function of \( \text{pH} \), stability curves followed by fluorescence were recorded from \( \text{pH} \) 1 to 12, one curve every 0.5 \( \text{pH} \) units. The \( m \) value and free energy change in buffer are represented as a function of \( \text{pH} \) in Figure 4b. The \( m \) value is remarkably constant from \( \text{pH} \) 5 to 12 and only a small increase, centered at \( \text{pH} \) 6.5, appears to take place towards the lower \( \text{pH} \) values. In the 5-12 \( \text{pH} \) interval, the stability is lowest in the alkaline region and sharply increases around \( \text{pH} \) 11 to reach a plateau from \( \text{pH} \) 10 to 8. Centered at \( \text{pH} \) 6.5, there is another sharp increase in stability leading to the maximal value exhibited at \( \text{pH} \) 5. Below \( \text{pH} \) 5, the stability decreases, which is accompanied by a concomitant pronounced decrease of the \( m \) value, likely representing the conversion of the native state into the monomeric molten globule (Figure 4b).

The thermodynamic parameters of reactions where proton uptake or release takes place are \( \text{pH} \)-dependent. In protein stability, such dependency provides information about the ionizable residues in the protein as well as about the change in protonation experienced by the residues involved when the protein unfolds (46-48). The apoflavodoxin stability data as a function of \( \text{pH} \) (Figure 4b) were fitted to equation 8. It should be noted that the equation only takes into account variations of the electrostatic contribution to the free energy change, which, if the native structure is not altered, is the most important contribution when \( \text{pH} \) is modified. Since apoflavodoxin appears to display at acidic \( \text{pH} \) a non-native conformation, the fit of the stability data from \( \text{pH} \) 2 to 5 should be considered an approximation. The fitting indicates that at least three sets of ionizable residues are responsible for the shape of the \( \text{pH} \) stability profile where three stability transitions are evident. In the first transition, at acidic \( \text{pH} \), the protein undergoes protonation of four ionizable groups during the unfolding, which destabilizes the native state. This acid-induced destabilization is characteristic in proteins because the \( K_a \) values of the acidic residues tend to be depressed in the native state with respect to the unfolded state (see for example Bartik et al. (49)). The two additional stability transitions, at neutral and basic \( \text{pH} \), correspond to unfolding-linked deprotonations in which two and three residues, respectively, are involved. These residues exhibit higher \( K_a \) values in the folded protein than in the unfolded one, allowing an easier deprotonation of the unfolded state, which is favored at basic \( \text{pH} \). This contributes to make proteins less stable at basic \( \text{pH} \). The transition at neutral \( \text{pH} \) involves 2 residues, with an average \( K_a \) of 5.8 in the unfolded state, close to the typical \( K_a \) of histidine residues in unfolded proteins (6.5) (34) and consistent with the fact that there are two histidines in \( H. \text{pylori} \) flavodoxin (His 102 and His 122). His 102 is partly buried in the native structure (44% solvent exposure) and establishes a cation/π interaction (35,50) with Phe 101 (11), which justifies the observed increase in the \( K_a \) (Table 2). On the other hand, His 122 is located in a very acidic environment, which can also raise the \( K_a \). As of the basic \( \text{pH} \) transition, there are at least 3 residues involved, with an average \( K_a \) in the unfolded state of 10.3, which points to either tyrosines or lysines. Apoflavodoxin contains 3 tyrosine and 14 lysine residues. Likely candidates are the buried Tyr121, Tyr104 and Lys79 (11%, 10% and 5% solvent exposures, respectively). Two of them, additionally, establish native interactions that may contribute to raise their \( K_a \) values: Tyr104 is hydrogen-bonded (2.86 Å) to Glu130 and Lys79 forms a cation/π interaction (3.6 Å) with Phe42.

It is sometimes assumed that the number of excess protons bound to the native or to the unfolded state equals the number of ionizable residues involved in the differential binding. However, this is only true when the \( K_a \) difference between the native and unfolded states is large, thus making possible that at certain \( \text{pH} \) value one state is completely protonated at a given site and the other state is fully unprotonated. In apoflavodoxin (insert of Figure 4b), such an extreme case is not achieved and the maximal proton excess is 2.9 at \( \text{pH} \) 3 (for a native conformation at this \( \text{pH} \)), 1.4 at \( \text{pH} \) 6.7 and 1.7 at \( \text{pH} \) 10.8.
Histidine pK\textsubscript{a} determination and tentative assignment-The pK\textsubscript{a} of the two histidine residues in \textit{H. pylori} flavodoxin (His 102 and His 122) have been determined by 1H NMR in both the apo and holoprotein (Figure 5). The pK\textsubscript{a} values of these residues (7.1 ± 0.1 and 7.3 ±0.1 in the apoprotein; and 7.1 ± 0.1 and 7.6 ± 0.1 in the holoprotein) are certainly higher than those expected for unfolded proteins (34) which indicates the charged forms are stabilized by favorable internal protein interactions. The pK\textsubscript{a} values agree with the averaged value of 7.6 ± 0.3 (Table 2) calculated for the two residues that give rise to the protein stabilization taking place below pH 7.0 (Figure 4b).

