Conformational stability of adrenodoxin mutant proteins

TATJANA V. BUROVA,¹ VITA BECKERT,² HEIKE UHLMANN,³ OTTO RISTAU,²
RITA BERNHARDT,³ AND WOLFGANG PFEIL⁴

¹ Institute of Biochemical Physics, Russian Academy of Sciences, Moscow, Russia

² Max-Delbrück-Centre for Molecular Medicine, Berlin-Buch, Germany

³ University of the Saarland, Department of Biochemistry, Saarbrücken, Germany

⁴ University of Potsdam, Institute for Biochemistry and Molecular Physiology, c/o Max-Delbrück-Centre for Molecular Medicine, Berlin-Buch, Germany

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Abstract

Adrenodoxin and the mutants at the positions T54, H56, D76, Y82, and C95, as well as the deletion mutants 4–114 and 4–108, were studied by high-sensitivity scanning microcalorimetry, limited proteolysis, and absorption spectroscopy. The mutants show thermal transition temperatures ranging from 46 to 56 °C, enthalpy changes from 250 to 370 kJ/mol, and heat capacity change $\Delta C_p = 7.28 \pm 0.67$ kJ/mol/K, except H56R. The amino acid replacement H56R produces substantial local changes in the region around positions 56 and Y82, as indicated by reduced heat capacity change ($\Delta C_p = 4.29 \pm 0.37$ kJ/mol/K) and enhanced fluorescence. Deletion mutant 4–108 is apparently more stable than the wild type, as judged by higher specific denaturation enthalpy and resistance toward proteolytic degradation. No simple correlation between conformational stability and functional properties could be found.

Keywords: ferredoxin; iron–sulfur protein; mutants; protein unfolding; scanning microcalorimetry

The importance of side-chain hydrogen bonds for protein stability was considered by Perutz and Raid (1975) by example of bacterial ferredoxins. Unfortunately, attempts to study ferredoxins by means of thermodynamic approaches failed due to destruction of the iron–sulfur cluster prior to protein unfolding. Only recently was an approach developed by example of adrenal ferredoxin that enables unfolding studies by means of high-sensitivity scanning microcalorimetry (Burova et al., 1995). Independently, *Anabaena* ferredoxin was studied by denaturant-induced unfolding (Hurley et al., 1995).

Adrenal ferredoxin (Adx) is a small acidic protein consisting of 128 amino acid residues and an iron–sulfur cluster of the [2Fe-2S] type (Okamura et al., 1985). The protein participates in the synthesis of steroid hormones by mediating the electron transport from the NADPH-dependent adrenodoxin reductase to mitochondrial cytochromes P450 (Estabrook et al., 1973; Lambeth, 1990; Usanov et al., 1990). Accordingly, most investigations on Adx

were devoted to electron transfer and interactions within the system consisting of adrenodoxin reductase, Adx, and cytochrome P450 (Estabrook et al., 1973; Kido & Kimura, 1979; Hanukoglu & Jefcoate, 1980; Lambeth & Pember, 1983; Lambeth & Kriengsiri, 1985; Usanov et al., 1986; Hara & Kimura, 1989). Because wild-type Adx was expressed in *Escherichia coli* (Palin et al., 1992; Sagara et al., 1992; Uhlmann et al., 1992) and site-directed mutagenesis was performed on Adx, specific contributions of various amino acids in protein–protein interaction could be specified (Coghlan & Vickery, 1991, 1992; Uhlmann et al., 1992, 1994; Beckert et al., 1994, 1995; Uhlmann & Bernhardt, 1995a, 1995b). Nevertheless, these results remain incomplete as long as the influence of amino acid replacements on protein stability is not quantified. Adrenodoxin is of particular interest because it is involved in an electron transport chain. Here it needs to be mentioned that Adx has to interact with adrenodoxin reductase as well as with the cytochromes P450_{sc} (CYP11A1) and P450_{11β} (CYP11B1). The question is whether this functional context requires a protein that is rather stable, in order to facilitate recognition by the partner molecules, or could it be advantageous to have a structure of marginal stability and high flexibility for the conformational changes accompanying interaction with proteins of the steroid converting enzyme? Furthermore, we speculate that the conformational stability might be irrelevant for complex formation because the latter is restricted to local events of few interacting amino acids. Therefore, scanning calorimetric investigations of Adx mutant proteins will be presented in this article to elucidate the influence of amino acid replacements on Adx conformational stability.

