Conformational stability of bovine holo and apo adrenodoxin—A scanning calorimetric study

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Abstract
Holo and apo adrenodoxin were studied by differential scanning calorimetry, absorption spectroscopy, limited proteolysis, and size-exclusion chromatography. To determine the conformational stability of adrenodoxin, a method was found that prevents the irreversible destruction of the iron-sulfur center. The approach makes use of a buffer solution that contains sodium sulfide and mercaptoethanol. The thermal transition of adrenodoxin takes place at \( T_m = 46-57 \, ^\circ\mathrm{C} \), depending on the \( \mathrm{Na}_2\mathrm{S} \) concentration with a denaturation enthalpy of \( \Delta H = 300-380 \, \text{kJ/mol} \). From \( \Delta H \) versus \( T_m \), a heat capacity change was determined as \( \Delta C_p = 7.5 \pm 1.2 \, \text{kJ/mol/K} \). The apo protein is less stable than the holo protein as judged by the lower denaturation enthalpy \( \Delta H = 93 \pm 14 \, \text{kJ/mol} \) at \( T_m = 37.4 \pm 3.3 \, ^\circ\mathrm{C} \) and the higher proteolytic susceptibility. The importance of the iron-sulfur cluster for the conformational stability of adrenodoxin and some conditions for refolding of the thermally denatured protein are discussed.

Keywords: ferredoxin; iron-sulfur protein; protein unfolding; scanning microcalorimetry; thermodynamics
garding protein folding. Furthermore, the importance of salt bridges was proposed in a classical paper by Perutz and Raid (1975) just on the example of ferredoxins from mesophile and thermophile bacteria. Unfortunately, the conformational stability of these proteins could not then be quantified.

Based on the DSC data, the thermodynamic analysis of protein unfolding allows us to elucidate features of the tertiary structure of proteins and to determine the contribution of interactions that maintain the stability of the native structure. This technique has been successfully applied to a great variety of proteins (Privalov, 1992). The aim of the present work is to carry out a first study of the thermal stability of wild-type Adx and the apo protein using DSC and related methods. The main problem to be solved is finding the appropriate experimental conditions for DSC studies in order to exclude possible artifactual connections with high chemical lability of the iron-sulfur cluster of Adx. Studies on mutant proteins of Adx will be presented in a separate paper. We hope that knowledge of the three-dimensional structure and the conformational stability of wild-type Adx and its mutants can be helpful in further understanding of the biological function of Adx.

Results

Holo adrenodoxin

Adx exhibits a characteristic absorption spectrum at physiological pH values (Estabrook et al., 1973). The spectrum shows peaks in the visible region at 415 and 455 nm and a wide near-UV absorption peak at 320 nm. Absorption bands of Adx are associated with the oxidized form of the iron-sulfur cluster. Accordingly, the thermal denaturation of Adx can be followed by measuring the absorbance at 415 nm during heating with a constant rate. Figure 1 shows a melting pattern obtained in glycine buffer, pH 8.5, corresponding to that at physiological pH, whereas in the spectrum of Adx at 70 °C all three absorption maxima disappear. If we take the absorption at 415 nm and 20 °C equal to 100%, the value is reduced by about 70% at 70 °C. Changes of absorbance are irreversible on cooling. The melting pattern shown in Figure 1 is complex and reflects at least two processes. At the same time, an exothermic process can be found in scanning calorimetry that overlaps the endothermic denaturation transition (Fig. 1, inset).

To find the reason for the unusual denaturation behavior of Adx, a great variety of solvent conditions were tested including pH values ranging from 6.0 to 9.2, use of different SH reagents, and the addition of oxygen-consuming enzymatic systems (glucose oxidase, lactate-2-monooxygenase). However, a different melting pattern was obtained only in the presence of sodium sulfide. In the presence of Na₂S, the complexity of the optically and calorimetrically monitored thermal transitions disappears (see below).