A preliminary assignment of the pK\textsubscript{a} values can be done from a comparison of the environment of the two histidine residues in the x-ray structures of \textit{H. pylori} apoflavodoxin (11) and holoflavodoxin (12). Both residues are slightly less exposed to solvent in the holo than in the apoprotein but the changes in exposure are similar for the two residues (not shown). However, while the environment of His 102 (located in the middle of helix α4 which exhibits high local stability (24)) hardly changes upon FMN binding, His 122 (located in the less stable flavodoxin long loop (24)) establishes in the holoprotein a new hydrogen bond with Asp 90 (2.7 Å). This bond, in fact a salt bridge, is expected to rise the pK\textsubscript{a} of the histidine in the holoprotein relative to the value exhibited in the apo form. We thus propose that the histidine residue exhibiting the same pK\textsubscript{a} value in the apo and holo forms (7.1±0.1) is His 102, while that changing from 7.3±0.1 in apoflavodoxin to 7.6 ±0.1 in holoflavodoxin is His 122.

Guanidinium-induced unfolding of apo and holoflavodoxin-\textit{H. pylori} holoflavodoxin is not fully denatured by urea after 24 hours of incubation (not shown). Indeed, a very slow urea unfolding has been reported for other flavodoxins (15,22), which has been attributed to a very slow limiting step consisting in the dissociation of FMN from the holoprotein. We have thus used guanidinium chloride to study the influence of FMN binding on \textit{H. pylori} flavodoxin stability. Although this ionic denaturant complicates, compared to urea, the physical interpretation of stability data at low denaturant concentrations, it is advantageous in this case not just because it is a stronger denaturant but also because it has been the denaturant chosen to study chemical unfolding in most flavodoxins (15,17,19).

Guanidinium chloride unfolding curves have been recorded for the apo form of the protein, for an equimolecular mixture of apoprotein and FMN (called here holoflavodoxin because at the working concentration of 20 μM about 99 % of the protein molecules are bound to the cofactor; see below) and for a mixture of apoprotein with 5.6 times FMN. Apoflavodoxin unfolding has been followed by far-UV CD and tryptophan fluorescence emission, while holoflavodoxin unfolding was followed by far-UV, near-UV and visible CD and by fluorescence emission at 350 nm and at 525 nm. In the experiment with excess FMN only CD (in the far-UV, near-UV and visible regions) could be used. The curves corresponding to apoflavodoxin unfolding are superimposable (Figure 6a). Apoflavodoxin thus seems, in principle, to follow a two-state model (equation 7) and the stability is calculated at 4.7 kcal/mol in MOPS 50 mM, pH 7.0. Similarly, the five curves corresponding to holoflavodoxin unfolding can be superimposed, which indicates that the unfolding of the complex is two-state, and the same is observed in the presence of excess FMN.

Binding of FMN to apoflavodoxin, modifies the far-UV CD spectrum (the signal at 225 nm increases 5 %, not shown), and strongly quenches tryptophan fluorescence emission (see inset in Figure 6). However, the ellipticity of the unfolded state at high guanidinium concentration is the same regardless of the presence of FMN (not shown) and the same applies to the fluorescence emission spectrum (inset in Figure 6). The spectroscopic evidence thus indicates that the chemical unfolding leads to a fully dissociated complex where the cofactor no longer interacts with the protein. To test this proposal, all the unfolding curves obtained for apoflavodoxin, holoflavodoxin, and holoflavodoxin with excess FMN were globally fitted to a two-state mechanism coupled to complex formation by specific ligand binding to the native state. The fitted curves, solid lines in Figure 6a, are in perfect agreement with the experimental data, and the global fit yields a stability in buffer of 4.7 ± 0.1 kcal mol\textsuperscript{-1} (with \(m = 3.9 ± 0.1\) kcal mol\textsuperscript{-1} M\textsuperscript{-1}) and a standard binding energy in buffer of -11.5 ± 0.2 kcal mol\textsuperscript{-1} (with a linear dependence of denaturant concentration of -1.6 ± 0.1 kcal mol\textsuperscript{-1} M\textsuperscript{-1}). From the fitted parameters, the population of each macroscopic state can be calculated as a function.
of denaturant concentration (equations 14, 15). As Figure 6b illustrates, an equimolar (20 μM) amount of FMN promotes the formation of 98.6% holoflavodoxin in the absence of denaturant and leads to a marked stabilization of the native state.