Reprint requests to: Wolfgang Pfeil, Max-Delbrück-Centre for Molecular Medicine, Robert-Rössle-Street 10, D-13125 Berlin-Buch, Germany; e-mail: wpfeil@orion.rz.mdc-berlin.de.

Abbreviations: Adx, adrenal ferredoxin (adrenodoxin); DSC, differential scanning microcalorimetry; EPR, electron paramagnetic resonance; GuHCl, guanidine hydrochloride; K_M , Michaelis–Menten constant; V_{max} , Michaelis–Menten constant; T_m , transition temperature (°C); ΔE , redox potential (mV); ΔH , denaturation enthalpy, calorimetric value (kJ/mol); Δh , specific denaturation enthalpy (J/g); CR, cooperative ratio; ΔC_p , heat capacity change (kJ/mol/K); $C_{ex,max}$, molar excess heat capacity; σ , standard deviation, percent of $C_{ex,max}$.

Results

Scanning calorimetric measurements of Adx mutant proteins were performed according to the protocol described by Burova et al. (1995). The procedure makes use of a buffer solution that contains sodium sulfide and mercaptoethanol, thus preventing destruction of the iron-sulfur cluster during heating before reaching the thermal transition temperature of the intact holo protein. Under these conditions, similar to the reconstitution protocol for the ferredoxin (Suhara et al., 1974), thermal transition of wild-type Adx approaches the two-state criterion, i.e., the ratio of calorimetric and van't Hoff heat (the cooperative ratio) becomes $CR = 1.0$ (Burova et al., 1995). For all the mutant proteins studied in this article, the two-state fit represents an almost perfect mathematical solution (Figs. 1, 2). The deviation from two-state behavior, quantified by the standard deviation of the fit in percent of the excess heat capacity maximum according to Chaires and Sturtevant (1986), does not exceed 1.6% on average. Therefore, all ΔH values given below refer to the two-state fit of scanning calorimetric recordings according to the procedure described in Materials and methods.

Compared with wild-type Adx, which exhibits $T_{tr} = 51.39 \pm 0.13^\circ\text{C}$, $\Delta H = 355 \pm 13 \text{ kJ/mol}$, and $\Delta C_p = 7.5 \pm 1.2 \text{ kJ/mol/K}$ (Burova et al., 1995), the mutants with amino acid replacements in positions T54, H56, D76, Y82, and C95, as well as the deletion mutants (residues 4–108, 4–114) show remarkable differences in transition temperature and enthalpy. The observed thermal transition temperatures T_{tr} range from about 46 to 56°C, and enthalpy changes ΔH at T_{tr} vary from about 250 to 370 kJ/mol (Table 1). When regarding the temperature dependence of the enthalpy change of the wild-type protein, most ΔH values of the mutants at their individual T_{tr} are close to the temperature function of ΔH of the wild type (Fig. 3). Substantial differences in changes in ΔH , however, can be found for T54A, H56Q, H56T, and H56R. The average of the heat capacity change as deduced from the individual scans amounts to $\Delta C_p = 7.28 \pm 0.67 \text{ kJ/mol/K}$ over all mutants except H56R (Table 1). Additionally, the heat capacity change of mutant H56Q was determined from ΔH versus T_{tr} varying the sulfide concentration as in Burova et al. (1995). The procedure yields $\Delta C_p = 7.49 \pm 1.19 \text{ kJ/mol/K}$ for adrenodoxin H56Q. Therefore, the heat capacity change ΔC_p is in fair agreement with the

Fig. 1. Molar heat capacity of mutants H56Q, C95S, and wild type (broken line) determined by differential scanning calorimetry at pH 8.5 in the presence of sulfide and mercaptoethanol. Open circles, experimental raw data; straight line, two-state fit.