To exclude side reactions, in particular the formation of polysulfides, when using Na₂S, a protocol was elaborated that suggests the use of the following buffer system: 40 mM glycine, X mM Na₂S, 1 mM ascorbic acid, pH 8.5. Buffer and protein handling were carried out under argon. Furthermore, after filling the calorimetric cell and applying 1 atm nitrogen pressure, the buffer and sample were preheated up to 33 °C prior to the calorimetric run. As will be shown below, the procedure can be further improved when adding mercaptoethanol to the buffer.

Using this protocol, calorimetric recordings of Adx obtained at various Na₂S concentrations resemble the melting pattern usually observed for small globular proteins (Fig. 2). The exothermic process that was found in the absence of Na₂S (Fig. 1, inset) is no longer visible. Similarly, in the Na₂S ascorbate buffer system, the optically monitored transition comes close to a sigmoidal curve when regarding the parabolic pre-denaturation slope (Fig. 3). The decrease in optical density at thermal Adx denaturation is less pronounced than the value obtained in the absence of Na₂S (Fig. 1) and amounts to about 25%. On cooling, the thermal transition is noncooperative as shown by the downslope in Figure 3.

The denaturation peak position of Adx is dependent on the Na₂S concentration (Fig. 2). The calorimetric transition tem-
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Fig. 3. Thermal denaturation of Adx monitored by optical absorption at 415 nm. Buffer: 40 mM glycine, pH 8.5, with 10 mM Na₂S and 1 mM ascorbate. Open circles, upscan with 0.5 K/min; crosses, downscan with −0.5 K/min. Nonlinear fit of the upscan by a two-state model with second-order exponentials for the pre- and postrenaturation slope gives \( T_{m} = 52.9 \pm 0.7 \) °C and \( \Delta H^{\text{rev}} = 600 \text{ kJ/mol} \).

Temperature varies from about 46 to 57 °C when increasing the Na₂S concentration from 2.5 to 30 mM. With an increasing Na₂S concentration, calorimetric and van’t Hoff enthalpies of denaturation diverge, thus indicating a complexity of the thermal transition (Table 1). At 30 mM Na₂S, even a shoulder of the calorimetric peak becomes visible (Fig. 2).

The complexity of the thermal transition of Adx as indicated by the cooperative ratio \( CP > 1 \) (Table 1) can be suppressed in the presence of mercaptoethanol. A calorimetric recording of Adx in the sulfide/ascorbate buffer system containing additionally mercaptoethanol is shown in Figure 4 along with a two-state fit. The calorimetric denaturation enthalpy \( (\Delta H^{\text{cal}} = 355 \pm 13 \text{ kJ/mol at } T_{m} = 51.39 \pm 0.13 \text{ °C as obtained from three measurements}) \) coincides with the value obtained by van’t Hoff treatment of the optically monitored transition under the same conditions (Fig. 5). Moreover, the shoulder of the calorimetric transition observed at 30 mM Na₂S disappears in the presence of mercaptoethanol (Fig. 6). Thus, mercaptoethanol has a significant influence on the cooperativity of the thermal Adx transition. However, it has no effect on the calorimetric enthalpy when comparing data obtained in the presence and in the absence of mercaptoethanol (Table 1).

As shown in Figure 7, the calorimetrically determined denaturation enthalpy \( \Delta H^{\text{cal}} \) becomes linearly dependent on \( T_{m} \). From \( \Delta H^{\text{cal}} \) versus \( T_{m} \), the heat capacity change was determined as \( \Delta C_{p} = 7.5 \pm 1.2 \text{ kJ/mol/K} \). From the individual calorimetric recordings, a mean value \( \Delta C_{p} = 6.4 \pm 2.2 \text{ kJ/mol/K} \) was obtained. \( \Delta C_{p} = 7.5 \pm 1.2 \text{ kJ/mol/K} \) from \( \Delta H^{\text{cal}} \) versus \( T_{m} \) (Fig. 7) is regarded as the more reliable value. The data are derived here varying the Na₂S concentration from 2 to 40 mM because there is no pronounced pH dependence of the transition temperature (Fig. 8).

