The role of quaternary interactions on the stability and activity of ascorbate peroxidase

DAVID MANDELMAN,¹ FREDERICK P. SCHWARZ,² HUIYING LI,¹ AND THOMAS L. POULOS¹

¹University of California at Irvine, Department of Molecular Biology and Biochemistry, Irvine, California 92697-3900

²Center for Advanced Research in Biotechnology/National Institute of Standards and Technology, 9600 Gudelsky Drive, Rockville, Maryland 20850

(RECEIVED June 4, 1998; ACCEPTED June 15, 1998)

Abstract

Point mutations at the dimer interface of the homodimeric enzyme ascorbate peroxidase (APx) were constructed to assess the role of quaternary interactions in the stability and activity of APx. Analysis of the APx crystal structure shows that Glu112 forms a salt bridge with Lys20 and Arg24 of the opposing subunit near the axis of dyad symmetry between the subunits. Two point mutants, E112A and E112K, were made to determine the effects of a neutral (alanine) and repulsive (lysine) mutation on dimerization, stability, and activity. Gel filtration analysis indicated that the ratio of the monomer to dimer increased as the dimer interface interactions went from attractive to repulsive. Differential scanning calorimetry (DSC) data exhibited a decrease in both the transition temperature (T_m) and enthalpy of unfolding (ΔH_c) with $T_m = 58.3 \pm 0.5^\circ\text{C}$, $56.0 \pm 0.8^\circ\text{C}$, and $53.0 \pm 0.9^\circ\text{C}$ and $\Delta H_c = 245 \pm 29$ kcal/mol, 199 ± 38 kcal/mol, and 170 ± 25 kcal/mol for wild-type APx, E112A, and E112K, respectively. Similar changes were observed based on thermal melting curves obtained by absorption spectroscopy. No change in enzyme activity was found for the E112A mutant, and only a 25% drop in activity was observed for the E112K mutant which demonstrates that the non-Michaelis-Menten kinetics of APx is not due to the APx oligomeric structure. The cryogenic crystal structures of the wild-type and mutant proteins show that mutation induced changes are limited to the dimer interface including an alteration in solvent structure.

Keywords: ascorbate peroxidase; differential scanning calorimetry; quaternary structure; salt bridges; solvent structure; subunit interface; thermostability

Ascorbate peroxidase (APx) is a homodimeric, heme containing enzyme that is used by plants to catalyze the reduction of hydrogen peroxide to water at the expense of ascorbate. This function is important for the plant to regulate the levels of potentially damaging oxidative radicals that can accumulate in photosynthetic tissues. As is the case with many heme peroxidases, APx shows little substrate specificity, and demonstrates appreciable activity in the presence of other electron donors such as guaiacol and potassium ferrocyanide. Limited kinetic analyses of APx have been carried out (Marquez et al., 1996) to analyze the rates of formation of the various catalytic intermediates. The initial work of Mittler and Zilinskas (Mittler & Zilinskas, 1991) showed that the reaction of APx with ascorbate does not give normal Michaelis-Menten ki-

netics, a result we have confirmed in the present study. While the overall fold of APx is the same as other nonmammalian heme peroxidases, APx is unusual in forming a homodimer. Therefore, the nonhyperbolic curves obtained when activity is measured as a function of substrate concentration suggests, possibly, cooperative kinetics due to subunit interactions. Nevertheless, a thorough mechanistic model has not been established to determine whether or not cooperativity plays a part in APx.

In addition to the dimeric structure of APx possibly contributing to enzyme activity, it is of interest also to understand the contribution of the dimer interface to stability. Studies with various multimeric proteins such as the repressor protein, ROP (Steif et al., 1993), aspartate transcarbamylase (Vickers et al., 1978), and rabbit aldolase A (Beernink & Tolan, 1994) illustrate how quaternary interactions provide varying levels of stability to the overall molecule. ROP, for example, is only partially stabilized by dimer interactions and can form stable monomers in 2.5 M guanidine. Arc repressor, on the other hand, seems to be completely stabilized by dimer interactions (Silva et al., 1992) since the ΔG of unfolding is equal to ΔG of dissociation. Oligomeric proteins such as arc repressor, tryptophan repressor (TR) (Shao et al., 1997), and triose

Reprint requests to: Thomas L. Poulos, University of California, Irvine, Department of Molecular Biology and Biochemistry, Irvine, California 92697-3900; e-mail: poulos@uci.edu.

Abbreviations: APx, ascorbate peroxidase; DSC, differential scanning calorimetry; E112A, APx Glu112 to Ala mutant; E112K, APx Glu112 to Lys mutant; RMSD, root-mean-square deviation; VAS, visible absorbance spectroscopy; ΔH_c , calorimetric enthalpy; ΔH_v , Van't Hoff enthalpy.

phosphate isomerase (TIM) (Borchert et al., 1994; Schliebs et al., 1997) utilize primarily buried, hydrophobic interactions at the dimer interface. Monomeric mutants of TR and TIM were made by residue substitution and/or deletion at the interface that rendered the interfaces less hydrophobic and more stable to a solvated environment. In the case of TR, a single residue substitution at the hydrophobic interface was sufficient to generate a monomeric variant. APx is different from these other well-studied multimeric proteins since ionic interactions and hydrogen bonding dominate at the dimer interface (Patterson & Poulos, 1995). Moreover, solvent molecules play an important role by bridging ionic groups at the interface. Therefore, disruption of ionic pairs at the interface of APx should decrease the affinity of the subunits for one another similar to changing the hydrophobic interface in other multimeric proteins.