Fig. 2. Molar heat capacity of mutants T54A and T54S at pH 8.5 in the presence of sulfide and mercaptoethanol. Open circles, experimental raw data; straight line, two-state fit.

value obtained for the wild-type protein, $\Delta C_p = 7.5 \pm 1.2 \text{ kJ/mol/K}$. Remarkably, the heat capacity change of the mutant H56R, $\Delta C_p = 4.29 \pm 0.37 \text{ kJ/mol/K}$, is significantly different.

The stability differences of the mutant proteins relative to wild type expressed in terms of increment Gibbs energy change $\Delta(\Delta G)$ at physiologic temperature are listed in Table 2, along with relative data of enzymic activity of the reconstituted P450 system. The most pronounced decrease in conformational stability is observed for the histidine mutants in position 56 and for T54A. In those cases, $\Delta(\Delta G)$ parallels the reduced transition temperature and enthalpy change. C95S, 4–108, and D76E show increased conformational stability compared with wild type. At the same time, transition temperature and enthalpy change are increased. Only minor effects on $\Delta(\Delta G)$, T_{tr} , and ΔH were observed for all tyrosine mutants, T54S, and the deletion mutant 4–114.

Additionally, all the mutant proteins were tested by limited proteolysis using thermolysin at 45°C, i.e., just at the beginning of

Fig. 3. Denaturational enthalpy change versus transition temperature for the studied mutant proteins. 1, Wild type; 2, H56Q; 3, H56T; 4, H56R; 5, T54S; 6, T54A; 7, Y82S; 8, Y82L; 9, Y82F; 10, Y82W; 11, C95S; 12, 4–108; 13, 4–114; 14, D76E. The broken line was constructed from ΔH and ΔC_p of the wild-type protein (see text).

Table 1. Calorimetric data for adrenodoxin mutant proteins (two-state fit)^a

Mutant	T_r (°C)	$\Delta H(T_r)^b$ (kJ/mol)	ΔC_p^b (kJ/mol/K)	$\Delta S(T_r)$ (J/mol/K)	$\Delta G(37^\circ\text{C})^c$ (kJ/mol)
WT ^d	51.39	355	7.5	1,090	13.31
H56Q	46.6	249.1 ± 0.8	6.99 ± 0.1	780	6.42
	46.3	249.7 ± 0.6	7.20 ± 0.1	780	6.28
H56T	48.5	265.6 ± 0.8	6.07 ± 0.18	830	7.98
	49.0	276.6 ± 0.7	5.78 ± 0.23	860	8.66
H56R	49.2	246.0 ± 0.8	4.13 ± 0.27	760	8.31 ^e
	48.8	244.8 ± 0.6	4.21 ± 0.21	760	8.04 ^e
	49.5	260.9 ± 0.6	4.84 ± 0.20	810	9.06 ^e
	49.6	242.8 ± 0.7	4.00 ± 0.24	750	8.41 ^e
T54S	51.3	350.9 ± 0.9	7.04 ± 0.28	1,080	13.14
	51.9	357.4 ± 0.6	8.92 ± 0.18	1,100	13.86
T54A	46.8	275.0 ± 0.7	6.12 ± 0.28	860	7.32
	47.4	295.4 ± 0.5	7.47 ± 0.15	920	8.35
Y82S	50.6	334.3 ± 0.5	8.19 ± 0.13	1,030	11.94
Y82L	50.7	330.0 ± 0.5	8.04 ± 0.12	1,020	11.82
Y82F	51.5	350.0 ± 0.7	7.54 ± 0.23	1,080	13.25
Y82W	51.7	328.8 ± 0.9	7.83 ± 0.30	1,010	12.43
C95S	56.0	376.0 ± 0.4	7.23 ± 0.11	1,140	17.64
	55.5	371.0 ± 0.4	6.34 ± 0.13	1,130	17.03
4-108	55.3	376.0 ± 0.4	8.10 ± 0.18	1,150	17.17
	55.1	372.2 ± 1.2	5.74 ± 0.34	1,130	16.83
4-114	50.9	313.7 ± 1.1	7.49 ± 0.30	970	11.26
	51.3	325.4 ± 1.1	4.27 ± 0.34	1,000	12.24
D76E	53.2	366.0 ± 0.7	9.17 ± 0.16	1,120	15.20
	53.6	360.8 ± 0.8	11.3 ± 0.24	1,100	15.21