The Adx thermal transition, when monitored by optical absorption at 415 nm, appears to be reversible (Fig. 5). The same \( \Delta H \) is even obtained on reheating. Compared with measurements in the absence of mercaptoethanol, the decrease of absorption during the scan is reduced to 14 ± 4%. Only slight differences exist in the spectra of the protein before and after the scan (inset in Fig. 5). The downscan shows noncooperative behavior of the renaturation process (Fig. 5). On the other hand, the Adx thermal transition is only partly reversible as judged by reheating the sample in the calorimetric cell (Fig. 4, dotted line). Nevertheless, protein taken from the calorimetric cell after completing the scan shows the same CD spectrum in the visible and near-UV region as the native protein. Furthermore, the protein entering the calorimetric cell after completion of the scan shows the same CD spectrum in the visible and near-UV region as the native protein. Furthermore, the protein

Table 1. Calorimetric data of Adx denaturation in the presence of Na₂Sa

<table>
<thead>
<tr>
<th>Na₂S (mM)</th>
<th>( T_{m} ) (°C)</th>
<th>( \Delta H^{\text{cal}} ) (kJ/mol)</th>
<th>( \Delta H^{\text{rev}} ) (kJ/mol)</th>
<th>( CR )</th>
<th>( \Delta C_{p} ) (kJ/mol/K)</th>
</tr>
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<tr>
<td>2.0</td>
<td>46.17 ± 0.37</td>
<td>324 ± 20</td>
<td>270 ± 10</td>
<td>0.88</td>
<td>5.72</td>
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<tr>
<td>2.5</td>
<td>46.83 ± 0.11</td>
<td>296 ± 29</td>
<td>270 ± 22</td>
<td>0.79</td>
<td>n.d.</td>
</tr>
<tr>
<td>5.0</td>
<td>50.51 ± 0.16</td>
<td>352 ± 21</td>
<td>320 ± 17</td>
<td>1.10</td>
<td>6.48</td>
</tr>
<tr>
<td>10</td>
<td>52.64 ± 0.06</td>
<td>354 ± 16</td>
<td>311 ± 8</td>
<td>1.14</td>
<td>6.01</td>
</tr>
<tr>
<td>10</td>
<td>53.08 ± 0.07</td>
<td>352 ± 15</td>
<td>316 ± 11</td>
<td>1.11</td>
<td>3.41</td>
</tr>
<tr>
<td>10b</td>
<td>51.31 ± 0.09</td>
<td>370 ± 6</td>
<td>351 ± 5</td>
<td>1.06</td>
<td>5.63 ± 2.85</td>
</tr>
<tr>
<td>10b</td>
<td>51.32 ± 0.1</td>
<td>348 ± 6</td>
<td>353 ± 5</td>
<td>0.98</td>
<td>6.20 ± 1.77</td>
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<tr>
<td>10b</td>
<td>51.55 ± 0.17</td>
<td>348 ± 12</td>
<td>346 ± 12</td>
<td>1.00</td>
<td>8.71 ± 1.05</td>
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<td>15</td>
<td>54.11 ± 0.13</td>
<td>380 ± 29</td>
<td>323 ± 14</td>
<td>1.18</td>
<td>9.01</td>
</tr>
<tr>
<td>20</td>
<td>55.60 ± 0.07</td>
<td>379 ± 11</td>
<td>306 ± 5</td>
<td>1.24</td>
<td>6.30</td>
</tr>
<tr>
<td>20</td>
<td>55.50 ± 0.07</td>
<td>366 ± 28</td>
<td>314 ± 17</td>
<td>1.16</td>
<td>10.28</td>
</tr>
<tr>
<td>30</td>
<td>57.50 ± 0.06</td>
<td>376 ± 20</td>
<td>308 ± 8</td>
<td>1.23</td>
<td>9.45</td>
</tr>
<tr>
<td>30</td>
<td>57.46 ± 0.05</td>
<td>376 ± 22</td>
<td>307 ± 10</td>
<td>1.23</td>
<td>7.72</td>
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<tr>
<td>30</td>
<td>55.68 ± 0.4</td>
<td>404 ± 19</td>
<td>352 ± 15</td>
<td>1.15</td>
<td>6.20 ± 1.32</td>
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<tr>
<td>30c</td>
<td>55.57 ± 0.17</td>
<td>409 ± 12</td>
<td>352 ± 6</td>
<td>1.16</td>
<td>4.19 ± 3.07</td>
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<tr>
<td>30b</td>
<td>55.27 ± 0.05</td>
<td>383 ± 14</td>
<td>355 ± 8</td>
<td>1.08</td>
<td>4.18 ± 0.81</td>
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<tr>
<td>40</td>
<td>56.56 ± 0.15</td>
<td>413 ± 21</td>
<td>366 ± 10</td>
<td>1.13</td>
<td>2.97 ± 2.14</td>
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<tr>
<td>50</td>
<td>55.35 ± 0.1</td>
<td>275 ± 10</td>
<td>335 ± 8</td>
<td>0.82</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*Mean ± standard error of the mean for the data treatment of individual scanning calorimetric recordings.

