Effect of replacing a conserved proline residue on the function and stability of bovine adrenodoxin

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A proline residue in the C-terminal part of the polypeptide chain is highly conserved among many [2Fe-2S] ferredoxins. To investigate the requirement for proline at this position, we constructed steric (4–108W), charged (4–108K), polar (4–108S) and non-polar (4–108A) truncated mutants of adrenodoxin and studied them for biological function and stability. Although the variants were expressed in Escherichia coli with a significantly lower yield compared with wild-type adrenodoxin, successful incorporation of the iron–sulfur cluster suggested their proper folding. Similar absorption, CD and EPR spectra indicated that the cluster environment was not affected by the mutations. No evidence for an essential role of Pro108 in determining the redox potential of adrenodoxin or its interactions with the redox partners was found. However, replacement of this residue results in a dramatic decrease in the overall protein stability. The differences in the Gibbs energy of unfolding at 37°C, Δ[ΔG(37°C)], are –5.0, –7.8, –10.1 and –10.7 kJ/mol for 4–108A, 4–108S, 4–108W and 4–108K mutants, respectively, compared with 4–108P as a control. We conclude that the principle function of Pro108 is to stabilize adrenodoxin threefold: (i) through limitation of the conformation of the polypeptide chain in this region, (ii) through a hydrogen bond to Arg14 and (iii) favorable hydrophobic contacts.

Keywords: ferredoxin/iron–sulfur cluster/protein unfolding/site-directed mutagenesis/thermodynamic stability

Introduction

The [2Fe-2S] ferredoxins comprise a family of small iron–sulfur proteins that are widely distributed in bacteria, plants and animals and participate in a broad variety of electron transfer reactions. The bacterial [2Fe-2S] ferredoxins, e.g. putidaredoxin and terpredoxin, are components of the hydroxylation systems of camphor and α-terpineol, the carbon sources for these organisms. In plants, [2Fe-2S] ferredoxin transfer electrons from photosystem I to NADP⁺ via ferredoxin-reductase in the process of carbon assimilation. The hydroxylation systems of vertebrates utilize ferredoxins to carry electrons from ferredoxin-reductase to various cytochromes P450. Bovine adrenodoxin (Adx) functions as an electron shuttle from an NADPH-dependent Adx-reductase (AdR) to cytochrome P450csc (CYP11A1), which in bovine adrenals catalyzes the side-chain cleavage of cholesterol, the initial step of adrenal steroidogenesis and to cytochrome P45011β (CYP11B1), being involved in the formation of cortisol and aldosterone (Lambeth and Kamin, 1979).

Despite the rather low sequential homology among the ferredoxins of different types, there are some residues that appear to be highly conserved throughout evolution (Figure 1). Among them is the proline residue that was presumed to be important for the integrity of the iron–sulfur cluster and for correct folding of Adx (Uhlmann et al., 1994). Thus, the deletion mutant 4–107, which lacks Pro108, is not able to incorporate the cluster and could be expressed in the form of an apo-protein in protease-deficient Escherichia coli strains only. In contrast, the deletion mutant 4–108P, where proline is reserved, folds correctly, successfully assembles the iron–sulfur cluster and retains its function in the electron transport pathway. Moreover, this mutant demonstrated the increased stability that made it a favorable object for crystallization. However, no proper experimental explanation for this phenomenon has been demonstrated so far.

The occurrence of a proline residue in a protein sequence often has a strong influence on the protein folding and stability (Nemethy et al., 1966; Matthews et al., 1987; Yutani et al., 1991). Theoretically, this effect is attributed to the limitation of the backbone entropy of unfolding supported by a proline. Indeed, the replacement of prolines has been shown to induce a large drop in the stability of lysozyme and phage λ repressor (Reidhaar-Olson et al., 1990; Yutani et al., 1991; Herning et al., 1992). However, in some cases the decrease in the stability upon proline replacements was only marginal (Alber et al., 1998; Chen et al., 1992). Surprisingly, for the α-subunit of tryptophan synthase and staphylococcal nuclease, protein stabilization has been observed upon proline replacement (Green et al., 1992; Nakano et al., 1993; Ogasahara and Yutani, 1997). These results indicate that the role of proline in stability and folding of a protein depends on the location of each proline residue and needs detailed investigation for every protein class or the individual protein.