The focus of the present paper is to investigate the effects of quaternary structure perturbation on the overall stability and function of APx.

Results

The dimer interface

Analysis of the crystal structure of APx (1APX) (Patterson & Poulos, 1995) reveals the primarily electrostatic nature of the dimer interface (Fig. 1). The single water molecule H-bonded with Glu106 in each monomer is at the center of symmetry relating the two monomers. In Table 1 are listed these and other intersubunit interactions ≤ 4.0 Å. One water bridges between the carbonyl O atom of Gly110 and the side chain of Arg24 in the opposing subunit. In addition, two water molecules bridge between the two Glu106 residues. These interactions also are maintained in the second dimer pair in the asymmetric unit. In addition to polar interactions, there are potentially important nonpolar interactions. Ile107 and Ala28 interact with their counterparts in the opposing subunit thereby providing important nonpolar interactions (Fig. 1). Nevertheless, since it appears that polar interactions dominate the

Fig. 1. A backbone ribbon trace of the crystal structure of APX. A water molecule (red sphere) lies at the noncrystallographic twofold axis. The lower figure is a magnification of this region highlighting some of the critical noncovalent interactions holding the subunits together. Figures 1–3 were made with SETOR (Evans, 1993) or MOLSCRIPT (Kraulis, 1991).

Table 1. Subunit interactions ≤ 4.0 Å in wild-type APX

Interface waters				
Subunit A	Distance (Å)	H ₂ O	Distance (Å)	Subunit B
E106–O,G110–O,NH	2.7,2.7,3.5	Y68	3.0	R24-NH1
R24–NH1	3.1	Y3	2.7,2.8,3.6	E106-O,G110-NH,G110-O
R21–NH1	2.9	X557	3.1	E112-OE2
E106-OE2	2.8	Y33	3.4,2.9	R24-NE,E106-OE1
R21–NH1	3.6	X71	3.0	E112-NH
E112-OE2	3.0	X231	3.3,2.8,3.0	E17-O,E17-OE2,R21-NH1
R24-NH2	3.6	X448	3.7,3.0,3.6	R24-NH2,E106-OE2,E112-OE1
G109-O	2.7	Y449	3.5	K22-NH
D229-OD2	2.8	Y71	3.0,3.0	R21-NH,NH2
E228-OE2	3.4	Y72	2.9	K22-NZ
L184-O	2.7	Y6	2.8,3.4	G25-O,Q29-N
L184-NH	3.1	X56	2.8	E29-OE1
R24-NH1,NH2	3.0,3.6	X564	2.8	E106-OE2
E106-OE1	3.2	Y209	3.5	R24-NH1
R24-NH1	3.1,3.4	X262	2.9	E106-OE2
Charge–charge interactions				
Subunit A	Distance (Å)		Subunit B	
E112-OE2	3.0		R24-NH1	
E112-OE2	3.1		R24-NH2	
R24-NH1	2.9		E112-OE2	
R24-NH2	3.0		E112-OE2	
K14-NZ	3.0		D229-OD1	
K18-NZ	3.5		D229-OD2	
D229-OD1	3.2		K18-NZ	
D229-OD2	2.7		K18-NZ	
Subunit C			Subunit D	
E112-OE1	3.0		K20-NZ	
K20-NZ	3.1		E112-OE1	
Nonpolar contacts				
Subunit A	Distance (Å)		Subunit B	
A28-CB	3.6		I107-CG2	
I107-CD1	3.6		I107-CD1	
T108-C	3.4		G25-CA	
G25-CA	3.3		T108-C	
G25-C	3.7		I185-CD1	
I185-CD1	3.6		G25-CA	

group of noncovalent forces at the dimer interface, perturbation of these interactions should have a major impact on the stability of the dimeric molecule.

Glu112 was chosen as the electrostatic partner to mutate. Analysis of the two dimers in the asymmetric unit reveals that Glu112 forms a salt bridge with Arg24 in dimer A/B and Arg24 and Lys20 in C/D. Thus, Glu112 participates in 4 of the 10 ionic interactions at the dimer interface due to the dyad symmetry of APx. If electrostatic interactions are important in holding the dimer together, then disruption of the Glu112–Arg24/Lys20 interactions should

have a major impact on the stability of the dimer. Two substitutions were chosen for this site: a neutral substitution with alanine, E112A, and a repulsive substitution with a lysine, E112K.

Cryocrystallography

Because crystals of the mutants were relatively unstable in the X-ray beam at room temperature, it was necessary to collect X-ray intensity data at cryogenic temperatures by flash freezing crystals in a stream of cold nitrogen gas. As a basis for comparison, a

cryogenic data set also was obtained for wild-type APx. Flash frozen crystals diffract to higher resolution so it was possible to extend the data from the room temperature limit of 2.2 to 1.8 Å.

For the native APx cryo data set, a round of rigid body refinement was carried out using the room temperature model to properly position the four APx subunits in the asymmetric unit (two dimers per asymmetric unit). Very little movement of the molecules was required to find a solution because the reduction in unit cell dimensions for the cryo data was very small (Table 2). As expected, the number of ordered solvent molecules visible in the electron density maps substantially increased. Five hundred thirty-two additional waters were added to the model giving 1,181 water molecules. In addition, two ethylene glycol molecules were located. The refinement of the wild-type structure required only a few rounds of positional refinement, model building, and temperature factor refinement to give a final *R*-factor of 0.19 (Table 2). The cryogenic wild-type structure was used as the starting model for the mutant structures. The site of mutation was initially modeled as Ala followed by refinement. In the case of the E112K mutant, the Lys side chain was modeled into $2F_o - F_c$ and $F_o - F_c$ maps followed by positional refinement. The RMS deviation (RMSD) of main-chain atoms between the room temperature and cryo wild-type models is 0.42 Å. The RMSDs between the cryo wild-type and E112A and E112K mutants are, respectively, 0.17 and 0.19 Å.