* Measured in the presence of 10 mM mercaptoethanol.

* Measured in the presence of 10 mM ascorbic acid.

Fig. 4. Calorimetric recording of Adx in 40 mM glycine, pH 8.5, with 10 mM Na₂S, 1 mM ascorbic acid, and 10 mM mercaptoethanol. Open circles, raw data; solid line, two-state fit with \( T_{m} = 51.43 ± 0.03 \) °C, \( \Delta H = 360.1 ± 0.9 \text{ kJ/mol} \), and \( \Delta C_{p} = 5.02 ± 0.53 \text{ kJ/mol/K} \). Protein concentration \( c = 3.05 \text{ mg/mL} \). Dotted line, reheating of the sample; broken line, reheating after urea treatment, see text.
Fig. 5. Adx denaturation monitored by optical absorption at 415 nm. Buffer: 40 mM glycine, pH 8.5, with 10 mM Na₂S, 1 mM ascorbate, and 10 mM mercaptoethanol. Solid circles (curve S), upscan with 0.5 K/min; crosses (curve D), downscan with −0.5 K/min; open circles (curve R), reheating with 0.5 K/min. Inset: Absorption spectra of the sample: 1, Adx at 20°C before the first scan; 2, Adx at 70°C after the first scan; 3, recooled Adx at 20°C after the first scan. Nonlinear fit of the first and second upscan by a two-state model with second-order exponentials for the pre- and postdenaturational slope gives $T_{m} = 53.6 \pm 0.9$ °C, $\Delta H_{m}^{	ext{opt}} = 351 \pm 36$ kJ/mol and $T_{m} = 52.1 \pm 1.3$ °C, $\Delta H_{m}^{	ext{opt}} = 350 \pm 27$ kJ/mol, respectively.

Fig. 6. Calorimetric recordings of Adx in 40 mM glycine, pH 8.5, with 30 mM Na₂S, 1 mM ascorbic acid measured in the presence and in the absence of mercaptoethanol. Open circles, measured in the presence of mercaptoethanol, raw data, protein concentration $c = 2.03$ mg/mL; solid line, measured in the absence of mercaptoethanol, protein concentration $c = 1.58$ mg/mL; broken line, two-state fit of the recording obtained in the presence of mercaptoethanol with $T_{m} = 54.14 \pm 0.04$ °C, $\Delta H = 356.0 \pm 1.2$ kJ/mol, and $\Delta C_p = 5.86 \pm 1.17$ kJ/mol/K.

Fig. 7. Calorimetrically determined molar denaturation enthalpy of Adx versus transition temperature (Na₂S concentration from 2 to 40 mM). Slope corresponds to $\Delta C_p = 7.5 \pm 1.2$ kJ/mol/K. Inset: Dependence of the transition temperature on sodium sulfide concentration.

Fig. 8. Dependence of the Adx transition temperature $T_{m}$ on pH. Open circles, optically monitored transitions, first scan; triangles downward, optically monitored transitions, reheating; triangles upward, calorimetric data.

