Calorimetric Analysis of Thermodynamic Stability and Aggregation for Apo and Holo Amyotrophic Lateral Sclerosis-associated Gly-93 Mutants of Superoxide Dismutase*§

Received for publication, August 29, 2005, and in revised form, December 13, 2005 Published, JBC Papers in Press, January 3, 2006, DOI 10.1074/jbc.M509496200

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Differential scanning calorimetry was used to measure changes in thermodynamic stability and aggregation for glycine 93 mutants of human copper, zinc-superoxide dismutase (SOD). Glycine 93 is a conserved residue at position i + 3 of a tight turn and has been found to be a mutational hot spot in familial amyotrophic lateral sclerosis (fALS). The fALS-associated mutations, G93A, G93S, G93R, and G93V, were made in a pseudo wild-type background containing no free cysteines, which prevented the formation of aberrant disulfide bonds upon thermal unfolding, and enabled quantitative thermodynamic analysis of the effects of the mutations. Thermal unfolding was highly reversible for all the SODs in both the fully metallated (holo) and metal-free (apo) forms. The data for all the holo-SODs and for the apo-pseudo-wild-type SOD were well fit by a 2-state unfolding model for native dimer (N2) to two unfolded monomers (2U), N2 ↔ 2U. The holo- and apo-forms of the mutants are significantly destabilized (by 1.5–3.5 kcal mol−1 monomer) relative to the corresponding forms of pseudo wild-type, with the relative stabilities being correlated with statistical preferences for amino acids in this structural context. Although van’t Hoff (ΔH/measured) to calorimetric (ΔH/cal) enthalpy ratios are close to unity for all the holo-SODs and for apo-pseudo-wild-type, consistent with a 2-state transition, ΔH/cal is considerably larger than ΔH/measured for all the apo-mutants. This suggests that the mutations cause apo-SOD to have an increased propensity to misfold or aggregate, which may be linked to increased toxic mutant SOD aggregation in fALS.

SOD is a highly stable homodimeric metalloenzyme (1–4). Each subunit forms a Greek key β-barrel structure that binds one zinc and one copper ion (5, 6); the zinc contributes to the structural stability of the active site, and the copper is involved in the reox dismutation of the toxic superoxide free radical (7). ALS is a common, rapidly progressive adult motor neuron disease for which there is very little in the way of treatment and no cure (8). Mutations in SOD were linked to fALS in 1993 (9); to date, over 110 SOD mutations have been associated with the disease. Although mutant SOD fALS represents only a few percent of all ALS cases, this is the major known cause of the disease (10, 11). Mutant SODs have a gain of toxic function, the molecular mechanism of which remains unresolved (8). Recent attention has focused on increased toxic misfolding of mutant SOD as a cause for fALS, analogous to other protein misfolding diseases (12–15). Increased misfolding has been correlated with decreased protein stability; hence, a better understanding of the thermodynamic effects of fALS mutations may lead to valuable insights into the disease process.

Wild-type human SOD contains 4 cysteines per monomer; in the native protein two of these (Cys-57 and Cys-146) form a conserved intra-subunit disulfide bond, and the other two (Cys-6 and Cys-111) remain as free cysteines. Thermal unfolding of wild-type SOD is highly irreversible because of the oxidation of the free cysteines (1), which precludes thermodynamic analysis. However, substitution of the cysteines at position 6 and 111 by alanine and serine, respectively, results in much higher reversibility (2, 16); alanine at position 6 is also found in other mammalian SODs (17). The structure, activity, and stability of the protein lacking free cysteines (pseudo-WT) are very similar to wild-type SOD (2, 6, 16, 18). This pseudo-WT protein has been used extensively in folding and structural studies (19–22) in order to avoid complications of free cysteine oxidation and is also used here.

Glycine 93 is a mutational hotspot for fALS; disease-associated mutations include substitutions to alanine, arginine, aspartic acid, valine, serine, and cysteine (www.alsod.org) (11). Glycine 93 is situated at position 3 of a type I tight turn (loop V, connecting β-strand 5 and 6), where it adopts a left-handed backbone conformation (supplemental Fig. S1) (23). This glycine is conserved in sequence and structure among many SODs (5).