Figures 2A and 2B are omit maps of the E112A and E112K mutants, respectively. There is a clear loss of density for the alanine mutant at position 112 with a minor bump in the density for the beta carbon. The lysine mutant, on the other hand, has defined density up through the gamma carbon of the side chain but the remainder of the density is disordered and cannot be modeled. In the wild-type structure, Glu112 is well ordered owing to the strong interaction with Arg24 in the opposing subunit. The ionic partner to the missing glutamate, Arg24, is still well ordered in the mutants owing to intrasubunit ionic interactions with Glu106 (Fig. 1).

The loss of the Glu side chain in the mutants leaves space that fills with new water molecules. The location of these water molecules is highlighted in Figure 3. The E112A mutant retains the bridging water molecules found in the wild-type structure but the new water molecules do not form a continuous hydrogen bonded bridge between subunits. In addition, the temperature factors for these water molecules range from 26–41 Å² in comparison with the conserved bridging waters that have temperature factors <9.0 Å². Therefore, these new water molecules in the mutant are relatively mobile and do not form important intersubunit interactions but simply take up the space once occupied by the Glu112 side chain.

Concentration dependence of dimerization

A highly reproducible method of gel filtration using an FPLC system and an analytical Superdex-75 column was employed to determine the apparent molecular weight of APx at different protein concentrations (Fig. 4). Wild-type APx exhibits a slight decrease in apparent molecular mass as the concentration of protein is decreased from 14 to 0.25 μM, the lower bounds for reliable detection. At the highest concentration measured, 14 μM, the apparent molecular mass of APx is 54.4 kDa, a close approximation to the previously determined molecular weight of 57.5 (Mittler & Zilinskas, 1991) using gel filtration. At the lowest concentration, 0.25 μM, the apparent molecular mass of APx is 45.2 kDa, which is only a 17% decrease from its dimeric molecular mass. The E112K mutant has an apparent molecular mass of 51 kDa at a concentration of 50 μM and drops to 31.4 kDa at 0.25 μM approaching the molecular mass of an APx monomer, 27.7 kDa, as determined by amino acid analysis plus heme content. The range of molecular masses for the E112K curve proceeds from monomer to dimer and allows an estimation of 2–5 μM, the approximate midpoint of the curve, for the dissociation constant, *K_D*, of the dimer. The elution behavior of the E112A mutant falls between that of wild-type APx and the E112K mutant. At 44 μM, the

Table 2. Summary of data collection statistics and crystallographic refinement

	rt APX ^a	cryo APX ^b	cryo E112A ^b	cryo E112K ^b
<i>a</i> (Å)	132.4	130.8	131.0	131.0
<i>b</i> (Å)	53.0	51.9	52.1	52.1
<i>c</i> (Å)	170.6	168.3	168.3	168.2
β (°)	107.1	107.5	107.5	107.5
Reflections observed	184,416	185,312	160,794	159,556
Unique reflections	73,104	88,355	68,858	66,861
<i>R</i> _{sym} ^c	7.3	7.4	7.1	5.5
% Overall completeness	91	94	93.3	90.6
% Complete (highest resolution shell)	91 (2.2)	77 (2.0–1.8)	80.4 (2.2–2.0)	66 (2.15–2.0)
<i>R</i> -factor ^d	19	19.1	18.8	19.4
<i>R</i> _{free}	—	24.5	23.5	24.6
% Reflections with <i>I</i> / σ <i>I</i> ≥ 2	90.7	80.4	76.1	70.5
Reflections used for refinement $ F > 2\sigma F $	51,337	85,971	68,032	66,114
RMSDs ^e bonds (Å)	0.007	0.007	0.007	0.007
RMSDs ^e angles (°)	1.3	1.3	1.3	1.3

^aPatterson and Poulos (1995).

^bCryogenic conditions as described in Materials and methods.

^c $R_{\text{sym}} = \sum |I_i - \langle I_i \rangle| / \sum I_i$, where *I_i* is the intensity of the *i*th observation and $\langle I_i \rangle$ is the mean intensity.

^d $R = \sum |F_o| - |F_c| / \sum |F_o|$; *R*_{free} = free *R* factor based on 5% of the reflections.

^eThe RMSDs of bond parameters represent the RMSDs from ideal values.

Fig. 2. Omit maps of (A) E112A and (B) E112K. Electron density maps were contoured to 1σ .

protein is completely dimeric, but at $0.25 \mu\text{M}$ the apparent molecular mass is 45 kDa, approximately the same as wild-type APx at $0.25 \mu\text{M}$.

APx activity

Figure 5 shows k_{cat} vs. [ascorbate] curves for wild-type APx and the 112 mutants. As noted (Mittler & Zilinskas, 1991), APx does not exhibit normal Michaelis–Menten kinetics. Therefore, V_{max} and K_m values cannot be obtained from traditional double reciprocal plots. Instead, V_{max} is reported as the maximal velocity observed once saturation is achieved. The activities of wild-type APx and E112A are essentially the same with $V_{\text{max}} \cdot 160 \text{ s}^{-1}$. The apparent V_{max} of E112K is $\approx 120 \text{ s}^{-1}$, that is, about 25% less active than wild-type APx.

