Stabilization of apoflavodoxin by replacing hydrogen-bonded charged Asp or Glu residues by the neutral isosteric Asn or Gln

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Knowledge of protein stability principles provides a means to increase protein stability in a rational way. Here we explore the feasibility of stabilizing proteins by replacing solvent-exposed hydrogen-bonded charged Asp or Glu residues by the neutral isosteric Asn or Gln. The rationale behind this is a previous observation that, in some cases, neutral hydrogen bonds may be more stable that charged ones. We identified, in the apoflavodoxin from Anabaena PCC 7119, three surface-exposed aspartate or glutamate residues involved in hydrogen bonding with a single partner and we mutated them to asparagine or glutamine, respectively. The effect of the mutations on apoflavodoxin stability was measured by both urea and temperature denaturation. We observed that the three mutant proteins are more stable than wild-type (on average 0.43 kcal/mol from urea denaturation and 2.8°C from a two-state analysis of fluorescence thermal unfolding data). At high ionic strength, where potential electrostatic repulsions in the acidic apoflavodoxin should be masked, the three mutants are similarly more stable (on average 0.46 kcal/mol). To rule out further that the stabilization observed is due to removal of electrostatic repulsions in apoflavodoxin upon mutation, we analysed three control mutants and showed that, when the charged residue mutated to a neutral one is not hydrogen bonded, there is no general stabilizing effect. Replacing hydrogen-bonded charged Asp or Glu residues by Asn or Gln, respectively, could be a straightforward strategy to increase protein stability.

Keywords: charged residues/conformational stability/flavodoxin/hydrogen bond/thermostability

Introduction

There are many instances where increasing the conformational stability of a protein at room temperature or increasing its thermostability may be required. Different rational approaches have been exploited to that end that, in some cases, have proved useful (Nicholson et al., 1988; Matsumura et al., 1989; Shih and Kirsch, 1995; Zhang, et al., 1995; Villegas et al., 1996; Grimsley et al., 1999; Williams et al., 1999). As the knowledge on the interactions influencing protein stability increases, new strategies may be devised that, ideally, should be general and easy to implement.

We studied protein stability and folding principles using the apoflavodoxin from Anabaena PCC 7119 as a model protein (Maldonado et al., 1998b). The structure and energetics of the wild-type form have been described (Genzor et al., 1996a,b; Maldonado et al., 1998a). Its urea unfolding follows a two-state mechanism (Genzor et al., 1996b). We have recently focused on the role of hydrogen bonds in protein stability. Despite the fact that these interactions were very early identified as playing a most important role in shaping protein structure (Pauling et al., 1951), their contribution to protein stability has been and is still widely debated (Fersht, 1987; Honig and Yang, 1995; Lazaridis et al., 1995; Sippl et al., 1996; Myers and Pacé, 1996). Using apoflavodoxin, we quantified the strength of a side-chain/side-chain hydrogen bond involving a histidine residue (Fernández-Recio et al., 1999) and we found, unexpectedly, that the hydrogen bond is stronger when the histidine is neutral. If this were applicable to other hydrogen bonds, a simple strategy towards protein stabilization would involve engineering mutations to transform charged hydrogen bonds into neutral ones. This can be best done by replacing aspartate or glutamate residues by their corresponding neutral isosteric amides, that retain hydrogen-bonding capabilities. To test the feasibility of this strategy, we selected, in apoflavodoxin, three surface-exposed hydrogen bonds involving acidic residues and prepared the appropriate mutants. In all cases, the mutant proteins are more stable at both 25°C and higher temperatures. Additional mutational and buffer-condition control experiments ruled out that the stabilization observed is due to removal of electrostatic repulsions upon mutation.