In this study, all of the above substitutions, with the exception of cysteine which would oxidize, were engineered into pseudo-WT. Highly homogeneous solutions of holo- and apo-pseudo-WT and mutant SODs were prepared and found to undergo highly reversible thermal unfolding. We show that DSC can be used to quantify the effects of SOD mutations on both thermodynamic stability and on aggregation.

MATERIALS AND METHODS

SOD Expression and Purification—Human fALS-associated mutant SODs were engineered into the pHSOD1ASlacIq vector, and the protein was expressed in Escherichia coli and purified as described previously (20, 24). Apoproteins were prepared by dialyzing the holoproteins against EDTA (7). The metal content of the purified proteins was assessed by inductively coupled plasma atomic emission spectroscopy.
Stability and Aggregation of fALS-associated Gly-93 Mutant SOD

High Reversibility of Thermal Unfolding of Apo-pseudo-WT SOD—DSC—Measurements were performed using a Microcal LLC VP-DSC (Microcal, Northampton, MA). Unless otherwise stated, SOD samples were in 20 mM HEPES, pH 7.8. Under these nonreducing conditions, the native disulfide remains intact. Protein concentrations for samples typically ranged from 0.25 to 1.5 \( \mu \text{g/ml} \). A negligible scan rate dependence on excess specific heat absorption was observed for SOD between 0.25 and 1.5 \( ^\circ \text{C} \) min \(^{-1} \) and so experiments routinely used a scan rate of 1 \( ^\circ \text{C} \) min \(^{-1} \). Buffer/buffer base lines were determined for each experiment and subtracted from protein/buffer scans, followed by data normalization for protein concentration.

Thermodynamic Analysis of DSC Data—DSC data were fit to a dimer 2-state model for equilibrium between native dimer (\( N_2 \)) and two unfolded monomers (\( 2U \)), as described elsewhere (26), according to Equation 1,

\[
C_p^{\text{tot}}(T) = \frac{\beta \Delta h^f(T) f_U(1 - f_U)}{R T^2} \left( 2 - f_U \right) + \left( 1 - f_U \right) (A + BT) + f_U (C + DT) \quad \text{(Eq. 1)}
\]

where \( C_p^{\text{tot}}(T) \) is the total specific heat absorption at temperature \( T \) (in Kelvin); \( R \) is the universal gas constant; \( f_U \) is the fraction of unfolded protein at \( T \); \( \Delta h^f(T) \) is the specific enthalpy of unfolding at \( T \); and \( A \) and \( C \) are the intercepts; \( B \) and \( D \) are the slopes of the folded and unfolded base lines, respectively; \( \beta \) is a temperature-independent constant equal to the ratio of the van’t Hoff to calorimetric enthalpies of unfolding, \( \Delta H_{\text{cal}}/\Delta H_{\text{vH}} \), multiplied by the molecular weight of the dimer; and \( T_m \) is the temperature where \( f_U = 0.5 \) (\( T_m \) is the temperature in Kelvin where the fraction unfolded is 0.5). For comparison, data were also fit to a monomer 2-state transition between the native and unfolded state, \( N \leftrightarrow U \), where there is no change in molecularity upon unfolding (MN2State in Origin, version 5.0, Microcal), after subtraction of base-line heat capacity, using Equation 2,

\[
C_p^{\text{ex}}(T) = \frac{\gamma \Delta h^f(T) f_U(1 - f_U)}{R T^2} \quad \text{(Eq. 2)}
\]

where \( C_p^{\text{ex}}(T) \) is the excess specific heat absorption at \( T \); \( \Delta h^f(T) \) is the enthalpy of unfolding at \( T \), and \( \gamma \) is \( \Delta H_{\text{cal}}/\Delta H_{\text{vH}} \). DSC data were fit by nonlinear least squares using Origin.

Aggregation Measurements—Aggregation propensity of apo-SODs was measured by monitoring 90° light scattering at 450 nm, caused by protein aggregation as temperature was increased from 5 °C at ~0.7 °C/min as described previously (20) and applied to many SOD mutants (19, 20). Aggregation was found to be extremely sensitive to solution pH (19) and to conditions of the apo stock solutions; highly reproducible temperatures for onset of aggregation were obtained by using stock apo-SOD solutions buffered in 20 mM HEPES, pH 7.8, and then diluted to 0.024 mg/ml in 20 mM MES, with a final pH of 5.5. The temperature (in °C) of onset of aggregation, \( T_{\text{agg}} \), was taken as the intercept of the lines for base-line scattering in the pre-aggregation temperature range and for scattering in the initial aggregation region.