The data were also fit to a cooperative model using the Hill equation to describe the catalytic rates (data not shown). The Hill model involved an exponential term, the Hill coefficient, n , to

Fig. 3. Stereo view of the dimer interface showing the bridging water molecules. Monomer A is green; monomer B is yellow. The bridging water molecules are red while the water situated at the center of symmetry between the two Glu106 residues is orange. (A) Wild-type APx and (B) E112A mutant. The new water molecules in the mutant taking the place of the missing Glu112 side chain are blue.

describe the sigmoidicity, or cooperativity, of the curve. All three curves were linear and resulted in fits with a Hill coefficient of 1.3–1.4. A Hill coefficient >1.0 might be suggestive of some cooperative behavior between subunits. The sensitivity of the FPLC results did not allow for the reliable detection of protein concentrations $<250 \text{ nM}$ and, since wild-type APx is still a dimer at 250 nM , we do not know if wild-type APx is a monomer or dimer at the dilute concentrations used for enzyme assays. However, the E112K mutant must be a monomer at the concentration used in the enzyme assays (20 nM) since the FPLC results clearly show that the E112K mutant is a monomer at 250 nM . Therefore, the non-Michaelis–Menten kinetics and Hill coefficients >1.0 are not due to the dimeric structure of APx.

Fig. 4. Concentration dependence on apparent molecular weight. APX (○), E112A (△), and E112K (□) were diluted in column equilibration buffer to the indicated concentrations and elution was monitored by absorbance at 280 nm.

Differential scanning calorimetry

To directly measure the thermodynamic parameters of conformational stability, DSC was used to determine the enthalpy and transition temperature of thermal denaturation for APx and the 112

Fig. 5. The kinetics of ascorbate oxidation by APX and the 112 mutants. Enzyme concentrations: 19 nM APX (○), 22 nM E112A (△), and 23 nM E112K (□). Results are the average of three independent measurements $\pm$ the standard deviation ($n = 3$).

Fig. 6. Thermograms of solutions of APX and the 112 mutants from DSC (solid lines). The samples were 0.08 mM (monomer) of APX, 0.12 mM E112A, and 0.1 mM E112K. The buffer was 50 mM KPO₄ buffer containing 0.15 M NaCl at pH 7.0 and the scan rate was 15 K/h. The fit of the two-state transition model (dashed lines) to the thermograms is also shown.

mutants. Figure 6 shows the DSC scans of APx and the two mutants at concentrations where the protein exists exclusively as a dimer at room temperature. To apply thermodynamic theory to the protein unfolding problem, reversibility had to be tested by rescanning the samples after cooling. The lack of any detectable thermogram upon rescanning indicated that the proteins were thermally unfolding irreversibly. Equilibrium thermodynamic theory has been applied to irreversible systems (Manly et al., 1985) in which the transition is calorimetrically irreversible, but the irreversible step takes place at a temperature above the transition and therefore the transition can be analyzed by equilibrium thermodynamics (Sanchez-Ruiz, 1995; Privalov & Potekhin, 1986). This was originally proposed as the Lumry-Eyring model:

where F = folded, U = unfolded, and Ag = aggregated protein. The irreversible reaction $U \rightarrow Ag$ effectively decreases the concentration of U in the reversible reaction, $F \leftrightarrow U$, and concomitantly affects the T_m of the protein. This effect becomes greater as the scan rate decreases. But, if $k_1, k_2 \gg k_3$ then T_m will be independent of scan rate. The APx and 112 mutant thermograms at scan rates of 15 and 30 °C/h are indistinguishable and thus the transition temperature (T_m), Van't Hoff enthalpy (ΔH_v) and calorimetric enthalpy (ΔH_c) for each protein are independent of scan rate and can effectively be analyzed by equilibrium thermodynamic theory. In addition to scan rate independence on T_m , protein concentration has no effect on the T_m of the transition. This suggests the dimer does not dissociate when it unfolds. The thermograms were analyzed with a two-state, $N_2 \leftrightarrow D_2$ model, which varies parameters of ΔH_v and T_m to fit the raw data (Kirchhoff, 1993). For cooperative, two-state denaturation, ΔH_c estimated from the area under the DSC curves should equal the ΔH_v . For a dimeric protein this ratio would be one-half, because the calorimetric enthalpy per subunit would be one-half the predicted Van't Hoff enthalpy for unfolding of a dimer as is indeed observed by the ratio of 0.7 ± 0.2 (Table 3).

Table 3. Thermodynamic parameters for the thermal unfolding of APX and the 112 mutants

	T_m (°C)		ΔH_v (kcal/mol) ^a		ΔH_c (kcal/mol) ^a	$\Delta\Delta G^b$ (kcal/mol) ^a	$\Delta H_c/\Delta H_v$
	DSC	VAS	DSC	VAS	DSC		DSC
APX ^c	58.3 ± 0.5	57.9	330 ± 26	191 ± 5	245 ± 29	0.0	0.75 ± 0.14
E112A ^c	56.0 ± 0.8	55.1 ± 1.2	295 ± 18	145 ± 9	199 ± 38	-1.4	0.68 ± 0.15
E112K ^c	53.0 ± 0.9	53.3 ± 1.5	232 ± 32	124 ± 19	170 ± 25	-2.8	0.75 ± 0.18

^aValues are calculated per mol of monomer.^b $\Delta\Delta G = \Delta G_{mut}(T_{ref}) - \Delta G_{wt}(T_{ref}) = \Delta H_{m,mut}(1 - T_{ref}/T_{m,mut}) + \Delta C_p[T_{ref} - T_{m,mut} - T_{ref}\ln(T_{ref}/T_{m,mut})]$.^cResults are the average of at least four independent measurements and reported as mean ± standard deviation.