Materials and methods

Choice of mutations

Three mutations were designed (E16Q, D96N and D100N) to replace aspartic and glutamic residues involved in surface exposed hydrogen bonds (E16–Y8, D96–N128 and D100–N97) by their isosteric neutral equivalents, asparagine or glutamine (Figure 1). Three additional control mutations were designed (E61A, D75A, D126A) where surface-exposed but not hydrogen-bonded aspartic or glutamic residues are replaced by alanine. For these control mutants, mutation to alanine was preferred because, although the charged mutated residues are not hydrogen bonded in the wild-type structure, a replacement by their corresponding amides could have resulted in new interactions occurring with previously unsuitable hydrogen bond acceptors in the neighbourhood, since the amides can act as donors.

Mutagenesis

All Anabaena PCC 7119 flavodoxin mutants were prepared by the method of Deng and Nickoloff (1992) directly on the expression plasmid pTrc 99a (Fillat et al., 1991). The wild-type protein was mutatcd to D126A and a wild-type variant carrying the mutation W120F (pseudo-wild-type) was mutated to D100N, E16Q, D96N, E61A and D75A. The pseudo-wild-type form (which is sometimes used in our laboratory because
it shows certain advantageous kinetic properties not related to this work) displays a structure and stability behaviour very similar to those of wild-type (not shown). The stability of each mutant apoflavodoxin was compared with that of its appropriate reference wild-type protein. Double-stranded DNA was isolated from the plasmid and the entire flavodoxin gene was sequenced to detect the mutations and to ensure that no additional mutations occurred elsewhere.

Protein expression and purification

Flavodoxin mutants were purified by an adaptation of the method described by Fillat et al. (1991). A 100 ml culture of *Escherichia coli* TG1 cells was grown overnight in Luria broth medium supplemented with ampicillin (50 mg/ml). Thirteen 1 l bottles containing 300 ml of the same medium with ampicillin were inoculated with 3 ml of culture and cells were grown at 37°C, pH 7, 150 r.p.m. until the optical density of the culture at 600 nm reached 1.2. At this point, isopropyl-β-thiogalactopyranoside was added at a 1 mM final concentration. Cultures were grown overnight at 150 r.p.m., 37°C. After cooling, cells were harvested by centrifugation in a JA-14 rotor from Beckman. The cell-paste (7–8 g) was washed into 0.15 M NaCl and frozen. The cell-paste was resuspended in 70 ml of 50 mM Tris–HCl, pH 8 (containing 1 mM EDTA,
Fig. 3. Chemical denaturation of wild-type apo flavodoxin at pH 7.0, 25.0 ± 0.1°C and an ionic strength of 0.18 M (100 mM sodium phosphate). Circles, guanidinium hydrochloride denaturation; squares, urea denaturation; open symbols, circular dichroism at 222 nm; closed symbols, fluorescence. (a) Raw data; (b) fraction folded.

Fig. 4. Urea unfolding curves of wild-type and mutant apo flavodoxins in 50 mM MOPS, pH 7.0, at 25.0 ± 0.1°C, followed by fluorescence emission at 320 nm (excitation at 280 nm). The raw data are represented. Solid lines are fits to the two-state Santoro–Bolen equation. (a) Pseudo wild-type and mutants involved in hydrogen bonds; (b) wild-type, pseudo-wild-type and mutants not involved in hydrogen bonds.

Table I. Hydrogen bonds mutated

<table>
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<tr>
<th>Residue</th>
<th>H-bond</th>
<th>Length (Å)</th>
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<td>E16-Y8 (COO^−...HO)</td>
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<td>D96-N128 (COO^−...H2N)</td>
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<td>D100N</td>
<td>D100-N97 (COO^−...H2N)</td>
<td>2.88</td>
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≥0.16 were pooled. All preparations were homogeneous by SDS–PAGE.

Apo flavodoxin preparation

The flavin mononucleotide group was removed from the holoprotein by treatment with trichloroacetic acid (Edmondson and Tollin, 1971).
the urea unfolding data were analysed assuming a two-state hydrochloride is used as denaturant (see Results). Accordingly, holds at higher ionic strength when either urea or guanidinium et al., 1996b). We show here that the two-state behaviour also.

