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

Asya V. Grinberg and Rita Bernhardt

Fachbereich Pharmazie und Umwelttechnologie, Fachrichtung 12–4 Biochemie, Universität des Saarlandes, PO Box 15 11 50, D-66041 Saarbrücken, Germany

1To whom correspondence should be addressed. E-mail: ritabern@rz.uni-sb.de

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 and sources for these organisms. In plants, [2Fe-2S] 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., 1988; 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.

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., 1988; 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.

© Oxford University Press 1057
Adx, CYP11A1 and CYP11B1 were isolated from bovine adrenals as described previously (Akrem et al., 1979). The concentration of AdR was estimated using ε_{414} = 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.

Circular dichroism spectroscopy

CD measurements were performed on a Jasco 7000 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. The thermal denaturation of the mutant proteins was monitored 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. All spectra were averaged to obtain each final spectrum.

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 chromato-graphically 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 mediated by the adrenodoxin mutants were measured 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 holo and apo forms. Western blot analysis with safranin T as indicator and mediator according to Sligar (1994). In the far-UV region all the mutants studied showed 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 flavins 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.
A.V. Grinberg and R. Bernhardt

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.

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, \( \Delta_d H \), 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 \( T_d = 48.9°C \). 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
accept the slope of the line with a correlation coefficient of 0.97 (Figure 4) according 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 \( 10.35 \pm 1.1 \) kJ/mol/K.

The stability differences between the mutant proteins expressed in terms of an increment of the Gibbs energy of unfolding, \( \Delta \Delta G(37^\circ C) \), 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, $ΔT^*$, 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, $α$, 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 (Beckert 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 (Beckert 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)

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

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 $\Delta S_{\text{p}}$ have been observed (Figure 4), the solvation term of Equation I seems

![Fig. 5. Close-up view of the three-dimensional structure of the truncated adrenodoxin 4–108P in the vicinity of C-terminal Pro108. The hydrogen bond between Pro108 O and Nε Arg14 (2.9 Å) is indicated as a dotted line. Spheres represent water molecules.](https://academic.oup.com/peds/article-abstract/11/11/1057/1478303/1)
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 \( \Delta \Delta G_{\text{conf}}(37^\circ \text{C}) = -5.4 \text{ kJ/mol} \) is similar to the experimental value \( \Delta G(37^\circ \text{C}) = -5.0 \text{ kJ/mol} \). 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 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 Pro108. The special arrangements of the sequence component that may contribute to changes in the stability of 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 \( \text{G} \) with Leu58 C\( \delta \) (4.09 Å) and His56 \( \text{G} \) (3.8 Å), Pro108 C\( \beta \) with Tyr82 C\( \varepsilon \) (4.94 Å) and Tyr82 C\( \delta \) (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 preserved. 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 functional properties as 4–108, being one residue longer. In fact, the loss of the formal charge in position 108 leads to a destabilization of 3.75 kJ/mol compared with 4–108 (A.V. Grinberg and R. Bernhardt, unpublished results). At the same time, the complete elimination of H-bonding events between Arg14 and Pro108 (mutation R14A) results in the decrease in \( \Delta \Delta G(37^\circ \text{C}) \) by 5.5 kJ/mol (A.V. Grinberg and R. Bernhardt, unpublished results). In general, hydrogen bonding and hydrophobic effects both constitute the principal source of stabilization energy in folded proteins (Privalov and Gill, 1988; Makhatadze and Privalov, 1995). Fersht et al. (1985) reported favorable H-bonds with values from 2.1 to 7.5 kJ/mol for polar partners and up to 18.8 kJ/mol when one of the partners bears a charge. The local packing density and hydrophobicity also seem to play a significant role, as a destabilization of −10.1 kJ/mol has been observed for 4–108W, presumably because of significant perturbations in the local structure, needed to accommodate the large side-chain of Trp. In the case of the 4–108K mutant, the positive charge seems to destabilize the structure by repulsion from His56 and Arg14.

Taken together, these data offer an insight into some of the features underlying the properties of iron–sulfur proteins. 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.

Acknowledgements

The authors thank Dr Kappl for support in the EPR measurements and Professor Pfiehl for critical evaluation of the manuscript. A.G. is very grateful to Vita Beckert for permanent support and stimulating discussions. This work is supported by a grant from the Boehringer Ingelheim Fonds to A.G., by the Deutsche Forschungsgemeinschaft, Grant Be 1343/1-3, and by the Fonds der Chemischen Industrie.

References

A.V. Grinberg and R. Bernhardt


Received February 3, 1998; revised June 22, 1998; accepted June 29, 1998