Fig. 9. Apo adrenodoxin

To study the stabilizing effect of the iron–sulfur cluster, apo Adx was prepared by trichloracetic acid treatment (Suhara et al., 1974). In nondenaturing electrophoresis, apo Adx migrates in a broad band, thus suggesting a mixture of conformers differing in size (Fig. 9A). In this respect, the behavior of apo Adx contrasts with that of the holo protein. By comparison, α-lactalbumin reference samples are included in Figure 9A that represent distinct folded and unfolded forms migrating in rather narrow bands.

runs as one band with the characteristic spectrum in rechromatography (data not shown). Simple cooling of the sample in the calorimetric cell after completing the scan does probably reestablish the local environment of the iron–sulfur cluster but not completely the tertiary structure. However, the sample taken from the calorimeter after completing the scan is able to refold in the presence of urea (Fig. 4, broken line). Therefore the thermal transition of Adx in the presence of sulfide/ascorbate and mercaptoethanol can be regarded as reversible, even if the renaturation procedure was not particularly optimized.
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Fig. 9. Electrophoretic characterization of holo and apo Adx. A: Non-denaturing electrophoresis at 18°C of: 1, holo adrenodoxin; 2, apo adrenodoxin; 3, bovine α-lactalbumin; 4, unfolded S-carboxymethyl-α-lactalbumin. B: SDS gel electrophoresis pattern after limited proteolysis of holo and apo Adx with TLCK-treated α-chymotrypsin at 25°C (chymotrypsin:Adx = 1:500, w/w). Duration of protease treatment is indicated in minutes. M, molecular weight standard.

In calorimetry, apo Adx shows a melting profile that is completely different from the holo protein (Fig. 10). The heat effect is marginal. A rough estimation for apo Adx gives ΔH = 93 ± 14 kJ/mol at T_m = 37.4 ± 3.3°C (mean of six measurements), with some uncertainty because the initial heat capacity of the protein is not measurable. The thermal transition of apo Adx is reversible as judged by reheating. The CD spectrum of apo Adx (inset in Fig. 10) is similar to that of the holo protein having mainly β- and random structure (Greenfield & Fasman, 1969; Brahms & Brahms, 1980; Manning & Woody, 1987; Manning et al., 1988). However, apo Adx shows a higher content of random structure, which is in agreement with the calorimetric behavior of the protein.

The partial specific heat capacities of apo and holo Adx show differences (Table 2). Because the denaturational heat capacity increase originates from exposure of nonpolar groups to the solvent (Privalov & Makhatadze, 1992), apo Adx seems to be less densely packed than the holo protein. In fact, the Stokes radius of apo Adx was found to be about 24 Å, a value close to that of carbonic anhydrase having a molar mass of about 29 kDa. At the same time, apo Adx is much more susceptible to limited proteolysis. The results in Figure 9 show a picture that is representative of the various proteolytic enzymes tested (see the Materials and methods). Holo Adx remains rather stable against chymotrypsin, except for the cleavage of terminal residues. Apo Adx is rapidly degraded. Here, a wide spectrum of fragments, in particular those having a molecular mass between 2 and 8 kDa, can be observed. Therefore, parts of the molecule that are buried in the holo protein are accessible for proteolytic cleavage in apo Adx.

Discussion

The experimental problems in studying the thermal denaturation of adrenodoxin seem to arise from the [2Fe-2S] cluster. The two -S- bridges between the iron ions can probably be disrupted upon heating, thus producing the complicated irreversible transitions shown in Figure 1. The exothermic transition observed in calorimetry disappears in the presence of sodium sulfide. Under the influence of Na2S both the transition temperature T_m and denaturation enthalpy ΔH increase. In this respect, the stabilization of the protein by Na2S can be considered a consequence of the Le Chatelier principle.

In the presence of Na2S, the thermal transition of Adx comes close to a two-state transition. However, at a Na2S concentration above 5 mM, the analysis of the cooperative ratio CR = ΔH^off/ΔH^eff shows some deviation from unity, the value expected for a real two-state transition (Table 1). At 30 mM Na2S, even a shoulder of the calorimetric peak can be found (Figs. 2, 6). A further hint for the complexity of Adx thermal denaturation is the noncoincidence of van’t Hoff heat derived from calorimetry and optical transitions. Usually, ΔH^eff from the optical transition is higher than the corresponding ΔH^off value derived from calorimetric recordings (see, e.g., the example in Fig. 3 and the data in Table 1).