To examine how the conserved Pro108 residue may tune the protein conformation of adrenodoxin and the properties of the iron–sulfur cluster, we replaced it by a series of different amino acids and the biological and chemical properties of the mutated forms were investigated. The amino acid replacements studied here were divided into four main groups: (1) steric, (2) charged, (3) polar and (4) non-polar, which are represented by the variants 4–108W, 4–108K, 4–108S and 4–108A, respectively. The primary emphasis was concentrated on the thermodynamic consequences of the proline substitutions. The techniques that provide deep insight into protein structure, folding and conformational stability could not be applied for studies of iron–sulfur proteins for a long time, owing to the high chemical lability and irreversible chemical destruction of the cluster. Only recently was an approach developed in our laboratory that allows one to study conformational stability of Adx by means of differential scanning calorimetry (Burova et al., 1995, 1996). Independently, the GuHCl induced unfolding of Anabaena ferredoxin was investigated by Hurley et al. (1995) by means of the circular dichroism (CD) technique.
Our results will be discussed in the light of the very recently obtained high-resolution (1.85 Å) X-ray structure of the truncated form of Adx (4–108P) (Müller et al., 1998). We will show that the principle selective pressure on maintaining proline at position 108 is the stabilization of the protein and promotion of a specific fold of this region through both limitation of the conformational entropy and favorable contacts including hydrophobic interactions and a hydrogen bond connecting different secondary structure elements of the Adx polypeptide chain.

Materials and methods

Nomenclature

We used a truncated mutant 4–108 of Adx that is fully active in its electron transporting function (Uhlmann et al., 1994). It is named according to the residues of intact mature adrenodoxin (128 residues in total) with deletion of three N-terminal and 20 C-terminal amino acids. Amino acid substitutions in mutant proteins are named using the single letter amino acid code. For example, 4–108A indicates alanine in position 108.

Construction and purification of proteins

Mutants were constructed in a pKKHC plasmid bearing the cDNA that codes for residues 4–108 of Adx under control of the IPTG inducible trc promoter (Uhlmann et al., 1992). E.coli strains HB101 and BL21 were used as host strains. The 3' polymerase chain reaction mixed primer 5'-GGGAAGCTTA-XXXTACTCGAACAGTC3' with HindIII cloning site was chemically synthesized by BioTez GmbH. PCR, ligation, plasmid preparation, transformation and dideoxy sequencing of the mutant plasmids were carried out according to standard protocols (Sambrook et al., 1989). The optimum expression levels of the 4–108W, 4–108S and 4–108K mutants were achieved at 30°C. Wild-type Adx (WT), 4–108P and 4–108A mutants were expressed at 37°C. The mutant proteins were isolated essentially as described by Uhlmann et al. (1992), with the exception that hydrophobic interaction chromatography was abandoned in favor of an additional ion-exchange chromatographic step on a DEAE-Fractogel column. The concentration of recombinant Adx was calculated using ε₄₁₄ = 9.8 mM⁻¹ cm⁻¹ for bovine Adx (Kimura, 1968).

AdR, CYP11A1 and CYP11B1 were isolated from bovine adrenals as described previously (Akrem et al., 1979). The concentration of AdR was estimated using ε₄₅₀ = 11.3 mM⁻¹ cm⁻¹ (Hitawashi et al., 1976). The concentrations of CYP11A1 and CYP11B1 were determined as described by Omura and Sato (1966).

Spectral studies

The absorption spectra of the proteins were recorded at room temperature using a Shimadzu UV2100 spectrophotometer. Electron paramagnetic resonance (EPR) measurements were carried out under the following conditions: modulation frequency 100 kHz, microwave power 10 mW, modulation amplitude 10 G, time constant 0.3 s and temperature –163°C on a Bruker ESP300 spectrometer after reducing the samples with Na₂S₂O₄.