Discussion

Native and molten globule conformations of H. pylori apoflavodoxin-The apoflavodoxin native conformation of known crystal structure (11) is dominant between pH 5 and 10. In this pH interval, the spectroscopic properties of the apoprotein change little. The fluorescence spectrum is consistent with the presence of two buried tryptophan residues (W64 and W155). The far-UV CD spectrum is very similar to that of Anabaena apoflavodoxin and, as previously noticed for the latter (3), it contains contributions from aromatic residues. The near-UV CD spectrum confirms that there are aromatic residues in asymmetric environments. Although the intensity of the spectrum is much lower than that of Anabaena apoflavodoxin (which contains two additional tryptophan residues (3)), it is similar to that observed in Anabaena apoflavodoxin mutants where any of those additional tryptophans have been removed (51). Finally, the 1H-NMR spectrum is rich in up-field shifted signals (Figure 2), characteristic of stable contacts between aliphatic and aromatic side-chains, and the hydrodynamic volume is very close to that expected for an approximately spherical, hydrated protein of its size.

Below pH 5, most spectroscopic properties change significantly. In the far-UV, the CD spectrum is typical of an α/β protein, which indicates that the contribution of aromatic residues (4,39) observed at pH 7 has disappeared. Consistent with this fact, the near-UV CD spectrum is flat. Moreover, although the 1H-NMR spectrum (Figure 2) still presents some shoulders at 0.4, 0.65 and 0.75 ppm that disappear in the presence of 6 M and are absent at pH 12, the vast majority of the high field peaks observed at pH 7, are absent. The apoflavodoxin conformation at acidic pH seems thus to have lost most of the spectroscopic indications of having native contacts, but still preserves the secondary structure. Not surprisingly, the fluorescence spectrum indicates that the tryptophan residues are still buried. A conclusive proof of compaction is the value of the hydrodynamic radius, which is consistent with a monomeric conformation with a volume expansion of around 30% relative to the native conformation at pH 7. Such a volume increase, which has been observed in other partly unfolded proteins (43), exposes apolar surface to solvent allowing an efficient interaction with ANS (Figure 3). That the low pH conformation exposes more surface to solvent than the native one is also indicated by the smaller value of the m slope in equilibrium chemical denaturation (see below). H. pylori apoflavodoxin at acidic pH thus exhibits all the characteristics of a molten conformation (52). Molten globules often appear in proteins at low pH (53-56) but they have also been reported under physiological conditions (57) and have been proposed to play roles in protein folding kinetics (58,59), enzymatic inactivation (60,61), and membrane translocation (61,62). For Anabaena apoflavodoxin, a molten globule conformation has also been observed at low pH (3), but it aggregates and has not been characterized in detail. However, a truncated variant of Anabaena apoflavodoxin adopts a monomeric molten globule conformation at neutral pH (63) and a low resolution structural characterization by equilibrium Φ-analysis indicates its structure is quite homogenously destabilized (36). Neither these molten globules observed in Anabaena nor the one described here for H. pylori apoflavodoxin seem to resemble the equilibrium thermal intermediate observed in several flavodoxins (1), including H. pylori (24) and whose structure consists in a large native-like region and a smaller unfolded one (14). The monomeric molten globule of H. pylori apoflavodoxin at acidic pH behaves well also towards thermal unfolding and it is being further investigated in our laboratory.

Above pH 12, H. pylori apoflavodoxin has lost both the secondary structure and the tertiary interactions, as judged by the CD and 1H-NMR spectra. However, the fluorescence maximum, which is blue-shifted relative to that in fully denaturing conditions, indicates that the tryptophan residues are not completely exposed to solvent. When the protein is heated to 363 K, the helical content seems higher and the tryptophan exposure less complete than when the protein is dissolved in 6 M urea (see Figure 1), which could be related to the presence of a highly stable α-helix that would remain folded at 363 K (24,25).

Stability of apoflavodoxin-The conformational stability of native apoflavodoxin
markedly changes from 4.5 kcal.mol\(^{-1}\) at pH 9 to a maximal value of 9.2 kcal.mol\(^{-1}\) at pH 5 (Figure 4b), close to the theoretical isoelectric point of 4.5.