An almost perfect approximation of Adx denaturation to the two-state behavior can be reached when using mercaptoethanol in addition to the sulfide/ascorbate-containing buffer system (Fig. 4). Under these conditions, the shoulder observed at 30 mM

Table 2. Partial specific heat capacity of apo and holo adrenodoxin

<table>
<thead>
<tr>
<th>Form</th>
<th>C_p at 25°C (J/g·K)</th>
<th>C_p at 80°C (J/g·K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holo</td>
<td>1.58 ± 0.15</td>
<td>2.38 ± 0.16 (n = 12)</td>
</tr>
<tr>
<td>Apo</td>
<td>2.31 ± 0.29</td>
<td>2.49 ± 0.31 (n = 8)</td>
</tr>
<tr>
<td>Apo</td>
<td>2.09</td>
<td>2.27</td>
</tr>
</tbody>
</table>

^a C_p at 20°C.

^b Calculated C_p value, based on the amino acid composition according to the approach of Makhatadze and Privalov (1990).
Na₂S disappears (Fig. 6). Furthermore, the value of the effective denaturation enthalpy obtained from independent optical measurements coincides with the calorimetric one (see the Results). In contrast to the action of Na₂S that really stabilizes Adx, mercaptoethanol has, within experimental uncertainty, no influence on the transition parameters. It can be argued that mercaptoethanol prevents an interchange of cysteine residues. Adx possesses five cysteine residues, four of them are involved in the formation of the iron-sulfur cluster (Cys-46, -52, -55, and -92) (Uhlmann et al., 1992). The problem remains to be solved by studying the stability of mutant proteins in which Cys-95 is replaced.

Adx thermal denaturation in the absence of Na₂S is accompanied by drastic changes in optical absorption at 415 nm due to the destruction of the iron-sulfur cluster. The process is irreversible. Adx thermal denaturation in the presence of Na₂S and mercaptoethanol is rather different and suited to determine the conformational stability of the protein. The transition is accompanied by moderate changes in the optical properties. After the scan, the protein regains nearly the original absorption and CD spectrum. Refolding, however, is noncooperative (Fig. 5). According to chromatography, the iron-sulfur cluster remains associated with the protein. These properties suggest that the local environment of the iron-sulfur center in renatured protein is similar to that in native Adx. Calorimetry, however, indicates that the polypeptide chain does not refold properly. On the other hand, thermally denatured Adx can be renatured in the presence of urea. That means Adx is capable of equilibrium folding under slightly modified experimental conditions. Cooling after the calorimetric scan is probably not sufficient to establish optimal refolding conditions. It is worth mentioning that Adx can be successfully reconstituted in the presence of Na₂S, FeSO₄, mercaptoethanol, and urea near 0°C (Suhara et al., 1974). The observed ability of Adx to unfold reversibly without dissociation of the iron-sulfur cluster raises the question of whether the holo or the apo protein is involved in vivo processes such as intracellular transport and folding. The observation that functionally active Adx can be expressed in the periplasm of E. coli (Uhlmann et al., 1992) supports the possibility of transport of the holo protein from the cytoplasm, where the iron-sulfur cluster can be assembled under reductive conditions, to the periplasm.

When the iron-sulfur cluster is removed from Adx, a completely different calorimetric recording is observed (Fig. 10). Apo Adx shows a reversible thermal transition at Tₜₐ₅ = 37.4 ± 3.3°C with ΔH = 93 ± 14 kJ/mol. The transition temperature and denaturational enthalpy are considerably reduced compared with holo Adx, which undergoes a thermal transition at 51.39°C with ΔH = 355 kJ/mol in the presence of 10 mM Na₂S. At the same time, the apo protein is more susceptible to protease digestion than the holo protein (Fig. 9). The pattern of fragments produced by the protease treatment is different for holo and apo protein. Similarly, as it was demonstrated for cytochrome b₅ and cytochrome P₄₅₀ (Pfeil, 1993; Pfeil et al., 1993), parts of the apo Adx molecule that are buried in a core region of the holo protein are accessible to protease digestion.