Circular dichroism spectroscopy

CD measurements were performed on a Jasco 700 spectropolarimeter equipped with a Jasco PTC348 temperature controller. Spectra were collected at 20°C between 185 and 650 nm at a step resolution of 0.5 nm with a bandwidth of 1 nm. All samples were solutions in 10 mM potassium phosphate buffer (pH 7.4). In the near-UV and visible range, the spectra were recorded at a protein concentration of 40 µM using a 0.5 cm pathlength. For the far-UV region, a pathlength of 0.1 cm was used and the protein concentration was 10 µM. Three scans were averaged to obtain each final spectrum. The recorded values were converted into molar ellipticity (visible and near-UV region) and mean residue ellipticity (far-UV region). Spectra were smoothed according to the Savitzky–Golay procedure.

Thermal denaturation

The thermal unfolding of the mutant proteins was monitored in a 1 cm hermetically closed cuvette by following the ellipticity at 440 nm over the temperature range 10–70°C with a temperature increment of 0.2°C at a heating rate of 50°C/h. Adx solutions for CD experiments were prepared chromatographically prior to use on a 0.5×10 cm Sephadex G-25 column using a carefully degassed buffer containing 40 mM glycine, 10 mM Na₂S, 1 mM ascorbate, 10 mM β-mercapto-
ethanol (pH 8.5). The protein concentrations used were 40 µM. The thermal denaturation was found to be 90% reversible under these conditions. The mathematical treatment of CD scans was made by a non-linear regression fit using the two-state model (Privalov, 1979) with SigmaPlot software.

Proteolytic digestion
Unspecific controlled limited proteolysis at elevated temperatures was carried out using thermolysin (EC 3.4.24.4) from Sigma. Wild-type and mutant proteins were incubated with thermolysin in a ratio of 100:1 and 250:1 (w/w) in the temperature range 30–60°C. The reaction was stopped by addition of an equal volume of gel-loading buffer containing 200 mM dithiothreitol and 2% SDS and boiling. The samples were analyzed by SDS–PAGE in a 15% gel.

Cytochrome c reduction
The specific activity of the recombinant proteins with adrenodoxin reductase was assayed at room temperature as described by Beckert et al. (1994).

Enzyme activity
The cholesterol side-chain cleavage activities of CYP11A1 were assayed at 37°C in the standard reconstituted assay system catalyzing the conversion of cholesterol to pregnenolone according to Beckert and Bernhardt (1997).

Optical titration
The differential spectral titration experiments with CYP11A1 were performed at room temperature as described by Kido and Kimura (1979) with modifications of Beckert et al. (1994).

Redox potential
The redox potentials of wild-type adrenodoxin and the mutant proteins were measured by the dye photoreduction method with safranin T as indicator and mediator according to Sligar and Gunsalus (1976). The data were analyzed using the Nernst equation.

Results
Expression and purification of the mutant proteins
The mutant proteins were constructed using a truncated Adx mutant 4–108 [minimal functional active unit of Adx (Uhlmann et al., 1994)] as a template. The desired mutations were introduced by means of PCR and the sequences of the mutant plasmids were confirmed by the dideoxynucleotide sequencing method. The mutants, carrying Trp, Ser or Lys in position 108, were shown to be unstable and for this reason were expressed at 30°C. Under these conditions the level of expression on a protein basis was comparable to that of mutant 4–108P. Nevertheless, the position 108 variants were produced as a mixture of holoh and apo forms. Western blot analysis revealed bands with a molecular mass of about 12.0 kDa for all mutant forms and the parent 4–108P variant. The final purification index \( A_{414}/A_{276} \) was \( \approx 0.9 \) for all variants, except 4–108W, for which it was 0.62 owing to the additional absorbance of Trp at 276 nm.

Properties of the iron–sulfur cluster
To measure the effect of the Pro108 substitution on the structure of the [2Fe–2S] cluster, UV–visible absorption and EPR spectra of the mutant proteins were recorded. The absorption spectra of oxidized WT adrenodoxin, the 4–108P form and the 4–108X mutants, with the exception of the 4–108W that has an enhanced maximum at 276 nm, were indistinguishable, exhibiting the characteristic peaks of Adx at 320, 414 and 455 nm (data not shown). This indicates that the incorporation of the iron–sulfur cluster was not disturbed by the proline replacement. Specific features of the reduced cluster can be sensitively reflected by EPR spectroscopy. A typical EPR spectrum of reduced Adx is characterized by two \( g \) values, \( g_\perp = g_\parallel = 1.94 \) and \( g_\perp = 2.03 \). The EPR signals of the mutants were not significantly changed, although the position of the low-field signal is slightly shifted and the high-field signal is somewhat broader compared with the wild-type (data not shown). For a further analysis of the structural differences between WT and mutants, CD spectroscopy was applied. The position 108 variants exhibited similar spectra, indicating that there is no significant difference in their conformations. Minor changes include increased ellipticities of the signal at 440 nm, representing some structural rearrangements of the polypeptide chain in the surrounding of the iron–sulfur cluster (Figure 2A).