^a pH 8.5, 10 mM Na₂S, 1 mM ascorbate, 10 mM mercaptoethanol.^b Standard error for each parameter of the two-state fit obtained from a single scan.^c Calculated using the individual T_r and ΔH values of the mutant proteins and $\Delta C_p = 7.28 \pm 0.67$ kJ/mol/K.^d Reference value, $T_r = 51.39 \pm 0.13^\circ\text{C}$, $\Delta H = 355 \pm 13$ kJ/mol and $\Delta C_p = 7.5 \pm 1.2$ kJ/mol/K (mean of five measurements).^e Calculated using $\Delta C_p = 4.29 \pm 0.37$ kJ/mol/K.

thermal transition. As shown in Figure 4A and B, this experimental tool is well suited to distinguish between pairs of mutants that exhibit different stability as expressed in terms of Gibbs energy change. For all mutant proteins, it was found that the resistance against proteolytic degradation parallels the calorimetric transition temperature. At the same time, the thermal transition temperature and enthalpy coincide with the calorimetric values when the transition is followed by CD in the sulfide-containing protecting buffer system (Uhlmann & Bernhardt, 1995b).

In the protecting buffer system, optical absorption at 415 nm does not change significantly during the thermal transition for all mutants studied. Moreover, the optical changes do not coincide with the thermal transition as monitored by calorimetry. The optical changes take place at $55.8 \pm 0.7^\circ\text{C}$ ($n = 21$), i.e., above the calorimetric transition temperature. Furthermore, no specific stability differences of the mutants that range from 46 to 56°C can be observed. Therefore, the optical changes seem not to be related to the conformational transition of the polypeptide chain and probably reflect the destruction of the cluster after dissociation from the thermally denatured protein. It is worth mentioning that only subtle changes of absorption at 415 nm can be observed when the protein is denatured by guanidine hydrochloride at 25°C in the presence of the sulfide-containing buffer (Fig. 5). Thus, according to the optical criteria, the protecting buffer system is suited to maintain properties of the cluster after protein denaturation.

As shown by Burova et al. (1995), Adx in the used buffer system is able to renature after thermal treatment under the influence of urea. Thermally denatured Adx taken from the calorimetric cell after reaching 70°C is able to regain the original absorption spectrum (Fig. 6) and EPR spectrum even without urea when kept for at least 12 h at room temperature in the sulfide buffer. On the other hand, when Adx is taken at 70°C from the calorimetric cell and immediately supplied to rechromatography, a high content of apo protein can be observed, which means that thermal denaturation of Adx leads to displacement of the iron-sulfur cluster and the protecting buffer system facilitates the reconstitution after thermal denaturation.