Table II. Stability of wild-type and mutant apoflavodoxins at low ionic strength as measured by urea denaturationa

<table>
<thead>
<tr>
<th>Protein</th>
<th>m (kcal/mol·M)</th>
<th>ΔG (kcal/mol)</th>
<th>ΔGmut - ΔGmut b (kcal/mol)</th>
<th>ΔGmut - ΔGmut c (kcal/mol)</th>
<th>U1/2 (M)</th>
<th>ΔU1/2 d (M)</th>
<th>ΔΔG e (kcal/mol)</th>
<th>ΔΔGmut f (kcal/mol)</th>
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<td>–</td>
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<td>–</td>
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<td>4.64</td>
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<td>–</td>
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<tr>
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<td>–</td>
<td>1.91</td>
<td>–0.005</td>
<td>–0.01</td>
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<tr>
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<td>0.55</td>
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<td>–</td>
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</tr>
<tr>
<td>D126A</td>
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<tr>
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<td>2.00</td>
<td>0.055</td>
<td>0.12</td>
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</tr>
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</table>

a In 50 mM MOPS, pH 7.0, at 25.0 ± 0.1°C.

The concentrations of mutant and wild-type apoflavodoxins were determined from their absorbances at 280 nm using an extinction coefficient of 34.1 mM⁻¹ cm⁻¹ for the wild-type protein (Genzor et al., 1996b) and 27.7 mM⁻¹ cm⁻¹ for the W120F mutants (unpublished results).

Absorbance, fluorescence and circular dichroism (CD) measurements

Emission fluorescence and CD spectra were recorded in a Kontron SMF 25 fluorimeter and in a Jobin Yvon spectropolarimeter, respectively, at 25.0 ± 0.1°C in 50 mM MOPS, pH 7.0. The thermal and urea denaturation curves were recorded in the same instruments.

Equilibrium urea denaturation

Protein samples were prepared by mixing urea solutions (900 µl) of different concentrations with 100 µl aliquots of 20 µM apoprotein in 500 mM MOPS, pH 7.0. When stated, the urea solution contained choline chloride (0.5 M after mixing with the protein aliquot). The unfolding at 25.0 ± 0.1°C was followed, after equilibration for 45 min, by measuring the emission fluorescence at 320 nm (excitation at 280 nm). The urea unfolding of apoflavodoxin at pH 7.0 and low ionic strength (I = 0.019 M; 50 mM MOPS) is two-state (Genzor et al., 1996b). We show here that the two-state behaviour also holds at higher ionic strength when either urea or guanidinium hydrochloride is used as denaturant (see Results). Accordingly, the urea unfolding data were analysed assuming a two-state model (Pace et al., 1989). The free energy of unfolding, ΔG, is considered to be a linear function of urea concentration according to

\[ ΔG = ΔG_u - mD \] (1)

where ΔG_u is the free energy of unfolding in water, D is the molar concentration of urea and m is a proportionality constant. The spectroscopic signals of the folded (\( S_F \)) and unfolded states (\( S_U \)) are assumed to vary linearly with urea concentration (Santoro and Bolen, 1988), with slopes \( m_F \) and \( m_U \), respectively. Under these assumptions, the observed spectroscopic signal follows the equation

\[ S = \{ S_F + m_F D + (S_U + m_U D)\exp[-(ΔG_u - mD)/RT]\} \{ 1 + \exp[-(ΔG_u - mD)/RT]\} \] (2)

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

Since the far- and near-UV CD spectra of the proteins (Figure 2) and their fluorescence spectra (not shown), indicate that the proteins are very similar spectroscopically, great care was taken during the analysis that similar slopes for the urea dependency of the native and unfolded signals (\( m_F \) and \( m_U \)) were obtained. This contributes to reducing the well-known greater errors in m values (as compared with half urea values) that are so frequently found in urea unfolding data analysis (Pace, 1990; Serrano et al., 1992). This, in turn, increases the reliability of a direct comparison of ΔG_u values of different protein variants. Nevertheless, the difference in protein stability among protein variants is best calculated from the average m value of the different proteins together with the difference in half urea (provided the differences in \( m_U \) values among the protein variants are not large or systematic; see Serrano et al., 1992, for a discussion).