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The signals in the 250–350 nm region have slightly different amplitudes, pointing to some conformational changes around aromatic chromophores (Figure 2B). In the far-UV region all the mutants shown, and also the wild-type protein, present mainly β-sheet and random coil conformations (Figure 2C). However, the 4–108P mutant and all the proline-substituted mutants display pronounced positive ellipticity signals at 195 nm. The differential spectrum of the WT and the 4–108P deletion mutant demonstrated the spectrum typically attributed to a random coil structure, suggesting that the deleted three N-terminal and 20 C-terminal amino acid residues exist in a random coil conformation (A.V. Grinberg and R. Bernhardt, unpublished results).

Redox potential
The redox potential of an electron transport protein is an important parameter for its specific function, i.e. its ability to accept and donate electrons. Redox potentials of the mutants were determined using the dye safranin T for reduction of Adx, which is suitable as its midpoint redox potential (~289 mV) is close to that of Adx. The measured redox potential of the wild-type protein (~275 mV, see Table I) resembles the value for the native Adx isolated from bovine adrenals (~273 mV). Although the difference in redox potentials between WT Adx and the 4–108P mutant is dramatic (~70 mV), the replacement of Pro108 by other amino acids, except lysine, does not influence the redox potential in general. The mutant 4–108K displays a redox potential of ~368 mV, which is 25 mV lower than that of the 4–108P form.

Effect of the mutations on interactions with redox partners
In order to characterize interaction of the Adx mutants with AdR, the kinetics of cytochrome c reduction were measured. Although this reaction does not occur physiologically, it is widely used to characterize the electron transfer from reduced AdR to Adx, since flavin to iron transfer has been shown to be the rate-limiting step of this reaction (Hanukoglu and Jefcoate, 1980). In this assay all the mutants studied exhibited only slightly increased \( K_m \) and \( V_{max} \) values compared with those of WT Adx (Table II), suggesting the interactions with AdR to be only insignificantly changed.

The effect of Pro108 replacement on the Adx interaction with the native electron acceptor CYP11A1 was investigated by analyzing the products of the respective hydroxylation reaction, in which cholesterol is converted into pregnenolone (Table II). The most pronounced effects on \( K_m \) are demonstrated
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Fig. 2. CD spectra of wild-type and mutant adrenodoxins. CD spectra in visible (A), near-UV (B) and far-UV (C) ranges for WT (---), 4–108P (----), 4–108A (- - -), 4–108S (· · ·) and 4–108K (· ··) are shown. The data are reported as mean residue ellipticity (far-UV) and molar ellipticity (visible and near-UV region).

Table I. Redox potentials of wild-type and mutant adrenodoxins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Redox potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>-275</td>
</tr>
<tr>
<td>4–108P</td>
<td>-343</td>
</tr>
<tr>
<td>4–108A</td>
<td>-337</td>
</tr>
<tr>
<td>4–108S</td>
<td>-342</td>
</tr>
<tr>
<td>4–108W</td>
<td>-334</td>
</tr>
<tr>
<td>4–108K</td>
<td>-368</td>
</tr>
</tbody>
</table>

*Standard deviation for the measurements was ±5 mV.

Fig. 3. Thermal unfolding curves of wild-type Adx and mutant proteins, monitored by CD spectroscopy. Changes in molar ellipticity at 440 nm are shown as a function of temperature. WT (●), 4–108P (○), 4–108A (△), 4–108S (●), 4–108W (▲).

for the 4–108W and 4–108K mutants, where the steric bulk or positive charge is implicated. As demonstrated in the unfolding experiments, at physiological temperature a large fraction of these mutants is represented in the unfolded form. It is reasonable to suggest that only the folded fraction of the protein is active in the interactions with CYP11A1. We tried to evaluate these parameters more accurately, normalizing the activity to the concentration of the folded form in the sample. These apparent kinetic constants are also given in Table II. Although such an estimation is rather rough owing to the differences in sample buffers, nevertheless, it can be deduced that the Pro108 residue is not crucial for the CYP11A1–Adx interactions. The same tendency was demonstrated in the spectral binding assay, where the affinities of the mutants to CYP11A1 were changed in the same manner (Table II).