RESULTS

High Reversibility of Thermal Unfolding of Apo-pseudo-WT SOD—Do-WT SOD (2, 20), which demonstrated high reversibility upon thermal denaturation, and to chemical denaturation experiments on SOD, which have shown high reversibility of unfolding (data not shown). The reversibility was calculated from the areas for the unfolding endotherms in successive DSC heating scans (Fig. 1A). Nearly 100% reversibility was observed when apo-pseudo-WT was scanned to the end of the unfolding transition. Scanning 15 °C beyond the transition caused the reversibility to decrease to ~90%. Consequently, apo data were fit just to the end of the unfolding transition, where thermal unfolding was completely reversible.

Apo-pseudo-WT Thermal Unfolding Fits a Dimer 2-State (\( N_2 \rightarrow 2U \)) Model—The DSC unfolding endotherms for the apo-SODs studied here were clearly asymmetric, because changes in heat capacity, \( C_p^U \), were more gradual at lower temperatures and more abrupt at increased temperatures (e.g. see Fig. 1); such asymmetry is expected for 2-state unfolding of a dimeric protein (\( N_2 \leftrightarrow 2U \)) (26). Because apo-SOD...
Stability and Aggregation of fALS-associated Gly-93 Mutant SOD

monomers associate with very high affinity (K_{d} \approx 10^{-10} \text{M}, pH 7.8, 25 °C (27)), it is reasonable that the dimer persists until the protein thermally unfolds. The apo-DSC data were well fit by a dimer 2-state model (Fig. 1B), whereas systematic deviations were observed upon fitting to the MN2-state model with no change in molecularity upon unfolding (N \leftrightarrow U) (Fig. 1C). Furthermore, fitting of data normalized per mol of monomer to the monomer model gave \Delta H_{\text{m}}/\Delta H_{\text{cal}} ratios of ~2, indicative of a dimeric cooperative unfolding unit. Formation of monomer was also confirmed by dynamic light scattering measurements, which showed that count rate and protein hydrodynamic diameter decreased through the temperature range of the DSC transition (supplemental Fig. S2). Protein cross-linking using glutaraldehyde, monitored by SDS-PAGE, also indicated formation of monomer at increased temperature (data not shown).

For a dimer 2-state transition, the unfolding transition should shift to higher temperature with increasing protein concentration, as has been observed for other dimeric proteins (26). This behavior was observed for all the apo-SOD proteins. Fig. 2A shows the thermograms and the fitted lines for the dimer 2-state model for 0.05–3.0 mg ml\(^{-1}\) apo-pseudo-WT. The parameters obtained from the fits are summarized in Table 1. All the fALS-related substitutions (Ser, Arg, Ala, Asp, and Val) of Gly-93 cause thermal unfolding to be shifted to lower temperature, with decreases in \(t_{\text{m}}\) ranging from ~8 °C for G93S to ~16 °C for G93V.

\[
\Delta C_p = \left( \frac{d \Delta H_{\text{avr}}}{dT} \right) \quad \text{(Eq. 3)}
\]

Fig. 2C shows the DSC thermograms of apo-pseudo-WT for pH 3.0–4.4, which are well fit by the dimer 2-state model, with average \Delta H_{\text{cal}}/\Delta H_{\text{cal}} ratios of 1.04 and average fitted \Delta C_p values of 3.13 kcal mol\(^{-1}\) °C\(^{-1}\). The slope of the corresponding Kirchoff plot decreases somewhat with increasing protein concentration, with predicted line based on change in solvent-accessible surface area of the folded protein relative to the unfolded (31, 32). By using the solvent-accessible surface area of the native state based on the crystal structure of pseudo-WT (Protein Data Bank code 1sos (33)) and the average of the upper and lower limits for the solvent-accessible surface area of the unfolded monomer (34), the predicted \(\Delta C_p\) is 3.79 kcal mol\(^{-1}\) °C\(^{-1}\). Using the lower bound for the unfolded surface area gives \(\Delta C_p\) of 2.91 kcal mol\(^{-1}\) °C\(^{-1}\). Taking all the data into consideration, the \(\Delta C_p\) for apo-pseudo-WT is in the range of values expected for a globular protein of this size and containing a single disulfide bond (30, 32, 35).