Thermal denaturation followed by fluorescence: two-state analysis

Thermal unfolding curves were acquired using fluorescence emission. To minimize the strong temperature dependence of fluorescence, the ratio of emission at two different wavelengths, 320 and 360 nm (excitation at 280 nm) was used. The buffer was 50 mM MOPS, pH 7.0 and the protein concentration was 2 µM.

Thermal denaturation data were fitted to the equation for a two-state equilibrium:

\[ S = \{ S_F + m_F T + (S_U + m_U T)\exp[-ΔG(T)/RT]\} \{ 1 + \exp[-ΔG(T)/RT]\} \] (3)
an accurate determination of
in the near-UV region (simultaneously measured at 291 nm in
monitored by circular dichroism and absorbance measurements
Thermal unfolding followed by four different spectroscopic
type, pseudo-wild-type and mutants not involved in hydrogen bonds.
are represented. Solid lines are fits to the two-state Santoro-Bolen equation.
(Pace et al., 1989) and they will not be reported.
Spectral characterization of mutant structural integrity
In the absence of X-ray structures, spectroscopic techniques
provide means to investigate whether mutations performed in
a protein have caused marked perturbations in the overall
structure. We report in Figure 2 the near- and far-UV CD spectra of pseudo-wild-type and the three mutant proteins
where charged surface-exposed residues have been mutated to
spectra of pseudo-wild-type and the three mutant proteins
spectra of the mutants cannot be distinguished from those of
of secondary structure and the tertiary interactions responsible
the pseudo-wild-type protein. This indicates that the percentage
the wild-type reference (not
comparable with the I state to the U state. From the relationship
where \( Z \) is a constant that describes the spectroscopic
resemblance of the I state to the U state. From the relationship
between fractional populations and equilibrium constants, \( F_{app} \)
can be calculated using Equation 7:
\[
F_{app} = \{ Z + \exp[-\Delta G_{IU}(T)/RT]\}/
\{ 1 + \exp[\Delta G_{NI}(T)/RT] + \exp[-\Delta G_{IU}(T)/RT]\}
\] (7)
where
\[
\Delta G_{NI}(T) = \Delta H_{NI}(T_{mNI})[1 - T/T_{mNI}] - \Delta C_p \Delta T_{mNI}
\] (8)
\[
\Delta G_{IU}(T) = \Delta H_{IU}(T_{mIU})[1 - T/T_{mIU}] - \Delta C_p \Delta T_{mIU}
\] (9)
In each fitting, all thermodynamic parameters (\( \Delta H, \Delta C_p \) and
\( T_m \)) were globally constrained, while the \( Z \) values were allowed
to vary among the different spectroscopic techniques.
Results
Spectral characterization of mutant structural integrity
In the absence of X-ray structures, spectroscopic techniques
provide means to investigate whether mutations performed in
a protein have caused marked perturbations in the overall
structure. We report in Figure 2 the near- and far-UV CD spectra of pseudo-wild-type and the three mutant proteins
where charged surface-exposed residues have been mutated to
the neutral corresponding amides. As the figure shows, the
spectra of the mutants cannot be distinguished from those of
the pseudo-wild-type protein. This indicates that the percentage
of secondary structure and the tertiary interactions responsible
for the near-UV CD spectra are the same in the four proteins.
The same applies to the three control mutants and their
corresponding wild-type proteins (spectra not shown). Additionally,
the emission fluorescence spectrum of each mutant protein is identical with that of the wild-type reference (not shown), indicating that the degree of tryptophan solvent exposure is the same.
Two-state unfolding of apoavodoxin