*H. pylori* apoflavodoxin contains a more balanced number of acidic and basic residues (26 and 17, respectively) than most known flavodoxins (i.e. *Anabaena* apoflavodoxin contains 31 and 15 residues, respectively) and, other things equal, the *H. pylori* protein would be expected, at pH values above the isoelectric point, to be more stable than its homologues. This is indeed the case when it is compared with *Anabaena* flavodoxin. Throughout the 6.0-9.5 pH interval (Figure 4b in this manuscript and Figure 2 in Maldonado et al. (64)), *H. pylori* apoflavodoxin is around 1.5 kcal.mol\(^{-1}\) more stable than the *Anabaena* protein. However, at pH 5.5, close to the isoelectric point, the stability gap shrinks to just 0.6 kcal.mol\(^{-1}\) because the *Anabaena* protein experiences a more intense relieve of electrostatic repulsions. Since the more balanced distribution of charges in *H. pylori*, that seems useful to increase stability at neutral pH, does not help to achieve a similarly large stabilization as the isoelectric point is approached, *H. pylori* apoflavodoxin makes use of a different strategy which is based on the presence of two histidine residues in protein environments that promote stabilization of their protonated forms. One is His 102 that, when protonated, can establish a cation/pi interaction with a neighbouring residue. Cation/pi interactions are known to raise histidine pK\(_a\) and contribute to protein stability (35,50). The second histidine residue, His122, is surrounded by acidic groups which can similarly raise the pK\(_a\). The contribution of these two histidine residues to the observed increase in protein stability from pH 9 to 5 (Figure 4b) can be quantitatively determined from the difference in pK\(_a\) values exhibited in the native and in the unfolded states (34). The pK\(_a\) of His 102 and 122 in the native protein have been determined from NMR titrations to be 7.1 and 7.3 respectively (Figure 5). A conservative estimation of their contribution to protein stability can be obtained assuming that their pK\(_a\) values in the denatured state are around 6.5, as determined for the unfolded state of a model protein (34). With this value, the combined stabilising effect of the two histidines in *H. pylori* apoflavodoxin would be around 2 kcal mol\(^{-1}\). However, the pK\(_a\) of protein residues can depart from standard values of the unfolded state depending on their specific microenvironments. In fact, according to the data in Table 2, the average pK\(_a\) of the two histidines in the denatured state may be as low as 5.8, which would set the contribution of these histidines to stability at 4 kcal mol\(^{-1}\). It is thus clear that at least half and perhaps the entire stabilization experienced by *H. pylori* apoflavodoxin as the pH is lowered to 5.0 arises from the appropriate location of these two histidine residues in protein regions that stabilise their charged rings. This seems a simple an efficient mechanism to increase protein stability at moderately low pH values and it could be used by other proteins.

In sharp contrast, the single histidine residue in *Anabaena* apoflavodoxin (His 34), is placed so that its charged form is destabilized and thus destabilises the protein when the pH is lowered. In the isoelectric point, the two proteins cannot be compared because while *H. pylori* apoflavodoxin remains soluble to at least 40 μM, the *Anabaena* protein becomes highly insoluble (3).

In spite of the large differences in stability observed across the 5 to 12 pH interval, the m slope (equation 6) hardly varies (2.19 kcal.mol\(^{-1}\).M\(^{-1}\) ± 0.03 on average), which is consistent with the dominance of a single native conformation from pH 12 to 5. However, from pH 5 to pH 1.5, a remarkable decrease in m value takes place. At the lower pH, the m value is as low as 1.3 kcal.mol\(^{-1}\).M\(^{-1}\). It seems thus that at very acidic pH values the protein exhibits a significantly less compact conformation, as expected for molten globules. It is also possible that the unfolded state at very low pH is more compact than at neutral pH, which would also contribute to lower the m value.