Table 3 provides a summary of the DSC experiments. The average T_m for the proteins are 58.3, 56.0, and 53.0 °C for APx, E112A, and E112K, respectively. In accord with these results, the average calorimetric enthalpies of unfolding for the proteins are 245 ± 29, 199 ± 38, and 170 ± 25 kcal/mol monomer for APx, E112A, and E112K. The trend indicate a decrease in the stability of the protein as the level of ionic attraction at the dimer interface decreases.

Thermal unfolding monitored by spectroscopy

The thermal stability of the APx dimer was also monitored by spectroscopy. The heme of peroxidases absorbs strongly near 400 nm and was used as an indicator of protein unfolding. Difference absorbance spectra showed a maximal difference at 405 nm between folded and thermally unfolded APx. Figure 7 shows the results of monitoring the change in absorbance at 405 nm as a

function of temperature. The inflection point of each curve represents the midpoint, T_m , between folded and unfolded species. To compare the DSC data to the spectral technique, the same two-state model was applied to fit the data and determine the T_m and ΔH_v of unfolding.

The measured transition temperatures for unfolding are 57.9, 55.1, and 53.3 °C for APx, E112A, and E112K, respectively, which agree well with the DSC determined T_m (Table 3). The calculated ΔH_v for wild-type APx, E112A, and E112K are 191, 145, and 124 kcal/mol monomer, respectively. Although the T_m values agree very well with the DSC data, the enthalpies are lower but do follow the same trend. The generally good agreement between the spectral melting analysis and DSC data indicates that the relatively simple and rapid spectral method can be used to analyze the effect of mutations, ligands, and substrate on stability. Substrate binding very often provides additional stability to enzymes (Vickers et al., 1978; Fukada et al., 1983). However, a 100-fold molar excess of ascorbate had no effect on the melting curves.

Discussion

Dimeric structure and stability

On removing Glu112 and its associated inter-subunit ionic interactions, the APx dimer/monomer equilibrium shifts toward monomers. The FPLC gel filtration studies demonstrate that at low protein concentrations, the E112K mutant behaves as a monomer while wild-type APx is primarily a dimer at the lower limit of concentrations, 250 nM, detectable by the FPLC method. The interface mutants are also less stable than wild-type APx, as indicated by the lowering of T_m and a decrease in ΔH of unfolding. The DSC results show that the protein unfolding follows a simple two-state transition model where the folded protein dimer unfolds without any change in the oligomeric state.

The fits to the raw DSC data were obtained using an $N_2 \leftrightarrow D_2$ model of protein dimer unfolding without dissociation. The use of this model is supported by the fact that the T_m was independent of protein concentration indicating a monomolecular unfolding event. Ghosaini and coworkers (Ghosaini et al., 1988) noticed similar behavior in T_m vs. protein concentration with the tetrameric catabolite activator protein (CAP), and modeled it as unfolding without dissociation. But this does not preclude the possibility of another mode of APx unfolding. It is argued (Sturtevant, 1987) that the accuracy of data fitting alone is insufficient to distinguish between different unfolding models, especially if the dissociation and un-

Fig. 7. Thermal denaturation curves of APX and the 112 mutants monitored by visible absorbance spectroscopy at 405 nm. Proteins were diluted in 25 mM, degassed KPO₄ buffer, pH 7.0, to a final concentration of: 6.3 μM APX (○), 5.6 μM E112A (□), and 6.7 μM E112K (Δ). Curves were fit (solid lines) to Equation 7 in Materials and methods.

folding are close together in temperature. Thus, we do not rule out the possibility of a dissociative event buried beneath the enthalpy of unfolding curve in the DSC transitions. Nonetheless, similar DSC analyses were done on the wild-type and mutant proteins because qualitative examination of the DSC transitions did not reveal any changes in the symmetrical, bell shapes of the curves, only shifts in the midpoints as the dimer interactions decreased. Thus, the mutations destabilize the dimer under thermal conditions without causing it to undergo dissociation.

Using the enthalpies and transition temperatures obtained from the DSC experiments, comparisons can be made between the free energies of stability of the wild-type and the 112 mutants. The Gibbs–Helmholtz equation can be used to calculate ΔG values at any temperature (T) relative to the midpoint transition (T_m):

$$\Delta G(T) = \Delta H_m(1 - T/T_m) + \Delta C_p[(T_m - T) - T \ln(T/T_m)]. \quad (1)$$

This equation can be modified to calculate the differences in ΔG values of the wild-type protein at a reference temperature, $T_{\text{ref}} = T_m$, vs. the 112 mutants at their T_m 's ($T_{m;\text{mut}}$) (Liggins et al., 1994). Substituting Equation 1 into $\Delta\Delta G(T_{\text{ref}}) = \Delta G_{\text{mut}}(T_{\text{ref}}) - \Delta G_{\text{wt}}(T_{\text{ref}})$ yields

$$\begin{aligned} \Delta\Delta G(T_{\text{ref}}) = & \Delta H_{m;\text{mut}}(1 - T_{\text{ref}}/T_{m;\text{mut}}) \\ & + \Delta C_p[(T_{\text{ref}} - T_{m;\text{mut}}) - T_{\text{ref}} \ln(T_{\text{ref}}/T_{m;\text{mut}})]. \quad (2) \end{aligned}$$

ΔC_p in Equation 2 is obtained from the difference in heat capacities between the folded and unfolded states of the wt protein at the transition midpoint. Any error in the ΔC_p calculation by this method is compensated for by the negligible contribution of ΔC_p to the overall $\Delta\Delta G$ calculation. For example, the wt ΔC_p of 0.17 kcal/(mol K) was used to yield the values seen in Table 3 for $\Delta\Delta G$. A 10-fold increase in ΔC_p yields $\Delta\Delta G$ values of -1.40 and -2.83 kcal/mol for E112A and E112K, respectively. In addition, inaccuracies in ΔC_p extrapolated to other temperatures are minimized over the small temperature range from 58.3 to 53 °C.