The two-state unfolding behaviour of the apoavodoxin from *Anabaena* in 50 mM MOPS, pH 7.0, 0.5 M choline chloride, at 25.0 ± 0.1°C has been reported (Genzor et al., 1996b) from the perfect superposition of the fluorescence and far-UV CD unfolding curves. We extended the analysis to higher ionic strengths and to guanidinium hydrochloride unfolding. Figure 3 shows the fluorescence and far-UV CD apoavodoxin unfolding curves in a pH 7 phosphate buffer of ionic strength 0.18 M. As shown by the data (raw data in upper panel and fraction folded in lower panel), the superposition of the curves is very good for both the urea and the guanidinium chloride experiments. The two-state behaviour of apoavodoxin in the low ionic strength MOPS buffer (Genzor et al., 1996b) is thus retained at this higher ionic strength. We assume in our analysis that the presence of choline chloride in some of the experiments does not change this behaviour.

Conformational stability at 25°C, at low ionic strength

The stability consequences of replacing negatively charged hydrogen-bonded residues by neutral isosteric residues were investigated by urea denaturation equilibrium unfolding. Three such charged residues were individually mutated to either asparagine or glutamine (Table I) in order to remove the charge and retain the hydrogen bonding capability. Each mutated residue is involved in just one hydrogen bond.

Three mutant apoavodoxins (E16Q, D96N and D100N) can be unfolded by moderate urea concentrations (Figure 4a) and the fluorescence unfolding curves can be fitted to two-state transitions using Equation 2. The slope of the observed transitions (*m* in Equation 1) is similar for all these proteins (with a spread of ±4%, Table II), which suggests that no major structural rearrangements occurred upon mutation. A direct comparison of the calculated Δ*G* values in water indicates the mutants are more stable than pseudo-wild-type by 0.32–0.52 kcal/mol. Comparison of the differences in the more precisely determined urea concentrations of mid-denaturation shows that the mutants display higher values than pseudo-wild-type by 0.14–0.26 M (Table II). This indicates that the mutations stabilize the protein from 0.29 to 0.54 kcal/mol. The average stabilizing effect is 0.43 kcal/mol.

Three additional control mutants were designed so that surface-exposed, but not protein hydrogen-bonded, aspartate or glutamate residues were replaced by alanines. The mutant stabilities were measured and analysed in the same way (Figure 4b) and the results are shown in Table II. Two of the three mutations (E61A and D126A) did not significantly stabilize or destabilize the protein, their stability being very close to that of the corresponding wild-type. This rules out the possibility that the folded structure of apoavodoxin can be generally stabilized by simply removing negatively charged groups. One of the mutants (D75A), nevertheless, did show a stabilizing effect, which prompted additional stability measurements at high ionic strength.

Conformational stability at 25°C, at high ionic strength

In order to minimize electrostatic effects associated with the removal of charges from the protein, the influence of the six mutations on the stability of apoavodoxin was also measured in the presence of 0.5 M choline chloride. The choline salt was used to avoid cation binding to the protein, which seems to take place when smaller cations are used (unpublished results).

The results at this higher ionic strength (Figure 5, Table III) mimic closely those obtained at low ionic strength, which indicates that the stabilizations observed at low ionic strength are not due to relief of electrostatic repulsions. For the three hydrogen-bonded mutants, the stabilizations observed range from 0.35 to 0.60 (directly calculated from the difference in the determined Δ*G* values in water) or from 0.29 to 0.57 kcal/mol (average 0.46 kcal/mol) when determined from the differences in the urea concentrations of mid-denaturation. As for the control mutants, two of them show hardly any stabilization (as at low ionic strength) and the one