Although the chemical unfolding data are consistent with a two-state unfolding, it should be noticed that analysis of the thermal unfolding at pH 9.0 (24) indicates that the native state never reaches 100 % of the population at any temperature, the maximal accumulation (85%) taking place at 283 K. Actually, at 298 K, around 70 % of apoflavodoxin is in the native state, and 30 % populates a thermal intermediate. The fact that the fluorescence and far-UV CD urea-induced unfolding curves can be superimposed and can be fitted to a two-state model throughout the 5.5-12 pH interval deserves thus an explanation. To determine an approximation to the m value of the intermediate, a urea-induced unfolding curve at pH 9 followed by fluorescence was recorded at 320 K, which is the
temperature of its maximal accumulation (around 80 %) (24). The m parameter obtained at this temperature is of 1.9 ± 0.1 kcal.mol⁻¹.M⁻¹, thus very similar to the value obtained at 298 K (2.0 ± 0.1 kcal.mol⁻¹.M⁻¹). This adds to the facts that, at 298 K, the native and intermediate stabilities differ by only 0.5 kcal mol⁻¹ and that the intensities of their fluorescence emission spectra are very similar (24). It is therefore unsurprising that the unfolding curves at 298 K appear to be two-state. To estimate the uncertainty in the stability and m value of the chemical unfolding of the native conformation that arise from the presence of the intermediate we have simulated a fluorescence unfolding curve for a mixture of 70 % native state (with m = 2.0; ΔGᵢᵤ = 4.5 kcal/mol and fluorescence change of 1.0) and 30 % intermediate (with m = 1.9, ΔGᵢᵤ = 4.0 kcal/mol and fluorescence change of 0.85 (taken from a fluorescence spectra deconvolution, (24)). The fitting of this curve to a two-state model gives an apparent stability of 4.33 kcal/mol with and m value of 1.99, which are very close to the values of the native state. In addition, we have performed a global three-state fit of the fluorescence and far-UV CD curves at pH 9 (Figure 4a), assuming the fluorescence and far-UV CD signals of the intermediate are 0.85 and 0.83, relative to 1.0 for the native state (24) and that their m values differ by 0.1 kcal mol⁻¹M⁻¹. The global fit yields for the native state a stability of 4.4 kcal mol⁻¹ with an m value of 2.13, compared to 4.6 and 2.08, respectively, for the two-state fit. All these data indicate that the presence of an intermediate so close in stability, m value and fluorescence properties to the native state modifies only slightly the values recovered in the two-state fittings summarized in Figure 4b. Thus the stability data as a function of pH reported in Figure 4b are a good approximation to the actual values exhibited by the native conformation. The stability determined for H. pylori apoflavodoxin at pH 7 using guanidinium hydrochloride as denaturant is lower (4.7 kcal mol⁻¹ in a two-state fit and 4.2 considering the presence of the equilibrium thermal intermediate, not shown) than using urea (6.4 kcal mol⁻¹ and 6.1, respectively). For Anabaena apoflavodoxin, however, similar stability values were observed (15). The different behavior of the two proteins may be related to their different percentages of charged residues. In fact the stability response of Anabaena apoflavodoxin to ionic strength is complex and dependent of the specific cation used (64). Alternatively, the population of intermediate, than in urea denaturation experiments seems to remain essentially constant along the unfolding equilibrium (see above), could become larger in guanidinium unfolding thus significantly lowering the apparent m value and leading to lower apparent stability values in guanidinium experiments.

Stabilization by FMN binding to the native state—The presence of the FMN cofactor has been described to transform the two-state equilibrium guanidinium unfolding of Anabaena apoflavodoxin into three-state, and the arising intermediate has been characterized as exhibiting a low secondary structure content (15). In contrast, the two-state guanidinium unfolding of H. pylori apoflavodoxin is not altered by the cofactor and therefore, no equilibrium intermediate is observed. The stability of the protein is greatly increased by the cofactor. Compared to the apoprotein, the observed stability of a 20 μM equimolecular mixture of apoprotein and FMN is 2.4 kcal/mol higher, and a mixture of 20 μM apoflavodoxin and 113 μM FMN appears to be 5.5 kcal/mol more stable than the protein alone. To test whether the stability data here reported for both apo and holoflavodoxin in the presence of different amounts of free cofactor is consistent with a simple two-state mechanism where the FMN cofactor can only bind to the native state, we have performed a global fit of the data where the dissociation constant of the apoflavodoxin/FMN complex has been treated as an unknown (equations 7, 10-15). The fitted value of the constant is 3.7 ± 1 nM, which compares well with the experimentally determined value of 4.4 nM (24). The two premises of the model: that the equilibrium unfolding is two-state and that the FMN cofactor only binds to the native state (and not to the unfolded one) seems thus to hold. In this fitting, the binding free energy is treated as linearly-dependent on the guanidinium concentration and the value obtained for the slope is of −1.6 kcal.mol⁻¹.M⁻¹. That the affinity increases with the concentration of guanidinium hydrochloride salt may be related to the expected important contribution of the hydrophobic effect to the binding. In fact, the same trend has been described for A. vinelandii. apoflavodoxin (18), although the slope is smaller: −0.8 kcal.mol⁻¹.M⁻¹. The larger stabilizing effect of guanidinium hydrochloride on the H pylori complex could be due to the more negative electrostatic potential at the binding site of the FMN phosphate moiety in this protein. To further confirm that the affinity
of FMN for *H. pylori* apoflavodoxin increases with salt concentration, isothermal titration calorimetry of FMN binding (24) has been performed at different NaCl concentrations (not shown), and the slope of a linear regression of binding free energy versus [NaCl] was -1.3 ± 0.2 kcal.mol⁻¹.M⁻¹, in good agreement with the dependency of [GuHCl], obtained in the global fit of apo and holoflavodoxin guanidinium unfolding.