In addition to the thermodynamic studies performed in the presence of sulfide-containing buffer, the redox potential of Adx and its functional activity were tested in the absence of the protectant by following cytochrome *c* reduction, cytochrome P450-mediated pregnenolone conversion (CYP11A1 assay), and cytochrome P450-mediated corticosterone conversion (CYP11B1 assay) (Uhlmann et al., 1994; Beckert et al., 1995; Uhlmann & Bernhardt, 1995a, 1995b; V. Beckert & R. Bernhardt, in prep.). In most cases, no significant alteration of Michaelis-Menten parameter V_{max} was found. In contrast, K_M varies considerably, as shown in Table 2 on the example of 11β hydroxylase activity of adrenocortical cytochrome P450 (normalized values obtained by the CYP11B1 assay). However, these values and, in a similar manner, those of the

Table 2. Increments of thermodynamic parameters for unfolding of adrenodoxin mutant proteins (pH 8.5, 10 mM Na₂S, 1 mM ascorbate, 10 mM mercaptoethanol) compared with relative activity and redox potential determined in the absence of sulfide

Mutant	ΔT_{tr} (°)	$\Delta(\Delta H(T_{tr}))^a$ (kJ/mol)	$\Delta(\Delta G(37^\circ\text{C}))^b$ (kJ/mol)	Relative K_M^d	ΔE^c (mV)
WT	0	0	0	1.0	-274
H56Q	-5.0	-69	-7.0	6.1 ^e	-302 ^e
H56T	-2.7	-64	-5.0	4.1 ^e	-340 ^e
H56R	-2.1	-96	-4.8	6.3 ^e	-339 ^e
T54S	+0.2	-2	+0.2	0.32 ^f	-329 ^f
T54A	-4.3	-39	-5.5	0.52 ^f	-340 ^f
Y82S	-0.8	-15	-1.4	2.7 ^e	-282 ^e
Y82L	-0.7	-18	-1.5	0.4 ^e	-274 ^e
Y82F	+0.1	-6	-0.1	0.2 ^e	-274 ^e
Y82W	+0.3	-28	-0.9	n.d.	n.d.
C95S	+4.4	-14	+4.0	1.0 ^g	-287 ^g
4-108	+3.8	-9	+3.6	0.17 ^h	n.d.
4-114	-0.3	-33	-1.6	0.33 ^h	n.d.
D76E	+2.0	-6	+1.9	3.8 ^e	-274 ^e

^a $\Delta(\Delta H(T_{tr}))$ was calculated from the individual ΔH and T_{tr} values of the mutant proteins (Table 1) for $T_{tr} = 51.39^\circ\text{C}$ of the wild type using $\Delta C_p = 7.28 \pm 0.67$ kJ/mol/K except mutant H56R ($\Delta C_p = 4.29 \pm 0.37$ kJ/mol/K).

^b $\Delta(\Delta G) = (\Delta G)_{\text{mutant}} - (\Delta G)_{\text{wild-type}}$ was calculated for 37°C using ΔC_p as in footnote a.

^c Data from Beckert et al. (1994).

^d Relative activity expressed by K_M of the 11 β hydroxylase activity of adrenocortical cytochrome P450.

^e Data from V. Beckert and R. Bernhardt (in prep.).

^f Data from Uhlmann and Bernhardt (1995b).

^g H. Uhlmann (unpubl. results).

^h Uhlmann et al. (1994).

other assays (data not shown) do not parallel the thermodynamic data. That means the functional parameters are not modulated by overall protein stability, although the amino acid replacements seem to have influence on the interaction with adrenodoxin reductase and the cytochromes. The changes in redox potential observed on all T54 and H56 mutants also do not correlate with changes of thermodynamic parameters, but provide a hint for local changes affecting the ligand field around the iron-sulfur cluster.