### Table III. Stability of wild-type and mutant apoavodoxins at high ionic strength as measured by urea denaturation

<table>
<thead>
<tr>
<th>Protein</th>
<th><em>m</em> (kcal/mol·M)</th>
<th>Δ<em>G</em> (kcal/mol)</th>
<th>(Δ<em>G</em>mut − Δ<em>G</em>wt)b</th>
<th>(Δ<em>G</em>mut − Δ<em>G</em>wt)c</th>
<th><em>U</em>1/2 (M)</th>
<th>Δ<em>U</em>1/2d</th>
<th>Δ<em>G</em>mut (kcal/mol)</th>
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<td>4.43</td>
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<tr>
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<td>2.24</td>
<td>5.11</td>
<td>–</td>
<td>–</td>
<td>2.28</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.28</td>
<td>5.18</td>
<td>–</td>
<td>–</td>
<td>2.27</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>D126A</td>
<td>2.29</td>
<td>5.30</td>
<td>0.15</td>
<td>0.04</td>
<td>2.31</td>
<td>0.035</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>2.19</td>
<td>5.07</td>
<td>–0.08</td>
<td>0.04</td>
<td>2.32</td>
<td>0.045</td>
<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>

³In 50 mM MOPS, pH 7.0, 0.5 M choline chloride, at 25.0 ± 0.1°C.

²The average Δ*G* value for pWT (4.45 kcal/mol) or for WT (5.15 kcal/mol) is subtracted from the corresponding mutant Δ*G* values.

³Average of two values.

⁴The average *U*1/2 value for pWT (2.140 M) or for WT (2.275 M) is subtracted from the mutant *U*1/2 values.

⁵The mean *m* values for pWT and mutants thereof is *m* mutant = 2.08 kcal/mol·M and that of WT and D126A is *m* mutant = 2.25 kcal/mol·M. These mean values are used to calculate the difference in conformational stability in the standard buffer using the relationship Δ*G* = *m* mutant*/U*1/2.

⁶Average of two values.

⁷These proteins contain a W120F mutation.
Stabilization of apo flavodoxin by H-bond engineering

**Fig. 6.** Two-state analysis of the thermal denaturation of apo flavodoxin followed by fluorescence. All samples were in 50 mM MOPS, pH 7.0 and the protein concentration was 2 µM. (a) Pseudo-wild-type and mutants involved in hydrogen bonds; (b) wild-type, pseudo-wild-type and mutants not involved in hydrogen bonds.

**Table IV.** Thermal denaturation of apo flavodoxin followed by fluorescence: two-state analysisa

<table>
<thead>
<tr>
<th>Protein</th>
<th>$T_m$ (°C)</th>
<th>$\Delta H$ (kcal/mol)</th>
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</thead>
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<tr>
<td>pWTb</td>
<td>53.7 ± 1.0</td>
<td>47.5 ± 4.9</td>
</tr>
<tr>
<td>E16Qb</td>
<td>56.9 ± 0.4</td>
<td>61.1 ± 5.6</td>
</tr>
<tr>
<td>D96Nb</td>
<td>55.8 ± 0.1</td>
<td>60.5 ± 2.8</td>
</tr>
<tr>
<td>D100Nb</td>
<td>56.9 ± 0.5</td>
<td>47.9 ± 1.7</td>
</tr>
<tr>
<td>E61Ab</td>
<td>54.6 ± 1.7</td>
<td>45.6 ± 4.2</td>
</tr>
<tr>
<td>D75Ab</td>
<td>57.9 ± 0.3</td>
<td>60.8 ± 1.4</td>
</tr>
<tr>
<td>WT</td>
<td>50.7 ± 0.3</td>
<td>28.6 ± 6.0</td>
</tr>
<tr>
<td>D126A</td>
<td>50.2 ± 0.5</td>
<td>32.7 ± 2.6</td>
</tr>
</tbody>
</table>

a2.0 µM apo flavodoxin in 50 mM MOPS, pH 7.0. Errors are the standard deviations of two experiments. 
bThese proteins contain a W120F mutation.