The calorimetric data enable the determination of the Gibbs energy of denaturation, ΔG. The enthalpy of denaturation for holo Adx unfolding calculated for 37°C (from Fig. 7) amounts to ΔH = 244 kJ/mol compared with ΔH = 93 ± 14 kJ/mol for the apo protein. The difference in Gibb's energy between holo and apo Adx amounts to Δ(ΔG) = 14 kJ/mol at 37°C. Thus, the incorporation of the iron-sulfur cluster and the accompanying conformational rearrangements of the polypeptide chain represent a mainly enthalpy-driven reaction. The conformational stability of Adx amounts to ΔG = 21 ± 4 kJ/mol at 25°C and pH 8.5 in the presence of 10 mM Na₂S and mercaptoethanol. Because the stability of most globular proteins amounts to 25–60 kJ/mol (Privalov, 1992), Adx must be regarded as a protein having marginal stability.

Taking into account the marginal conformational stability of Adx, the importance of side-chain hydrogen bonds for the protein stability, as originally described by Perutz and Raid (1975) for bacterial ferredoxins, becomes understandable. In fact, replacements of glutamate at position 74 by either alanine (E74A) or glutamine (E74Q) in human ferredoxin produce marked instability of the holo protein, and apo protein formation (Coghlan & Vickery, 1991). The unusual thermostability of Adx in the alkaline medium, i.e., the absence of large changes in transition temperature up to pH 10.2 (Fig. 8), provides a further hint for the importance of side-chain hydrogen bonds of the lysyl-carboxylate type for the stabilization of the Adx folded conformation.

In previous studies, no distinction was made between ferredoxin stability based on the irreversible destruction of the iron-sulfur cluster, and conformational stability based on reversible denaturation in the presence of proteactants for the iron-sulfur center. The experimental conditions elaborated in this paper for studying the thermal transition of Adx provide a way for studying folding and stability of iron-sulfur proteins in general. The procedure will be applied to characterize mutant proteins that could be helpful in elucidating the particular role of single amino acid residues in Adx functioning and stability.

Materials and methods

Adx preparation

Adx was obtained as described by Uhlmann et al. (1992). The protein was functionally active with the cytochromes c, P₄₅₀₁₁A₁, and P₄₅₀₁₁B₁ as electron acceptors. The Adx samples were homogeneous as judged by mass spectrometry, electrophoresis, HPLC, and amino acid analysis (Uhlmann et al., 1994). The ratio of optical density at 415 and 276 nm was Q = 0.92 (Uhlmann et al., 1992). Adx was used immediately after its preparation. A few samples were stored at –20°C as a stock solution in a 50 mM potassium phosphate buffer.

Apo Adx was prepared by trichloracetic acid treatment according to Suhara et al. (1974), followed by rechromatography on a 60-cm HiLoad Sephacryl 100 column (Pharmacia, Sweden) in 25 mM potassium phosphate, pH 7.4, containing 1 mM DTT.

Calorimetry

Scanning calorimetric measurements were carried out using two instruments: the MicroCal MC-2D scanning calorimeter (MicroCal Inc., Northampton, Massachusetts) with the DA-2 data acquisition system, and the DASM-1M microcalorimeter (NPO "Biopribor," Pushchino, Russia), modified for automated data acquisition. Thermograms were in general obtained at a scan rate of 53.63 s/K with the MC-2D and 59.22 s/K with the DASM-1M microcalorimeter. Partial specific heat capacity was determined according to Makhadzade and Privalov (1990). Adx
Adrenodoxin stability

solutions for calorimetric measurements were prepared by dialysis of the stock protein solution against the carefully degassed buffer of choice for 24 h at 4°C or by chromatography prior to use on a 1 × 10-cm Sephadex G25 column. The concentration of Adx after dialysis was determined spectrophotometrically in 50 mM Tris-HCl buffer, pH 7.4, using \( E = 9.8 \text{ mM}^{-1} \text{ cm}^{-1} \) at 415 nm (Kimura, 1968) for the holo protein and \( E = 0.247 \text{ mM}^{-1} \text{ cm}^{-1} \) at 280 nm for apo Adx (Suhara et al., 1974). The molecular mass of the recombinant Adx, where the N-terminal serine was replaced by methionine, was determined by mass spectrometry to be 14,015.5 ± 2.2, which corresponds to the molecular mass of the recombinant Adx, where the N-terminal serine was replaced by methionine, was determined by mass spectrometry to be 14,017.8 (Uhlmann et al., 1994). Partial specific volume was assumed to be 0.72 mL/g as calculated from the amino acid composition (Uhlmann et al., 1994). The protein concentration in scanning calorimetric measurements was 0.5–3.1 mg/mL for holo Adx and 1.6–3.5 mg/mL for apo Adx.