Thermal unfolding of Adx mutants

Recently, the thermal unfolding of Adx was successfully studied using high-sensitivity differential calorimetry and a special buffer system with sodium sulfide and β-mercaptoethanol that prevents the destruction of the [2Fe-2S] cluster during heating was elaborated (Burova et al., 1995, 1996). Here we applied the same buffer system to study the stability of the Adx mutants by the CD technique, monitoring the unfolding of the mutants by ellipticity changes at 440 nm (Figure 3). The thermal transitions were analyzed assuming the two-state model, that represents the perfect mathematical solution for all mutants studied here. Values of the unfolding temperature, $T_d$, and the unfolding enthalpy, $\Delta_d H(T_d)$, derived from this analysis are listed in Table III. We carefully checked the reversibility of the unfolding of WT Adx and all the mutants. Hence the thermally unfolded Adx was able to regain the original absorption spectrum and 85–90% ellipticity of the signal at 440 nm after 12 h, when kept at 4°C in the sulfide-containing buffer. Moreover, the repeated scan of the once denatured protein had the same profile as the original one and gave the same transition temperature. The plot of thermal unfolding of WT Adx shows $T_d = 48.9^\circ$C. The $\Delta_d H(T_d)$ value for this transition is 332 kJ/mol (Table III). The deletion mutant 4–108P has an enhanced transition temperature of 51.7°C, while $\Delta_d H(T_d)$ is not significantly changed. Upon proline replacement, dramatic changes in the overall protein stability were induced, as judged by essentially decreased transition temperatures and enthalpies. Thus, the $T_d$ of the mutants 4–108A, 4–108S, 4–108K, 4–108W range from 46 to 38°C. The van’t Hoff enthalpies at
The role of Pro108 in bovine ferredoxin

Table II. Kinetic constants for some catalytic reactions of wild-type and mutant adrenodoxins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cytochrome c reduction</th>
<th>Cholesterol side-chain cleavage</th>
<th>Spectral binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (nM) $V_{max}$ b</td>
<td>$K_m$ (µM) $K_{app}$ c (µM) $V_{max}$ d</td>
<td>$K_s$ (µM)</td>
</tr>
<tr>
<td>WT</td>
<td>6.8 ± 0.1 29.8 ± 0.6</td>
<td>1.22 ± 0.05 1.20</td>
<td>6.45 ± 0.08 1.27 ± 0.03</td>
</tr>
<tr>
<td>4–108P</td>
<td>8.5 ± 0.1 47.6 ± 0.7</td>
<td>0.58 ± 0.06 0.57</td>
<td>6.33 ± 0.13 0.67 ± 0.03</td>
</tr>
<tr>
<td>4–108A</td>
<td>8.4 ± 0.1 45.0 ± 0.8</td>
<td>1.05 ± 0.06 0.99</td>
<td>6.65 ± 0.20 1.01 ± 0.04</td>
</tr>
<tr>
<td>4–108S</td>
<td>8.7 ± 0.2 48.2 ± 1.0</td>
<td>1.50 ± 0.07 1.22</td>
<td>6.50 ± 0.24 0.60 ± 0.05</td>
</tr>
<tr>
<td>4–108W</td>
<td>8.9 ± 0.2 39.2 ± 0.8</td>
<td>4.16 ± 0.11 2.74</td>
<td>4.20 ± 0.21 2.58 ± 0.04</td>
</tr>
<tr>
<td>4–108K</td>
<td>8.7 ± 0.2 41.0 ± 1.1</td>
<td>4.06 ± 0.10 2.16</td>
<td>5.13 ± 0.20 2.17 ± 0.06</td>
</tr>
</tbody>
</table>

*aInteraction with AdR was assayed by monitoring the reduction of cytochrome c at 550 nm. Catalytic activities of adrenodoxin mutants in CYP11A1-dependent substrate conversion were studied by analyzing the products of respective hydroxylation (pregnenolone). Binding of oxidized adrenodoxin to oxidized CYP11A1 was followed spectrophotometrically by the high-spin shift of the P450 heme iron in the Soret region (393–417 nm), caused by Adx-induced cholesterol binding.