Structurally, the changes at the interface are modest. In the E112K mutant, the new Lys112 side chain is disordered presumably due to electrostatic repulsions at the interface. The mutations also leave sufficient room for new waters to take the place of the missing Glu112 side chain. A similar phenomenon has been seen when a Trp is replaced by an Asp on the Fv binding fragment of a monoclonal antibody (Ysern et al., 1994). The void created in the lysozyme antigen-Fv interface by replacement of Trp by Asp resulted in two water molecules occupying the empty space. It appears that the polar dimer interface of APx has a greater ability to adapt to perturbations owing to the ability of solvent to substitute for side-chain–side-chain interactions. This contrasts sharply with the rigid structure of hydrophobic interfaces seen in many other oligomeric proteins.

Solvent structure at the dimer interface

The higher resolution cryogenic structures helped to clarify the role of solvent molecules at the dimer interface. Sixteen water molecules participate in 25 interactions ≤ 4.0 Å at the interface of the wild-type structure. In addition, there are eight strong charge interactions of ≤ 3.1 Å. *A priori* a polar dimer interface may seem

unusual since we might expect the packing of oligomers to be more similar to the packing of the protein interior. However, an examination of several dimeric structures in the Protein Data Base shows that the APx dimer interface is not so unusual. For example, the *E. coli* regulatory protein, AraC, forms a homodimer (1ARC) (Soisson et al., 1997) and has a water molecule near the center of the dimer interface with related Gln and Asn residues from each subunit hydrogen bonded with the central water. This is very similar to the interactions involving Glu106 in APx (Figs. 1, 3). Also similar to APx, the dimer interface of AraC is formed by related helices. Glutathione-S-transferase (1PGT) also follows the same pattern with waters bridging between the helical interface of the homodimer (Ji et al., 1997).

Enzyme activity and the dimer

The results of gel filtration studies indicate the E112K mutant is monomeric at submicromolar concentrations and yet its activity is essentially the same as the wild-type protein, both assayed at nanomolar levels. These results demonstrate that the non-Michaelis–Menten kinetics observed with APx and the Hill coefficient >1 is not the result of cooperative subunit interactions. Perhaps these data are limited to ascorbate's unusual properties as an anionic radical since more traditional hyperbolic plots are seen when guaiacol (2-methoxy phenol) is used as the substrate (data not shown).

Methods and materials

Materials

MilliPore filtered water was used in the preparation of all buffers and reagents. The potassium phosphate, trizma base, polyethylene glycol (PEG) 3350, H₂O₂ (30%), and sodium ascorbate were purchased from Sigma (St. Louis, Missouri). Mutagenic oligos were purchased from Operon Technologies (Alameda, California).

Mutagenesis and expression

The Transformer Site-Directed Mutagenesis kit (CloneTech, San Diego, California) was used to generate the point mutants E112A and E112K. The oligo sequences used were: (112A) 5'-CCGGTGG ACCTGCAGTTCCTTCC-3' with the selection primer (*NdeI* to *EcoRI*) 5'-CACACCGGATATCGTGCACTC-3' and (112K) 5'-CCGGTGGACCTAAAGTTCCTTCC-3' with the selection primer (*PstI* to *XhoI*) 5'-CAGTCGACCTCGAGGCAAGCTTGGC-3'. Mutations were verified by dideoxy sequencing (Sequenase). APx and the mutant clones were expressed and purified as previously described (Patterson & Poulos, 1994) using the pMal fusion protein system (New England Biolabs, Beverly, Massachusetts). Protein concentration was determined using an extinction coefficient of 9.1×10^4 L/mol cm at 403 nm. Proteins were flash frozen in a dry ice/ethanol bath and stored at -70 °C in 50 mM KPO₄, pH 7.0.

Gel filtration

A Superdex-75 (HR 10/30) column attached to an FPLC medium pressure chromatography system (Pharmacia, Uppsala, Sweden) was used to measure the molecular size of wild-type and mutant proteins. The column was equilibrated in 150 mM NaCl/50 mM KPO₄, pH 7.0 at a flow rate of 0.5 mL/min. The proteins were run

isocratically at this flow rate, and protein elution was monitored by absorbance at 280 nm. Prior to the experimental runs, low molecular weight chromatography standards (Pharmacia) were run on the column to generate a standard curve of k_{av} vs. molecular weight according to the formula:

$$k_{av} = (V_e - V_o)/(V_t - V_o). \quad (3)$$

With $V_t = 24$ mL, $V_o = 8.84$ mL, and V_e , the elution volume of the protein being measured, reported by the integration step at the end of the run. Protein standards used were: RNase, 13.7 kDa; chymotrypsinogen A, 25 kDa; cytochrome c peroxidase, 34 kDa; ovalbumin, 43 kDa; bovine serum albumin, 67 kDa; and void volume, V_o , was measured with blue dextran 2000, 2,000 kDa.