Once established that FMN only binds to the native state, the stabilization due to cofactor binding can be quantitatively predicted (equation 11) from the value of the dissociation constant. The incremental stability brought about by the cofactor in *H. pylori* flavodoxin is compared in Figure 7 to that exerted on the *A. vinelandii*, *Anabaena* and *D. desulfuricans* proteins. The stabilization of *H. pylori* flavodoxin is intermediate between those exerted on the two other long-chain flavodoxins (*Anabaena* and *Azotobacter*) and that on the short chain *Desulfovibrio* one. Figure 7 shows the stabilization expected for two different concentrations of apoprotein (5 and 20 μM), which is different, to emphasize that special care is needed to compare stability data of holoflavodoxin from different species. All available data on the stabilization exerted by the FMN cofactor on the different flavodoxins so far studied are compatible with a lack of interaction between the cofactor and the denatured state.

Functional flavodoxin at pH 5. *H. pylori* is the only living entity capable to survive in the human stomach, due to the presence of an H⁺-stimulated cytoplasmic urease (65,66). Some models of urease-dependent pH homeostasis have been proposed (67), and the value of the internal pH of the bacterium still remains controversial. However, the cytoplasmic pH seems to vary between 8 and 5 depending on the external pH and the concentration of urea in the medium (27,68). Significant variations in cytoplasmic pH are potentially challenging for protein function and organism facing those changes could benefit from a tailoring of protein solubility, stability or ligand binding capabilities so that function is not compromised when the pH changes. In this respect it is worth noting that while there is ample evidence indicating that a variety of flavodoxins studied are stable and soluble at neutral pH in both the apo and holo conformations (1), the same may not be true at pH 5. So far, only the flavodoxin from *Anabaena* has been studied at this and lower pH values (3).

Its apo form becomes essentially insoluble at the isoelectric point (3.9) and its solubility at pH 5 is already very low. Perhaps related with this, its molten globule conformation at lower pH is aggregated, unlike the monomeric molten globule of *H. pylori* here described. For *Anabaena* cells, a drop in cytoplasmic pH to 5.0 would pose a problem since newly synthesized apoflavodoxin molecules would be insoluble and thus formation of the functional complex with FMN would be difficult. Besides, the concentration of holoflavodoxin could begin to drop due to the non-covalent nature of the apoflavodoxin/FMN complex. In contrast, the apoflavodoxin from *H pylori* is soluble, and very stable, at pH 5, which allows newly synthesized apoflavodoxin molecules to readily fold into the native FMN binding-competent conformation and to remain soluble to accomplish cofactor binding, which further increases stability. On the other hand, our analysis shows that whenever a low pH regime is imposed on *H. pylori* cells previously growing at higher pH values, the existing flavodoxin molecules will remain folded and soluble and will retain the bound FMN cofactor thus remaining functional.

The higher stability of *H. pylori* apoflavodoxin in neutral and mildly acidic conditions, compared to that of the *Anabaena* protein, compensates the lower stabilizing effect of FMN binding in *H. pylori*, which is the lowest reported for long-chain flavodoxins. This lower affinity seems related to local weakening of the regions involved in cofactor binding (24) and to the presence of a natural pocket due to substitution of a conserved tryptophan residue by an alanine (12). Our preliminary analysis of more than 200 flavodoxin sequences available in UniProtKB (69) (not shown) indicates that this last structural feature only appears in *Helicobacter pylori*, *Helicobacter acynomichis* (both able to infect the stomach) and *Treponema pallidum*. However, *Helicobacter hepaticus*, not living in the stomach, display a tyrosine residue. The pocket related to the presence of the alanine residue could thus be required for functioning (either for modulation of redox potentials or for coupling with specific redox partners). Whatever the case, it seems that the higher stability of *H. pylori* apoflavodoxin counterbalances its lower affinity for FMN.

It would be interesting to know whether other *H. pylori* proteins have adapted to the acidic environment by displaying a higher stability below pH 7 than in neutral conditions.
Although the data available is very limited, two recent studies are consistent with it. The first one relates to an *H. pylori*-specific cysteine-rich protein, Hcps, that is maximally stable at pH 5 (70), and the second one, to a bacterial acyl carrier protein that in *H. pylori* is partially unfolded at neutral pH but it becomes fully folded at pH 6 (and more stable at pH 5) (71).

More protein stability data is needed to test the possibility that greater stability at acidic pH values is a common feature of *H. pylori* proteins relative to other microorganisms.
REFERENCES


**FOOTNOTES**

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**FIGURE LEGENDS**

**Fig. 1.** Spectroscopic characterization of apoflavodoxin. In sodium phosphate 23 mM, pH 2 (black); sodium acetate 16 mM, pH 5 (red); MOPS 30 mM, pH 7 (green); CHES 35 mM, pH 9 (blue); and sodium phosphate 2.5 mM, pH 12 (cyan); all buffers with an ionic strength of 10 mM. Three different conditions were used: native conditions, 298 K without denaturant (continuous lines) and denaturating conditions, samples either at 363 K (dot lines) or at 298 K with 6 M of urea (dash lines). Protein concentration was 2 μM for fluorescence (a), 20 μM for far-UV CD (b) and 40 μM for near-UV CD (c).