Discussion

Figure 7 provides a sketch of Adx structure that is based on the proposed similarity of Adx with putidaredoxin (Beckert et al., 1995), in agreement with ¹H-NMR spectroscopy (Greenfield et al., 1989; Miura & Ichikawa, 1991; Beckert et al., 1995). The amino acid replacements studied in this article concern, in particular, positions proposed to be involved in electron transfer and protein-protein interaction within the mitochondrial steroid converting cytochrome P450 system. The amino acid replacements in position of T54 concern an evolutionary conserved residue that is involved in redox potential modulation (Uhlmann & Bernhardt, 1995b). The replacements T54S and T54A resemble the situation demonstrated on the replacement of T157I in phage lysozyme T4 (Alber et al., 1987). Similarly, replacement T54A disrupts a side chain H-bond in which threonine is involved. The replacement is accompanied by a substantial reduction in conformational stability in contrast to the T54S mutant.

Fig. 4. Limited proteolysis of the Adx mutant proteins by thermolysin at 45°C , ratio protein:thermolysin = 1:250. **A:** Wild type and mutant 4-108. Lanes 1-5, wild type, incubation time 0, 3, 5, 10, and 15 min; lane 6, molecular weight marker; lanes 7-11, mutant 4-108, incubation time 0, 3, 5, 10, and 15 min. **B:** Mutants T54A and T54S. Lanes 1-5, mutant T54A, incubation time 0, 3, 5, 10 and 15 min; lane 6, molecular weight marker; lanes 7-11 mutant T54S, incubation time 0, 3, 5, 10, and 15 min.

Histidine 56 seems to play a key role in stabilization of Adx structure. Replacement by Q reduces the enthalpy change by 100 kJ/mol, and the Gibbs energy change by about 7 kJ/mol. These changes represent rather dramatic effects, bearing in mind that frequently small changes in $\Delta(\Delta G)$ observed on amino acid replacements may mask larger changes in transition enthalpy and entropy (Sturtevant, 1994). In the case of the replacement H56T, the changes of T_{tr} , ΔH , and $\Delta(\Delta G)$ remain significant, but less pronounced than those of H56Q, probably due to the potential of threonine to establish a hydrogen bond via side chain. Nevertheless, the order of magnitude in decrease of $\Delta(\Delta G)$ resembles the most drastic effects ever observed on histidine replacements, e.g., H18 of barnase (Matouschek et al., 1989; Serrano et al., 1992) and H36 of apomyoglobin (Barrick et al., 1994). Whereas the replacements H56Q and H56T disturb the balance between stabilizing and destabilizing contributions in Adx, the replacement of His 56 in Adx by arginine produces a conformational change. In that case, a moderate decrease of transition temperature of 2.4°C is accompanied by about 100 kJ/mol decrease in ΔH . At the same time, the heat capacity change is reduced for H56R to $\Delta C_p = 4.29 \pm 0.37$ kJ/mol/K compared with $\Delta C_p = 7.28 \pm 0.67$ kJ/mol/K for wild type and the other mutants. In the current understanding of heat

Fig. 5. Absorption spectra of Adx in sulfide and mercaptoethanol containing buffer in the presence of guanidine hydrochloride measured at 25 °C. 1, Adx without GuHCl; 2–4, in 6 M GuHCl. 2, 1 min; 3, 20 min; 4, 90 min after mixing with GuHCl. Inset: Absorption spectra of Adx without protectant buffer in the presence of 6 M GuHCl. 1, 1 min; 2, 20 min; 3, 90 min after mixing with GuHCl.

capacity changes (Makhatazde & Privalov, 1990; Privalov & Makhatazde, 1992), parts of the polypeptide chain are probably exposed to the solvent in H56R. This finding is further supported by the unusual fluorescence intensity of H56R (Fig. 8, inset), which indicates distortion of the region around Tyr 82. In fact, H56 is

Fig. 6. Absorption spectra of renatured Adx at 25 °C (see text). 1, Native protein; 2, renatured with urea for 24 h and then subjected to rechromatography; 3, subjected to rechromatography immediately after thermal denaturation; 4, renatured with urea for 24 h and then dialyzed; 5, dialyzed immediately after thermal denaturation.