Calorimetry

DSC measurements were performed with a Hart 7707 DSC heat conduction scanning microcalorimeter, which consists of three removable 1 mL solution cells and a reference cell. The measurements were usually performed at a scan rate of 15 K/h. To determine any dependence of the transition parameters on scan rate, scans were also performed at 30 K/h. Least-squares fit of the two-state transition model, $N \leftrightarrow U$ where N is the folded state and U the unfolded state, to the data was performed by the EXAM Program (Kirchhoff, 1993). The sample size was 0.5 mL and the scans were performed from 30 to 90 °C. The uncertainties in Table 3 are the standard deviations of each transition quantity determined from the number of independent determinations described in Table 3.

Spectroscopy

A Cary 3 UV-Vis spectrophotometer (Varian Inc.) equipped with a multicell, thermal block was used for the thermal denaturation experiments. The equipment was controlled by PC under the Cary OS software package, version 3.0. A special routine used for DNA thermal stability studies was employed to run the temperature controller and data collection. The temperature was incremented at 1 °C/min and absorbance was measured at 405 nm to monitor the electronic state of the heme iron. Data points were collected every 0.2 °C for wild-type APx and the 112K mutant and every 0.5 °C for the 112A mutant. The proteins were diluted in degassed, 25 mM KPO₄, pH 7.0.

To correct for minor differences in protein concentration, the visible spectra were converted to percent folded

$$\% \text{ folded} = [(Y - Y_u)/(Y_f - Y_u)] \times 100 \quad (4)$$

where Y is the observed absorbance at any point along the unfolding curve, Y_f and Y_u are the pretransitional and posttransitional intercepts for the folded and unfolded portions of the curve, respectively. The fraction of unfolded protein can be calculated anywhere on the curve based on the relation

$$f_u = (y_f - y)/(y_f - y_u). \quad (5)$$

Data were fit to a two-state model of protein unfolding, $N \xrightleftharpoons{k} U$, where N is the folded dimer, U is the unfolded dimer, and $k = [U]/[N] = f_u/(1 - f_u) = (y_f - y)/(y - y_u)$. Solving for y gives

$$y = y_f + y_u(K)/(1 + K). \quad (6)$$

At the midpoint transition temperature, T_m , $\Delta G = 0$ and $\Delta S = \Delta H_v/T$; the free energy relation $\Delta G = \Delta H - T\Delta S$ reduces to

$$\Delta G = \Delta H_v - \Delta H_v T/T_m = \Delta H_v(1 - T/T_m). \quad (7)$$

Substituting in $-RT \ln K$ for ΔG in Equation 7 and solving for K gives

$$K = \exp(-\Delta H_v(1 - T/T_m)/RT). \quad (8)$$

Substituting Equation 8 and the linear relation, $y = mT + b$, into Equation 6 gives

$$y = \frac{m_f T + b_f + (m_u T + b_u) \exp(-\Delta H_v(1 + T/T_m)/RT)}{1 + \exp(-\Delta H_v(1 + T/T_m)/RT)}. \quad (9)$$

Equation 9 was used in Sigmaplot 4.0 (Jandel Scientific Software, San Rafael, California) to perform nonlinear regression analysis on the visible spectra data and return values for ΔH_v and T_m .

Ascorbate peroxidase assay

The 50 mM sodium ascorbate, 9 mM H₂O₂, and enzyme dilutions were prepared in a buffer of 50 mM KPO₄, pH 7.0, 0.1 mM EDTA. The 1 mL reactions were initiated in the quartz cuvette by the addition of 90 μM H₂O₂ and ascorbate oxidation was monitored by the change in absorbance at 290 nm. Assays were performed at 23 °C. Activity was determined by dividing the ΔAbs_{290} by $\epsilon_{290} = 2.8 \text{ mmol}^{-1} \text{ cm}^{-1}$. One unit of activity is defined as 1 μmol ascorbate oxidized in 1 min. K_{cat} was calculated by dividing the units of activity by the mM concentration of enzyme used in the assay.

Cryocrystallography

Crystals of APx and the 112 mutants were grown as previously described (Patterson & Poulos, 1994) with one round of touch-seeding and one round of macroseeding to obtain diffraction quality crystals. Prior to diffraction experiments, crystals were soaked overnight at 4 °C in mother liquor plus 20% ethylene glycol to serve as a cryoprotectant against freezing induced cracking of the crystals. Ethylene glycol was empirically determined to be the best cryoprotectant because it minimized the mosaicity of the crystal lattice due to soaking and uneven freezing.

The crystals were mounted in nylon loops attached to the end of magnetic mounting pins (Hampton Research, Laguna Niguel, California) that were then mounted on the goniometer and rapidly frozen in a stream of liquid nitrogen. The liquid nitrogen stream was left on during data collection, maintaining the crystals at a temperature of ≈ -150 °C. Only one crystal was required per data set.

Data were collected using a Siemens X1000 multiwire area detector and a rotating Cu anode X-ray source equipped with focusing optics. Two orientations of data were collected per crystal with ϕ at 0 and 90°. The monoclinic crystal was rotated 180° around the w for each orientation with a $\Delta\omega = 0.3^\circ$. The data were indexed, integrated, and scaled with XDS (Kabsch, 1993) and crystallographic refinement was carried out with XPLOR (Brünger, 1992). Molecular modeling was carried out in TOM/FRODO 3.0 (Jones, 1982; Israel & Chirino, 1994). A summary of the refinement is presented in Table 2.

Acknowledgments

The authors would like to thank Dr. David J. Schuller and Dr. M. Sundaramoorthy for expert crystallographic advice, and Dr. C.S. Raman for many discussions and advice on the intricacies of protein stability. This work was supported by NIH Grant GM42614 (T.L.P.) and NIH training Grant GM07311-22 (M.D.).