High Reversibility of Thermal Unfolding of Gly-93 Mutant SOD—The reversibility of unfolding was very high for all the apo-mutants, close to 100% for scans to the end of the unfolding transition, and >90% for scans 15 °C beyond the transition. As for apo-pseudo-WT, data were fit just to the end of the unfolding endotherm. Thermograms for the apo-Gly-93 mutants and pseudo-WT and fits of the data to a dimer 2-state model are shown in Fig. 3A. Values for the fitted parameters are summarized in Table 1. All the fALS-related substitutions (Ser, Arg, Ala, Asp, and Val) of Gly-93 cause thermal unfolding to be shifted to lower temperature, with decreases in \(t_{\text{m}}\) ranging from ~8 °C for G93S to ~16 °C for G93V.
Stability and Aggregation of fALS-associated Gly-93 Mutant SOD

| Apo-SOD | [Protein] | $t_m$ | $\Delta H_{\text{st}}$ | $\Delta H_{\text{cal}}$ | $\Delta C_p$ |
|---------|-----------|-------|----------------------|----------------------|-------------|
|         | (mg ml$^{-1}$) | °C | kcal mol$^{-1}$ | kcal mol$^{-1}$ °C$^{-1}$ | kcal mol$^{-1}$ °C$^{-1}$ |
| Pseudo-WT | 3.00 | 61.9 ± 0.0 | 183.3 ± 1.9 | 1.44 | 3.93 |
| Pseudo-WT | 2.99 | 61.3 ± 0.0 | 178.5 ± 1.4 | 1.00 | 5.11 |
| Pseudo-WT | 1.50 | 60.3 ± 0.0 | 164.3 ± 4.0 | 0.96 | 4.13 |
| Pseudo-WT | 1.42 | 60.0 ± 0.0 | 163.6 ± 2.4 | 0.96 | 3.38 |
| Pseudo-WT | 0.73 | 58.8 ± 0.0 | 144.4 ± 2.5 | 0.94 | 4.30 |
| Pseudo-WT | 0.44 | 59.1 ± 0.2 | 137.6 ± 9.4 | 0.96 | 4.25 |
| Pseudo-WT | 0.27 | 57.8 ± 0.2 | 115.4 ± 2.7 | 0.78 | 3.85 |
| Pseudo-WT | 0.21 | 58.4 ± 0.1 | 116.7 ± 9.1 | 0.84 | 3.74 |
| Pseudo-WT | 0.20 | 58.3 ± 0.1 | 112.8 ± 6.7 | 1.02 | 2.95 |
| Pseudo-WT | 0.05 | 57.8 ± 0.6 | 106.5 ± 18.9 | 0.97 | 3.71 |
| Mean ± S.D. | | | 1.00 ± 0.18 | 4.13 ± 0.65 |

$\Delta H_{\text{st}}/\Delta H_{\text{cal}}$ Ratios for Apo-mutant SODs Are Greater Than 1—A particularly interesting result is that the $\Delta H_{\text{st}}/\Delta H_{\text{cal}}$ ratios for all the apo-mutants are significantly greater than 1 (Table 1). This is in marked contrast to apo-pseudo-WT that shows $\Delta H_{\text{st}}/\Delta H_{\text{cal}}$ ratios of ~1 for a wide range of pH values and protein concentrations. High ratios for $\Delta H_{\text{st}}/\Delta H_{\text{cal}}$ are often taken as an indication of protein aggregation (26, 36) and may be related to increased mutant protein misfolding in fALS.

Aggregation can cause high $\Delta H_{\text{st}}/\Delta H_{\text{cal}}$ ratios in a number of ways. Irreversible aggregation can distort DSC traces and cause increases in $\Delta H_{\text{cal}}$ and/or decreases in $\Delta H_{\text{cal}}$. This does not occur to a significant extent for apo-SODs, however, as these showed very high reversibility in routinely performed multiple reheating scans. Another cause of high $\Delta H_{\text{st}}/\Delta H_{\text{cal}}$ ratios is non-native intermolecular interactions during thermal unfolding, resulting in an apparent cooperative unfolding unit that is larger than the dimer, i.e., $\Delta H_{\text{st}}$ is increased and $\Delta H_{\text{cal}}$ calculated per mol dimer is too low. Another possibility is the formation of small, weakly associated reversible aggregates during the scan; if formation of such aggregates is slightly exothermic, the effects on fitted parameters could be similar to those for irreversible aggregation. Finally, if aggregates are measured by protein concentration measurements, but do not contribute to the DSC endotherm, then $\Delta H_{\text{cal}}$ will be systematically low.