Fig. 7. Location of the amino acid replacements studied in this paper based on a model of Adx structure from Beckert et al. (1995).

located close to Y82, as was shown in a previous NMR study (Beckert et al., 1995). Obviously, the Adx fold does not tolerate the introduction of a voluminous residue carrying a positive charge when replacing His 56 by arginine.

Point mutations of Y82 affect binding to the cytochromes P450 (Beckert et al., 1994), but produce little effect on T_m , ΔH , and $\Delta(\Delta G)$. Tyr 82 is proposed to be solvent exposed. In agreement with the proposal, replacement by serine, leucine, phenylalanine, and even by tryptophan does not produce large changes of transition temperature and enthalpy. Because Tyr 82 is involved in complex formation with cytochromes P450, any replacements produce changes in functional properties (Table 2).

Fig. 8. Molar heat capacity of mutant H56R and wild type (broken line). Inset: Fluorescence spectra of mutant H56R and wild type measured in 10 mM potassium phosphate, pH 7.4.

Cys 95 is, in contrast to the cysteine residues in positions 46, 52, 55, and 92, not involved in the complexation of the iron-sulfur cluster, as was shown by Uhlmann et al. (1992). In fact, no destabilization of the protein can be found on replacement C95S, thus further supporting the former findings.

Surprisingly, the deletion mutant consisting of residues 4–108 shows enhanced transition temperature, enthalpy change, and Gibbs energy change compared with wild type. Although the reduced chain entropy in the deletion mutant could be responsible for the enhanced stability compared with wild type, there is an additional increase in enthalpy change. The increase in enthalpy becomes more visible when comparing the specific values, i.e., Δh in J/g. Δh for the wild-type protein amounts to 26.9 J/g, compared with $\Delta h = 31.2$ J/g for the deletion mutant 4–108 (both values at $T_r = 55.2^\circ\text{C}$, the transition temperature for the 4–108 mutant). At the same time, the deletion mutant 4–108 shows slightly altered EPR and CD spectra compared with the wild-type protein (Uhlmann et al., 1994). Interestingly, the K_M value of this mutant decreased compared with the wild type. Together with the calorimetric data, these findings suggest structural differences between the deletion mutant and wild type that remain open until structure analysis is completed. On the other hand, the deletion mutant 4–114, which contains two additional aspartate residues in positions 109 and 113, is less stable and unfolds with lower expenditure in heat (the specific enthalpy Δh corresponds to the wild type), although its K_M value is also decreased compared with wild-type Adx. Thus, the hydrophilic C-terminal sequence 108–128, which could not be allocated in model building of Adx, produces unfavorable interactions rather than contributing to the overall protein stability. On the other hand, further cleavage of C-terminal residues, in particular removal of Pro 108, leads to loss of the capability for cluster assembly in Adx (Uhlmann et al., 1994).

Limited proteolysis parallels the calorimetric results. Pairwise comparison of mutants by thermolysin treatment slightly below the thermal transition temperature always shows higher degradability of those mutants that are less stable in calorimetry. This means that the higher the equilibrium concentration of the thermally denatured protein, the higher the degradability.

The transition temperature observed by CD in the far ultraviolet-region agrees with the calorimetric values (Uhlmann & Bernhardt, 1995b). Remarkably, the changes in optical absorption of Adx on heating do not completely coincide with the calorimetric scans. However, the changes of optical absorption at 415 nm take place around $55.8 \pm 0.7^\circ\text{C}$, i.e., distinctly above the conformational transition and at the same time identically for all the mutant proteins, irrespective of their different conformational stability. This suggests that the origin of the optical changes at 415 nm is not related to unfolding of the polypeptide chain.