Renaturation experiments with iernally denatured Adx were made on samples taken from the calicometer after completing the scan. The samples were incubated with 8 M urea for 40 min and dialyzed against glycine buffer, pH 8.5, containing sodium sulfide, ascorbate, and mercaptoethanol. The operation was carried out under argon at 4°C.

Spectroscopy

Optically monitored unfolding curves were obtained on a Kontron 930 UV-VIS spectrophotometer equipped with a Haake F3-C thermostat and PG20 temperature programmer. Adx absorbance at a fixed wavelength (415 nm) was followed over a temperature range from 20 to 80°C during heating with the rate 60 K/h. In some cases, the heating rate 30 K/h was used. A buffer change was made immediately before spectrophotometric measurements using a 1 × 10-cm Sephadex G25 column. The concentration of Adx was usually about 0.02 mM. Mathematical treatment of Adx denaturation (i.e., determination of \( T_{m} \) and \( \Delta H^{m} \)) was made by nonlinear regression using various models: a two-state transition with a linear pre- and postdenaturational slope, a two-state transition with parabolic pre- and postdenaturational slope, and a three-state transition with linear pre- and postdenaturational slope. The programs written in Turbo-Pascal were kindly provided by Dr. O Ristau (Berlin).

CD spectra were measured on a JASCO 720 spectropolarimeter. Measurements in the far-UV region were made in 5 mM potassium phosphate buffer, pH 7.5, containing 0.2 mM DTT. The protein concentration was about 0.2 mg/mL and the optical pathlength, 0.1 mm. Measurements in the visible and near-UV region were made in 10 mM potassium phosphate buffer, pH 7.5, using a protein concentration of 1.0–1.5 mg/mL and an optical pathlength of 5 mm. Temperature was 25°C if not otherwise indicated.

Stokes radius

The determination of a Stokes radius for apo Adx was performed by gel-exclusion chromatography on a high-resolution Hiload 16 × 60-cm Sephacryl 100 column using 25 mM potassium phosphate buffer, pH 7.4, with 1 mM DTT. The calibration was made with carbonic anhydrase from bovine erythrocytes, myoglobin from horse, cytochrome c from horse heart, trypsin inhibitor from bovine lung, DNP-L-α-alanine (all substances from Serva), blue dextran (Pharmacia), and cytochrome b_{5} tryptic fragment from rabbit (Pfeil, 1993). The Stokes radii of the standard proteins were taken from Rogers et al. (1965), Ackers (1970), and Corbett and Roche (1984).

Limited proteolysis and electrophoresis

Controlled proteolysis was performed according to Carrey's protocol (1989). The following enzymes were used without further purification: carboxypeptidase A (EC 3.4.17.1) (Miles), TLCK-treated α-chymotrypsin from bovine pancreas (EC 3.4.21.1) (Sigma), clostripain from Clostridium histolyticum (EC 3.4.22.8) (Boehringer), papain from Papaya carica (EC 3.4.22.2) (Serva), pepsin from porcine stomach (EC 3.4.23.1) (Wortaiington), pronase P from Streptomyces griseus (EC 3.4.24.4) (Serva), protease from Staphylococcus aureus V8 (EC 3.4.22.19) (Miles), and TPCK-treated trypsin from bovine pancreas (EC 3.4.21.4) (Merck). SDS-PAGE was conducted in a discontinuous Tris-Tricine system (Schägger & von Jagow, 1987) on a 16% gel. The molecular mass was identified relative to the Pharmacia myoglobin fragment standard.

Nondenaturing electrophoresis was performed using the discontinuous Tris-Tricine system on 16% polyacrylamide with bovine α-lactalbumin and S-carboxymethyl-α-lactalbumin as reference proteins.

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