Several additional approaches were used to try to distinguish between the various potential causes for high $\Delta H_{\text{st}}/\Delta H_{\text{cal}}$ values. Apo-G93R was stabilized using glycerol, to assess whether the high ratios were a consequence simply of decreased $t_m$. Addition of 20% glycerol increased the $t_m$ from ~49 to 59 °C, however, $\Delta H_{\text{st}}/\Delta H_{\text{cal}}$ was unchanged. Dynamic light scattering and chemical cross-linking by glutaraldehyde as a function of temperature did not clearly identify the formation of aggregates.

The molecularity of the unfolding transition can be determined from plots of lnP versus 1/tem as shown in Equation 4,

$$\text{molecularity} = \frac{mR}{\Delta H_{\text{st}}} + 1$$

where P is protein concentration; m is the slope of the plot; $\Delta H_{\text{st}}$ is taken as the average fitted $\Delta H_{\text{st}}$ at the various protein concentrations; and $R$ is the gas constant (37). If high $\Delta H_{\text{st}}$ is the reason for $\Delta H_{\text{st}}/\Delta H_{\text{cal}}$ ~1, then the molecularity should be larger than 2 (26, 37). However, the observed molecularities for apo-pseudo-WT and for G93A were found to be ~2, indicative of a dimeric cooperative unfolding unit. A Kirchhoff plot of $\Delta H_{\text{st}}$ versus $t_m$ for the apoproteins has less scatter than the same plot for $\Delta H_{\text{cal}}$ and has a higher slope that is more consistent with the average $\Delta C_p$ of 3.30 kcal mol$^{-1}$ °C$^{-1}$ (see below). This suggests that $\Delta H_{\text{st}}/\Delta H_{\text{cal}}$ ratios deviate from unity because of inaccuracies in the $\Delta H_{\text{cal}}$ values. Note that increased $\Delta H_{\text{st}}/\Delta H_{\text{cal}}$ is correlated with decreased $\Delta C_p$ (Table 1), as may be expected for the occurrence of some exothermic aggregation. Given the preceding considerations, plausible explanations for the high $\Delta H_{\text{st}}/\Delta H_{\text{cal}}$ ratios are the occurrence of reversible aggregation with small heat effects during the scan or the presence of undetected aggregates throughout the scans.

Gly-93 Mutations Decrease Apo-SOD Stability—Because $\Delta H_{\text{st}}$ likely reflects the unfolding of the dimer, this value was used to calculate the Gibbs free energy of unfolding, $\Delta G$ (kcal mol$^{-1}$), and the change in $\Delta G$ of the mutant proteins relative to the pseudo-WT, $\Delta \Delta G$. For these calculations, $\Delta C_p$ was taken to be independent of temperature, as is commonly assumed (38–40), and was set to 3.30 kcal mol$^{-1}$ °C$^{-1}$, which is an average of the directly fitted $\Delta C_p$ values for individual DSC scans and

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**Table 1**

Fitted 2-state dimeric parameters for apo-SODs

Data were acquired in 20 mM HEPES, pH 7.8.