**Fig. 7.** Three-state global analysis of the thermal denaturation of wild-type apo flavodoxin followed by fluorescence, near-UV circular dichroism, far-UV circular dichroism and absorbance measurements. Fluorescence conditions: 2 µM apo flavodoxin in 50 mM MOPS, pH 7.0; ratio of emission at 320 and 360 nm. Near-UV absorbance and near-UV circular dichroism conditions (curves obtained simultaneously): 40 µM apo flavodoxin in 50 mM MOPS, pH 7.0; signal recorded at 291 nm. Far-UV circular dichroism conditions: 1 µM apo flavodoxin in 5 mM MOPS, pH 7.0 with 15 mM NaCl; ellipticity at 222 nm. (a) Raw data represented in arbitrary units for comparison; (b) global fit to a three-state model. Data are shown as apparent unfolded fractions. The solid lines are the best fit, with all thermodynamic parameters being the same for the four curves.

that was more stable than wild-type at low ionic strength (0.52 kcal/mol) is similarly more stable at high ionic strength (0.46 kcal/mol).

**Fluorescence thermal unfolding with a two-state analysis**

The effect of the mutations on the thermostability of apo flavodoxin was first determined from thermal denaturation experiments using a ratio of fluorescence emission intensities at two different wavelengths to minimize non-structure-related
the four curves of each protein variant are globally fitted to the equations of thermal unfolding, keeping the real three-state thermal unfolding mechanism (M.P. Iruñ et al., 2001). As shown in Figure 7 (raw data in upper panel and Methods), as described in Luo et al. (2000), errors provided by the fitting program.

Three-state global analysis of fluorescence, far-UV CD, near-UV CD and absorbance thermal unfolding curves

We notice that the two-state fits of the fluorescence thermal unfolding curves (Figure 6) are an oversimplification of the real three-state thermal unfolding mechanism (M.P. Iruñ et al., 2001). As shown in Figure 7 (raw data in upper panel and apparent unfolded fraction after a three-state global analysis in lower panel), the fluorescence and near-UV CD curves display a temperature of mid-denaturation clearly lower than the absorbance and far-UV curves. This indicates that the two-state model does not apply to the thermal unfolding. We accordingly performed a global three-state analysis (see Methods), as described in Luo et al. (1995). In this analysis, the four curves of each protein variant are globally fitted to the equations of thermal unfolding, keeping ∆G, ∆Cp and Tm of the two equilibria (Nf/I and If/U) the same for the four curves. The results of such a fit for the wild-type protein are shown in Figure 7b and the calculated temperatures of mid-denaturation for the different protein variants are shown in Table V. The differences in Tm between the mutants with neutral hydrogen-bonding groups and the pseudo-wild-type protein are higher than those calculated from the simple two-state analysis of the fluorescence curves and are manifested as a stabilization of both the native state relative to the intermediate and of the intermediate relative to the unfolded state. Interestingly, the E61A and D126A control mutants display the same melting temperatures for the two equilibria as their corresponding reference proteins. Similarly, as already shown by the simple two-state fit, the D75A mutant is more stable than its reference. The results of the global three-state analysis thus reinforce the provisional conclusions derived from the simple two-state fits of the fluorescence thermal unfolding curves and agree well with the urea denaturation data.

Discussion

Apovlofavinoid stabilization by replacing hydrogen-bonded charged Asp or Glu residues by the neutral isosteric Asn or Gln

Inspired by the recent finding that neutral hydrogen bonds may be stronger than charged ones (Fernández-Recio et al., 1999), we wanted to test if proteins can be stabilized by neutralizing some of their charged hydrogen bonds. Although this could be studied in principle by measuring the stability of a given protein at different pH values, any change in pH will alter simultaneously many ionizing residues in almost every protein and interpretation of the results will be difficult. To avoid this problem, we devised a different strategy. Surface-exposed charged groups (aspartate and glutamate residues) that are hydrogen bonded to single partners in apovlofavinoid were mutated to their isosteric neutral amides (asparagine and glutamine), that retain hydrogen-bonding capability. The effect of the mutations on protein stability was then measured by urea and thermal denaturation. Three out of three engineered mutations consistently made apovlofavinoid more stable at both 25°C and higher temperatures, which is good evidence that the strategy is successful.