**Fig. 2.** Up-field 1D-NMR spectra of apoflavodoxin. From top to bottom, the spectra at pH 7, pH 5, pH 12, pH 2, and pH 2 in 6 M urea. The spectra were acquired in 100 % D2O at 298 K. The buffers used were: phosphate buffer (pH 7 and pH 12, 100 mM), deuterated acetic acid (pH 5, 50 mM) and deuterated glycine (pH 2, 100 mM).

**Fig. 3.** Binding of ANS to apoflavodoxin as a function of pH. Apoflavodoxin and ANS concentrations were 2 μM and 250 μM, respectively, and the ionic strength of all buffers was 10 mM. ANS emission fluorescence at 445 nm of 

**Fig. 4.** Urea-induced unfolding. (a) Urea unfolding curves of apoflavodoxin in 23 mM sodium phosphate, pH 2 (squares); 16 mM sodium acetate, pH 5 (circles); 30 mM MOPS, pH 7 (triangles); 35 mM CHES, pH 9 (stars). Solid symbols for fluorescence at 2 μM protein concentration; semi-solid symbols, near-UV CD at 40 μM protein concentration; and open symbols for far-UV CD at 20 μM protein concentration. For a better visual comparison, the data were normalized so that the signals are from roughly 0 to 1. The lines represent the fits to a two-state equation. (b) Conformational stability of apoflavodoxin as a function of pH. The experimental stabilities (closed circles) were obtained by fitting the urea-induced unfolding curves at a given pH to a two-state model. The line represents the fitting of all the stability data to equation 7. The fitting line is dashed at acidic pH to indicate that the protein adopts a conformation that differs from the known x-ray structure. The m parameter is shown as open circles. The inset shows the bounded proton excess in protein unfolding, calculated as a function of pH (equation 8).

**Fig. 5.** Flavodoxin histidines pKₐ determination. The pKₐ of the two histidine residues in 

**Fig. 6.** Guanidinium-induced unfolding of apo and holo flavodoxin. (a) Guanidinium chloride unfolding curves of apoflavodoxin (filled symbols), holoflavodoxin (empty symbols) and holoflavodoxin with 93 μM excess FMN (half-filled symbols) in MOPS 50 mM, pH 7. The protein concentration was in all cases 20 μM. UV-Fluorescence emission curves (excitation at 280 nm) at 320 nm for apoflavodoxin and at 350 nm for holoflavodoxin are represented as squares and visible fluorescence emission for holoflavodoxin (at 525 nm) as stars. The CD signals are represented as circles for far-UV CD (at 225 nm), as triangles for near-UV CD (at 291 nm) and as rhombus for visible CD (at 373 nm). The gray lines represent the global fit of all the curves to a two-state model coupled to ligand binding to the native state. The inset in Figure 5a shows the fluorescence spectrum of apoflavodoxin (continuous lines) and holoflavodoxin (dashed lines) at different guanidinium concentrations. (b) The relative populations of each state (apoflavodoxin in black, holoflavodoxin in red and unfolded state in green) during guanidinium-induced unfolding, as derived from the results of
the global fit: apoflavodoxin in absence of FMN (thick lines); apoflavodoxin plus an equimolar concentration of FMN (thin lines); apoflavodoxin in presence of 93 μM excess FMN (dash lines).

**Fig. 7.** Concentration-dependent FMN stabilization of several apoflavodoxins from different species. The stabilization by cofactor binding for the apoflavodoxins from different species (H. pylori: gray, Anabaena and A. vinelandii: black, and D. desulfuricans: light-gray) has been calculated for FMN concentrations between 0 and 500 μM and total protein concentrations of 5 μM (continuous line) and of 20 μM (dashed line).
Table 1. Stability of *H. pylori* apoflavodoxin from urea denaturation