*bExpressed by nmol cytochrome c reduced/min.

*cThe apparent constant calculated considering the fraction of the protein in the folded conformation at 37°C (Table III).

*dExpressed by nmol pregnenolone produced/min/nmol CYP11A1.

Table III. Thermodynamic parameters of unfolding and the temperature range of intensive proteolytic degradation for wild-type and mutant adrenodoxins

<table>
<thead>
<tr>
<th>Protein</th>
<th>$T_d$ (°C)</th>
<th>$\Delta H(T_d)$ (kJ/mol)</th>
<th>$\Delta T_d$ (°C)</th>
<th>$\Delta G_d$ $\Delta H(T_d)$ (kJ/mol)</th>
<th>$\Delta^*$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>48.9 ± 0.6</td>
<td>332 ± 15</td>
<td>0</td>
<td>0</td>
<td>47–51</td>
</tr>
<tr>
<td>4–108P</td>
<td>51.7 ± 0.4</td>
<td>336 ± 9</td>
<td>+2.8</td>
<td>+4</td>
<td>51–55</td>
</tr>
<tr>
<td>4–108A</td>
<td>46.3 ± 0.6</td>
<td>280 ± 11</td>
<td>−2.6</td>
<td>−52</td>
<td>42–47</td>
</tr>
<tr>
<td>4–108S</td>
<td>42.4 ± 0.3</td>
<td>256 ± 8</td>
<td>−6.5</td>
<td>−76</td>
<td>42–47</td>
</tr>
<tr>
<td>4–108W</td>
<td>39.6 ± 0.5</td>
<td>209 ± 11</td>
<td>−9.3</td>
<td>−123</td>
<td>37–42</td>
</tr>
<tr>
<td>4–108K</td>
<td>38.6 ± 0.6</td>
<td>214 ± 10</td>
<td>−10.3</td>
<td>−118</td>
<td>37–42</td>
</tr>
</tbody>
</table>

$T_d$ is the midpoint of the thermal unfolding transition. $\Delta H(T_d)$ is the denaturation enthalpy of the transition determined at $T_d$. $\Delta T_d$ and $\Delta G_d$ are the difference in transition temperatures and in unfolding enthalpies of a mutant and wild-type protein measured at the transition temperatures. Errors are estimated from the standard deviation of multiple measurements. $\Delta^*$ is the temperature range of intensive proteolytic degradation by thermolysin.

The unfolding temperatures of these mutants are reduced by 50–120 kJ/mol relative to the 4–108P value.

The temperature dependence of the enthalpy change of unfolding for the WT and the mutant proteins gives a straight line with a correlation coefficient of 0.97 (Figure 4) according to the Kirchhoff law (Privalov, 1979). Extrapolations of the enthalpies of unfolding of the mutants to 37°C using the experimental heat capacity increment, $\Delta C_p$, (Burova et al., 1995, 1996) and likewise using the temperature dependence give statistically indistinguishable results. Therefore, we can accept the slope of the $\Delta H(T_d)$ vs $T_d$ correlation plot as an averaged estimation of the heat capacity increment of unfolding for all mutant proteins, which is $10.35 ± 1.1$ kJ/mol/K.

The stability differences between the mutant proteins expressed in terms of an increment of the Gibbs energy of unfolding, $\Delta \Delta G(37°C)$, at the physiological temperature are given in Table IV. As shown before (Table III), the deletion of 24 amino acids (mutant 4–108P) induces an increase in the Gibbs energy of unfolding by 2.81 kJ/mol compared with WT Adx, while the enthalpy of unfolding is not changed significantly. This means that the decrease in the chain-size entropy of unfolding is primarily responsible for stabilization of this protein. By contrast, a dramatic drop in the Gibbs energy is observed for the position 108 mutants.
Relationship of conformational stability and susceptibility to proteolysis

The $T_d$ of a protein can also be approximated from the temperature range, $\Delta T^a$, at which it becomes sensitive to digestion by the unspecific protease thermolysin. The data from such experiments for the position 108 variants and WT Adx are summarized in Table III. There is a good correlation between the midpoint of this range and the $T_d$ obtained from CD experiments (correlation coefficient 0.98). Based on our stability data, we calculated the degree of unfolding, $\alpha$, at 37°C for the mutant proteins (Table IV). For the mutants 4–108K and 4–108W the degrees of unfolding are 0.399 and 0.347, respectively. This suggests that at physiological temperatures essential fractions of the thermolabile mutants are in the unfolded state. Therefore, the probability of their degradation by intracellular proteases increases.