The stabilization is not due to removal of electrostatic repulsions and could be due to a higher strength of neutral hydrogen bonds relative to charged ones

When the stability of protein variants is compared, possible different secondary structure propensities of the residues involved should be considered. To avoid such complications, we selected mutations that are located either in loops (four cases) or in helices (two cases) where Asp and Asn, on the one hand, and Glu and Gln, on the other, have very similar propensities (see wwwbio.unizar.es/helixsc.html).

Different explanations can be offered for the observed stabilization upon replacing charged hydrogen-bonded residues by their neutral amides, but the following two are the simplest. As mentioned above, there is recent evidence that neutral hydrogen bonds may sometimes be stronger than charged ones (Fernández-Recio et al., 1999). In this respect, mutations such as those we have introduced in apovlofavinoid are potentially stabilizing, provided that the hydrogen bond is retained. On the other hand, apovlofavinoid is a very acidic protein that at neutral pH bears a high excess negative charge. It is thus conceivable that lowering the negative excess charge by replacing aspartates/glutamates by their amides could have a general stabilizing effect that would explain the observed results. To investigate this second possibility, we studied the stability effect of three additional point mutations where aspartate or glutamate residues, solvent-exposed but not hydrogen-bonded, were replaced by alanine residues. Our data show that two of these three control mutants display essentially the same stability (both by urea and thermal denaturation) as wild-type. Based on this, a general stabilization of the apovlofavinoid structure by mutations involving non-hydrogen-bonded acidic groups can be ruled out. There is, however, one control mutant where a clear stabilizing effect is observed. Further analysis
was performed to clarify if, in this mutant, the stabilization is indeed due to relief of electrostatic repulsions. If this were the case, the stabilizing effect would be significantly decreased at high ionic strength. We measured the stability of this mutant, relative to wild-type, in the same buffer but in the presence of 0.5 M choline chloride and the stabilization observed was very similar, which indicates that this mutation does not stabilize apo flavodoxin by removal of electrostatic repulsions but by some other, so far unknown, mechanism. In fact, 0.5 M choline chloride has an almost negligible effect on the stability of all the mutants analysed (relative to wild-type), including the three mutants involved in hydrogen bonding (Table III). The stabilizations we measure are therefore not due to removal of electrostatic repulsions. The simplest explanation that remains is that surface-exposed hydrogen bonds involving amides are stronger than those involving the corresponding carboxylic groups, in line with previous findings on a His/Tyr hydrogen bond (Fernández-Recio et al., 1999). This interpretation, however, relies on the assumption that the hydrogen bonds remain formed upon mutation, which is reasonable owing to the isosteric nature of the mutations, but confirmation will require further testing by X-ray analysis.

In practice, and whatever the cause, it seems that replacing hydrogen-bonded aspartate or glutamate residues at the surface of proteins by asparagine or glutamine, respectively, could be reasonable owing to the isosteric nature of the mutations, but explanations that remain is that surface-exposed hydrogen bonds involving amides are stronger than those involving the corresponding carboxylic groups, in line with previous findings on a His/Tyr hydrogen bond (Fernández-Recio et al., 1999). This interpretation, however, relies on the assumption that the hydrogen bonds remain formed upon mutation, which is reasonable owing to the isosteric nature of the mutations, but confirmation will require further testing by X-ray analysis.

In practice, and whatever the cause, it seems that replacing hydrogen-bonded aspartate or glutamate residues at the surface of proteins by asparagine or glutamine, respectively, could be a simple approach towards increasing protein stability at 25°C and also towards increasing protein thermostability.

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References