<table>
<thead>
<tr>
<th>pH</th>
<th>D$_{1/2}$ (M)</th>
<th>m</th>
<th>ΔG$_0$ (kcal.mol$^{-1}$.M$^{-1}$)</th>
<th>D$_{1/2}$ (M)</th>
<th>m</th>
<th>ΔG$_0$ (kcal.mol$^{-1}$)</th>
<th>D$_{1/2}$ (M)</th>
<th>m</th>
<th>ΔG$_0$ (kcal.mol$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>0.84 ± 0.03</td>
<td>1.35 ± 0.04</td>
<td>1.13 ± 0.05</td>
<td>0.89 ± 0.05</td>
<td>1.28 ± 0.04</td>
<td>1.14 ± 0.07</td>
<td>0.85 ± 0.03</td>
<td>1.33 ± 0.04</td>
<td>1.13 ± 0.05</td>
</tr>
<tr>
<td>5</td>
<td>4.04 ± 0.01</td>
<td>2.32 ± 0.02</td>
<td>9.37 ± 0.08</td>
<td>4.1 ± 0.02</td>
<td>2.24 ± 0.04</td>
<td>9.2 ± 0.2</td>
<td>4.08 ± 0.01</td>
<td>2.18 ± 0.06</td>
<td>8.9 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>3.01 ± 0.02</td>
<td>2.18 ± 0.06</td>
<td>6.56 ± 0.2</td>
<td>2.94 ± 0.01</td>
<td>2.11 ± 0.08</td>
<td>6.2 ± 0.2</td>
<td>2.98 ± 0.01</td>
<td>2.15 ± 0.05</td>
<td>6.4 ± 0.1</td>
</tr>
<tr>
<td>9</td>
<td>2.21 ± 0.01</td>
<td>2.03 ± 0.07</td>
<td>4.5 ± 0.2</td>
<td>2.16 ± 0.01</td>
<td>2.19 ± 0.01</td>
<td>4.73 ± 0.03</td>
<td>2.19 ± 0.02</td>
<td>2.08 ± 0.09</td>
<td>4.6 ± 0.2</td>
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</table>

*a The units are: D$_{1/2}$ (M), m (kcal.mol$^{-1}$.M$^{-1}$), ΔG$_0$ (kcal.mol$^{-1}$). Each experiment was performed in triplicate. The errors reported are experimental errors.
b At 298.2 ± 0.3 K in 23 mM sodium phosphate, pH 2
c At 298.2 ± 0.2 K in 16 mM sodium acetate, pH 5
d At 298.2 ± 0.2 K in 30 mM MOPS, pH 7
e At 298.2 ± 0.2 K in 35 mM CHES, pH 9.
Table 2. Proton linkage in \textit{H. pylori} apoflavodoxin$^a$

<table>
<thead>
<tr>
<th></th>
<th>1$^{st}$ group$^b$</th>
<th>2$^{nd}$ group</th>
<th>3$^{rd}$ group$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>$3.8 \pm 0.7$</td>
<td>$1.8 \pm 0.3$</td>
<td>$3 \pm 3$</td>
</tr>
<tr>
<td>$pK_a^N$</td>
<td>$2.1 \pm 0.2$</td>
<td>$7.6 \pm 0.3$</td>
<td>$11.4 \pm 0.5$</td>
</tr>
<tr>
<td>$pK_a^U$</td>
<td>$3.9 \pm 0.2$</td>
<td>$5.8 \pm 0.4$</td>
<td>$10.3 \pm 0.5$</td>
</tr>
<tr>
<td>$\Delta pK_a$</td>
<td>$1.8$</td>
<td>$-1.8$</td>
<td>$-1.1$</td>
</tr>
</tbody>
</table>

$^a$ Parameters obtained from fitting the conformational stability data to the equation 7 (see Materials and Methods) at different pH values. $pK_a^U$ and $pK_a^N$ are the mean pK$_a$ values for a specific group of residues in the protein when the protein is unfolded and folded, respectively; $n_i$ is the number of exchanged protons for each group of residues $i$ and $\Delta G_0$ is the hypothetical stability value when the pH reaches an infinite value (protein is completely unprotonated).

$^b$ Due to the fact that at acidic pH there are conformational changes in the structure of the reference state ("native" state), the parameters obtained for the acidic transition have to be taken into account only as hypothetical ones.

$^c$ The larger errors for the third proton exchange process (at basic pH) are due to imprecision in the calculation of $\Delta G_0$: ($\Delta G_0 = 0 \pm 0.3$).

$^d$ $\Delta pK_a = pK_a^U - pK_a^N$. 

35
Figure 1

(a) Fluorescence signal (a.u.) vs. Wavelength (nm)

(b) M.R.E. (deg.cm$^2$.M$^{-1}$) vs. Wavelength (nm)

(c) M.R.E. (deg.cm$^2$.M$^{-1}$) vs. Wavelength (nm)
Figure 3

Fluorescence emission (495nm) vs pH
Figure 4

(a) Normalized signal (a.u.) vs [urea] (M)

(b) ΔG (kcal.mol⁻¹) vs pH

Inset: <ΔM> uptake vs pH
Figure 6

normalized signal (a.u.) vs. molar fraction of GuHCl (M)

(a) fluorescence intensity vs. wavelength (nm)
(b) molar fraction vs. [GuHCl] (M)
Figure 7

\[ \Delta G_{\Delta} \text{ (kcal/mol)} \]

\[ [\text{FMN}]_{T} (\mu \text{M}) \]
Conformational stability of helicobacter pylori flavodoxin. fit to function at pH 5
Nunilo Cremades, Marta Bueno, José Luis Neira, Adrián Velázquez-Campoy and Javier Sancho

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