Discussion

The unique Pro108 residue in bovine ferredoxin was once suggested to be an important prerequisite for the correct protein folding and cluster assembly (Uhlmann et al., 1994). The C-terminal part of the polypeptide chain following this residue is most variable in the structures of ferredoxins from different sources and was shown to rotate freely in solution (Miura and Ichikawa, 1991). This part is believed to have a modest significance in maintaining the folded conformation of Adx. However, the proline is highly conserved among all vertebrates, many bacterial, yeast and even some plant ferredoxins (Figure 1). Here we analyzed the substitution mutants, in which Pro108 is replaced by Ala, Ser, Lys and Trp, in order to study the role of this residue in Adx structure, function and stabilization mechanism. In contrast to the deletion mutant 4–107 that lacks the proline residue in position 108 and is not able to incorporate the iron–sulfur cluster during its expression in E.coli, the expression of the mutants with different substitutions in position 108 yields holo-proteins. Accordingly, proline in position 108 is not obligatory for the incorporation of the iron–sulfur cluster into adrenodoxin. Nevertheless, the mutants 4–108K, 4–108W and 4–108S are produced with significantly lower yields than the wild-type or the 4–108P mutant, suggesting that these proteins are targets of intracellular degradation, although the specific environment of the iron–sulfur cluster and the overall protein conformations are essentially similar to the 4–108P in both oxidized and reduced states, as shown by absorption spectroscopy, EPR and CD.

One of the most important parameters that displays the specific function of Adx, i.e. its ability to accept and donate electrons, is the redox potential. This reflects any perturbation in the surroundings of the cluster, including solvent accessibility, hydrophobicity and polarity of the immediate environment around the cluster, extent and localization of hydrogen bonds and the nature of coordinating ligands (Tsukihara et al., 1986; Gurbel et al., 1989). Based on the data given here, we can exclude a direct role of Pro108 in the modulation of the Adx redox potential. In fact, the changed size, hydrophobicity or polarity of the residue in position 108 do not cause any significant perturbation in the redox potential (Table I). At the same time, when proline is substituted by the positively charged Lys, the redox potential moves 25 mV lower. It is interesting that the deletion of 20 C-terminal amino acids, including two positively and five negatively charged groups, causes a decrease in the redox potential from −273 to −343 mV. Hence it is reasonable to speculate that the molecular electrostatic potential of the protein and/or charge distribution could be of importance in controlling the redox potential of ferredoxins. Alternatively, the deletion of the C-terminal part of the Adx polypeptide chain could alter the water accessibility to the protein core.

Our investigations of the functional properties of 4–108X mutants demonstrate that Pro108 is not directly involved in the Adx–AdR interaction and the mutations of this residue do not cause conformational changes affecting the affinity to AdR (Table II). The determination of hydroxylating activities of CYP11A1 reconstituted with Adx revealed a similar tendency. Although the redox potentials were decreased in all the mutants compared with WT Adx, which should theoretically make the electron transfer more favorable (Becket and Bernhardt, 1997), the kinetics of this reaction are only marginally changed, especially when considering that at physiological temperatures a large fraction of our unstable mutants is present in the unfolded form (Table IV). This indicates, in accordance with previous results (Becket and Bernhardt, 1997), that no correlation between the redox potential and the overall activity ($V_{max}$) can be observed in this system.