Ferredoxin denaturation in the presence of thiol reagents was first studied by Averill and Orme-Johnson (1978). It was found that the spectral properties of the cluster may remain almost unchanged even after displacement from the polypeptide chain (Averill et al., 1978). Similarly, both heat and guanidine hydrochloride denaturation produce only small changes in optical absorption at 415 nm in the presence of the sulfide-containing buffer. Without protectant, pronounced optical changes occur in protein unfolding studies due to simultaneous destruction of the cluster. Although in denaturation studies, prosthetic groups are frequently used to monitor protein unfolding, the procedure may fail in case of ferredoxins in determining thermodynamic quantities.

The buffer conditions used in the present article allow reconstitution of holo Adx in a slow process as judged by EPR and optical

spectroscopy (see Results). Moreover, the protein can be renatured under the influence of urea (Burova et al., 1995). Therefore, the equilibrium thermodynamic treatment applied in this article appears to be justified.

In conclusion, some findings should be emphasized. Adx and the mutants studied in this article show rather low conformational stability, ranging from about 6 to 17 kJ/mol at physiologic temperature. Probably, Adx, having marginal stability, is favorable in forming electron transfer complexes with the various macromolecules. However, no simple correlation between conformational stability and functional properties could be found (see Results). Pronounced structural changes in the region of H56 and Y82 that reduce even the heat capacity change are produced by the replacement H56R. Furthermore, the increased conformational stability of the mutant 4–108, which lacks a considerable part of the C-terminal residues, is remarkable. The differences in denaturation enthalpy observed on the mutant proteins are in agreement with the original findings by Perutz and Raid (1975). The denaturational transition in the protectant buffer is a two-state process. However, the mechanism of Adx denaturation is complex due to the properties of the cluster that render any studies by optical absorption more difficult.

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 (Beckert et al., 1994, 1995; Uhlmann et al., 1994; Uhlmann & Bernhardt, 1995b). The ratio of optical density at 415 and 276 nm was $Q \geq 0.92$ (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.

Calorimetry

Scanning calorimetric measurements were conducted on a DASM-4 microcalorimeter (NPO "Biopribor," Puschino, Russia) equipped with a DASM-1M gold cell. Thermograms were in general obtained at a scan rate of 60 K/h. Adx solutions for calorimetric measurements were prepared by chromatography prior to use on a 1×10 cm Sephadex G25 column using carefully degassed buffer. Sample handling was made under argon. The concentration of Adx after chromatography 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 $E = 0.247 \text{ mg}^{-1} \text{ cm}^{-1}$ 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 $14,015.5 \pm 2.2$, which corresponds to the theoretical one of 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 0.5–2.0 mg/mL for Adx.

Mathematical treatment of the calorimetric scans was made by nonlinear regression using a two-state model that approximates pre- and postdenatural slope by polynomial expression. The programs were written in Turbo-Pascal.

Optical 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 of 30 K/h. Further details of the procedure are given by Burova et al. (1995).

Fluorescence spectroscopy

Fluorescence emission spectra of Adx solutions in 10 mM potassium phosphate, pH 7.4, were measured at room temperature with an RF-5001 PC spectrofluorimeter by exciting at 270 nm. Concentration of holo protein was 0.07 mg/mL. Measurements were made in a 1-cm cell.

Electron spin resonance

EPR spectra were measured with Varian E3 spectrometer under following conditions: microwave power 5–10 mW; modulation amplitude 0.4–1.0 mT; scan rate 6.25 mT/min; time constant 0.3 s, temperature –163 °C. For recording EPR spectra, protein samples were reduced with dithionite.

Limited proteolysis and electrophoresis

Controlled proteolysis was performed at elevated temperature (45 °C if not otherwise indicated) by thermolysin (EC 3.4.24.4; Boehringer) as described by Pfeil (1993). SDS-PAGE was conducted in a discontinuous Tris-Tricine system (Schägger & von Jagow, 1987) on 16% gel. The molecular mass was identified relative to the Pharmacia myoglobin fragment standard.

Functional activity and redox potential

For the procedures, see Beckert et al. (1994) and Uhlmann and Bernhardt (1995b).

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