The identification of certain commercial equipment and materials in this paper does not imply a recommendation or endorsement by the National Institute of Standards and Technology that such equipment or material are necessarily the best available for the purpose.

References

- Beernink PT, Tolan DR. 1994. Subunit interface mutants of rabbit muscle Aldolase form active dimers. *Protein Sci* 3:1383–1391.
- Borchert TV, Abagyan R, Jaenicke R, Wierenga RW. 1994. Design, creation and characterization of a stable, monomeric triosephosphate isomerase. *Proc Natl Acad Sci USA* 91:1515–1518.
- Brünger AT. 1992. *A system for X-ray crystallography and NMR. Version 3.1*. New Haven, CT: Yale University Press.
- Evans SV. 1993. SETOR: Hardware-lighted three-dimensional solid model representations of macromolecules. *J Mol Graphics* 11:134–138.
- Fukada H, Sturtevant JM, Quijcho FA. 1983. Thermodynamics of the binding of L-arabinose and of D-galactose to the L-arabinose-binding protein of *Escherichia coli*. *J Biol Chem* 258(21):13193–13198.
- Ghosaini LR, Brown AM, Sturtevant JM. 1988. Scanning calorimetric study of the thermal unfolding of Catobolite Activator Protein from *Escherichia coli* in the absence and presence of cyclic mononucleotides. *Biochemistry* 27:5257–5261.
- Israel M, Chirino AJ. 1994. TOM/FRODO molecular modelling program for the Iris Version 3.0.
- Ji X, Tordova M, O'Donnell R, Parsons JF, Hayden JB, Gilliland GL, Zimiak P. 1997. Structure and function of the xenobiotic substrate-binding site and location of a potential non-substrate-binding site in class pi Glutathione-S-Transferase. *Biochemistry* 36:9690–96702.
- Jones TA. 1982. *Computational crystallography*, Oxford, UK: Oxford University Press, pp 303–317.
- Kabsch W. 1993. Automatic processing of rotation diffraction data from crystals of initially unknown symmetry and cell constants. *J Appl Cryst* 26:795–800.
- Kirchhoff WH. 1993. EXAM: A two-state thermodynamic analysis program. Washington, DC: National Institute of Standards and Technology.
- Kraulis PJ. 1991. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J Appl Cryst* 24:946–950.
- Liggins JR, Sherman F, Mathews AJ, Nall BT. 1994. Differential scanning calorimetric study of the thermal unfolding transitions of yeast iso-1 and iso-2 cytochromes *c* and three composite isozymes. *Biochemistry* 33:9209–9219.
- Manly SP, Matthews KS, Sturtevant JM. 1985. Thermal denaturation of the core protein of *Lac* repressor. *Biochemistry* 24:3842–3846.
- Marquez LA, Quitariano M, Zilinskas BA, Dunford HB. 1996. Kinetic and spectral properties of pea cytosolic ascorbate peroxidase. *FEBS Lett* 389:153–156.
- Mittler R, Zilinskas BA. 1991. Purification and characterization of pea cytosolic ascorbate peroxidase. *Plant Physiol* 97:962–968.
- Patterson WR, Poulos TL. 1994. Crystallization and characterization of ascorbate peroxidase. *J Biol Chem* 269:17020–17024.
- Patterson WR, Poulos TL. 1995. Crystal structure of recombinant pea cytosolic ascorbate peroxidase. *Biochemistry* 34:4331–4341.
- Privalov PL, Potekhin SA. 1986. Scanning microcalorimetry in studying temperature-induced changes in proteins. *Methods Enzymol* 131:4–51.
- Sanchez-Ruiz JM. 1995. Differential scanning calorimetry of proteins. *Subcell Biochem* 24:133–176.
- Schliebs W, Thanki N, Jaenicke R, Wierenga RW. 1997. A double mutation at the tip of the dimer interface loop of triosephosphate isomerase generates active monomers with reduced stability. *Biochemistry* 36:9655–9662.
- Shao X, Hensley P, Matthews CR. 1997. Construction and characterization of monomeric tryptophan repressor: A model for an early intermediate in the folding of a dimeric protein. *Biochemistry* 36:9941–9949.
- Silva JL, Silveira CF, Correia A Jr, Pontes L. 1992. Dissociation of a native dimer to a molten globule monomer. Effects of pressure and dilution on the association equilibrium of arc repressor. *J Mol Biol* 223:545–555.
- Soisson SM, McDougall-Shackleton B, Schleif R, Wolberger C. 1997. Structural basis for ligand-regulated oligomerization of AraC. *Science* 276:421–425.
- Steif C, Weber P, Flossdorf J, Cesareni G, Kokkinidis M. 1993. Subunit interactions provide a significant contribution to the stability of the dimeric four- α -helical-bundle protein ROP. *Biochemistry* 32:3867–3876.
- Sturtevant JM. 1987. Biochemical applications of differential scanning calorimetry. *Ann Rev Phys Chem* 38:463–488.
- Vickers LP, Donovan JM, Schachman HK. 1978. Differential scanning calorimetry of aspartate transcarbamoylase and its isolated subunits. *J Biol Chem* 253:8493–8498.
- Ysern X, Fields BA, Bhat TN, Goldbaum FA, Acqua WD, Schwarz FP, Poljak RJ, Marriaza RA. 1994. Solvent rearrangement in an antigen-antibody interface introduced by site-directed mutagenesis of the antibody combining site. *J Mol Biol* 238:496–500.