In contrast to the marginal effect on Adx structure and function, the Pro108 substitutions are shown to affect dramatically the overall conformational stability of the protein. It has been proposed previously that the effect of proline replacement can be attributed to the increased entropy of the unfolded chain that correlates with the local backbone mobility (Nemethy et al., 1966). If no secondary effects are introduced, all amino acid substitutions, except Gly, are expected to destabilize the protein structure by roughly the same value. However, a proline residue on the very end of the chain is supposed to have lesser effect than a proline somewhere in the middle of the chain. The entropy contribution into the stability of a protein is given by (Freire, 1994)

$$\Delta S = \Delta S_{\text{conf}} + \Delta C_p \ln(T/T_{\text{conv}})$$

where $T_{\text{conv}}$ is the convergence temperature at which the residue normalized entropies of proteins assume a similar value (Privalov and Gill, 1988). It includes the configurational and solvation entropy contributions (the first and the second terms, respectively). Since no changes in the $\Delta C_p$ have been observed (Figure 4), the solvation term of Equation 1 seems
to be the same for all mutants. Accordingly, the only entropic component that may contribute to changes in the stability of the mutants is the configurational entropy. However, for bovine adrenodoxin the hypothesis of the entropic cause of the decreased stability is quantitatively supported only in the case of Pro→Ala substitution, where the theoretically estimated $\Delta \Delta G_{\text{fold}}(37^\circ \text{C}) = -5.4 \text{ kJ/mol}$ is similar to the experimental value $\Delta \Delta G(37^\circ \text{C}) = -5.0 \text{ kJ/mol}$. For 4–108S, 4–108W or 4–108K mutants the falls of the Gibbs energy of unfolding, $\Delta \Delta G(37^\circ \text{C})$, are more pronounced. They are $-7.8$, $-10.1$ and $-10.7 \text{ kJ/mol}$, respectively, suggesting that factors other than the configurational entropy are responsible for the reduced stability of these mutants. For instance, an additional destabilization could be attributed to the destruction of the a priori rigid conformation and particular fold of the polypeptide chain around this residue can be examined using the very recently obtained X-ray data on the truncated Adx mutant 4–108P (Müller et al., 1998). The vicinity of Pro108 is plotted in Figure 5, showing that the structure is perfectly ordered around this residue. In fact, Pro108 occupies a key position in maintaining the Adx structure. Situated at the end of the $\beta$-strand J, it fixes the $\beta$-turn between strands A and B by forming a hydrogen bond to Arg14 (2.9 Å), and, on the other hand, hydrophobic interactions of Pro108 C$\gamma$ with Ile58 C$\gamma$ (4.09 Å) and His56 C$\gamma$ (3.8 Å), Pro108 C$\beta$ with Tyr82 C$\beta$ (4.94 Å) and Tyr82 C$\varepsilon$ (4.74 Å) form a link to strand D and to a $\beta$-turn, in which Tyr82 is situated. Losing these contacts results in a misfolded protein that is not able to incorporate an iron–sulfur cluster upon expression in E.coli (Uhlmann et al., 1992).

The steric requirements imposed by the proline residue direct the H-bonding between the C=O of Pro108 and Nε Arg14. At the pH used in this work, C-terminal Pro108 and Arg14 residues are expected to be ionized, so that this interaction could be described as a ‘salt bridge’. In the WT molecule the Pro108–Arg14 H-bond is supposed to be reserved. However, its strength is somewhat decreased, since a carbonyl oxygen is a less favorable H-bond acceptor than a carboxyl oxygen. This conclusion is strongly supported by studies of another truncated Adx mutant, 4–109, that has the same oxygen. This conclusion is strongly supported by studies of another truncated Adx mutant, 4–109, that has the same oxygen is a less favorable H-bond acceptor than a carboxyl oxygen is a less favorable H-bond acceptor than a carboxyl oxygen is a less favorable H-bond acceptor than a carboxyl oxygen is a less favorable H-bond acceptor than a carboxyl oxygen is a less favorable H-bond acceptor than a carboxyl oxygen.

Adrenodoxin (Adx) is a 129-residue iron–sulfur protein involved in the last step of the respiratory chain of the mitochondrial electron transport system in animals. The iron–sulfur proteins have attracted considerable attention over the past decades due to their importance in various biological processes. In the present case of bovine adrenodoxin, which can be considered as a prototype of vertebrate [2Fe–2S] ferredoxins, the replacement of the conserved proline by other amino acids does not influence the properties of the iron–sulfur cluster, but introduces drastic changes in the protein stability. We suppose that Pro108 serves as a ‘clip’, that brings into proximity different structural elements, providing the specific fold of the Adx molecule in this region.

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