| Apo-SOD | [Protein] | $t_m$ (°C) | $\Delta H_{\text{st}}$ (kcal mol$^{-1}$) | $\Delta H_{\text{st}}/\Delta H_{\text{cal}}$ | $\Delta C_p$ (kcal mol$^{-1}$ °C$^{-1}$) |
|---------|-----------|-----------|----------------------|----------------------|-------------|
| Pseudo-WT | 3.00 | 61.9 ± 0.0 | 183.3 ± 1.9 | 1.44 | 3.93 |
| Pseudo-WT | 2.99 | 61.3 ± 0.0 | 178.5 ± 1.4 | 1.00 | 5.11 |
| Pseudo-WT | 1.50 | 60.3 ± 0.0 | 164.3 ± 4.0 | 0.96 | 4.13 |
| Pseudo-WT | 1.42 | 60.0 ± 0.0 | 163.6 ± 2.4 | 0.96 | 3.38 |
| Pseudo-WT | 0.73 | 58.8 ± 0.0 | 144.4 ± 2.5 | 0.94 | 4.30 |
| Pseudo-WT | 0.44 | 59.1 ± 0.2 | 137.6 ± 9.4 | 0.96 | 4.25 |
| Pseudo-WT | 0.27 | 57.8 ± 0.2 | 115.4 ± 2.7 | 0.78 | 3.85 |
| Pseudo-WT | 0.21 | 58.4 ± 0.1 | 116.7 ± 9.1 | 0.84 | 3.74 |
| Pseudo-WT | 0.20 | 58.3 ± 0.1 | 112.8 ± 6.7 | 1.02 | 2.95 |
| Pseudo-WT | 0.05 | 57.8 ± 0.6 | 106.5 ± 18.9 | 0.97 | 3.71 |
| Mean ± S.D. | | | 1.00 ± 0.18 | 4.13 ± 0.65 |
Stability and Aggregation of fALS-associated Gly-93 Mutant SOD

The Kirchoff and calculated ΔC_p values. ΔG as a function of temperature was calculated as shown in Equations 5–8,

$$\Delta G(T) = \Delta H_{\text{m}}(T) - T \Delta S(T)$$  \hspace{1cm} (Eq. 5)

where

$$\Delta H_{\text{m}}(T) = \Delta H_{\text{m}}(T_m) + \Delta C_p(T - T_m)$$  \hspace{1cm} (Eq. 6)

$$\Delta S(T) = \Delta S(T_m) + \Delta C_p \ln \frac{T}{T_m}$$  \hspace{1cm} (Eq. 7)

and

$$\Delta S(T_m) = \frac{\Delta H(T_m) - \Delta G(T_m)}{T_m}$$  \hspace{1cm} (Eq. 8)

ΔG(T) is shown in Fig. 3B for the apo-mutant and pseudo-WT proteins. The validity of the ΔG calculations was checked by determining the temperatures where lines of (Equation 9)

$$\Delta G = -RT \ln P$$  \hspace{1cm} (Eq. 9)

intersect with the ΔG curves (Fig. 3B) (38). The temperature of intersection is the predicted t_m for that particular protein concentration. On average, the predicted t_m values were in good agreement with the fitted values.

Table 2 summarizes the calculated ΔG and ΔΔG values at the average t_m of all the proteins (t_m) and at 25 °C. The ΔG values determined at 25 °C are somewhat lower than the values determined at the t_m because the predicted temperatures of maximum stability for all isoforms are less than 20 °C (Fig. 3B). For the apo-mutants there are significant decreases in the ΔG values relative to pseudo-WT, with ΔΔG values ranging from −3.7 for G93S to −7.0 kcal mol⁻¹ for G93V at the t_m. The decreased stability of the mutants is caused by increases in the entropy of unfolding (ΔS). These increases are partially compensated by decreases in the enthalpy of unfolding (ΔH) (Table 2). Similar entropy-enthalpy compensation has been observed in many other systems (41).

Gly-93 Mutations Increase Apo-SOD Aggregation Propensity—The propensity of apo-SODs to aggregate was determined by measuring temperature of onset of aggregation (t_{agg}) with increasing temperature by light scattering, as has been done for many other mutant apo-SODs (19, 20). Raw data are shown in supplemental Fig. S3, and t_{agg} values are summarized in Table 2. As has been found in previous studies (19, 20), decreased apoprotein stability is correlated with increased aggregation propensity, as measured by a lower t_{agg}. It has been suggested that the stability correlation may be broken by mutations that increase (decrease) protein net charge and consequently slow (enhance) aggregation (19). Under conditions employed here, apo-pseudo-WT SOD will have a slight negative net charge (42). G93D will have increased net charge, which may account for its relatively high t_{agg}. The converse is not observed for G93R (decreased net charge); thus additional factors may also play a role in determining t_{agg}. In this respect, it is interesting that based on ΔH_{m}/ΔH_{calc} ratios, G93D aggregates more than G93R.

Oliveberg and co-workers (19) have further proposed that lower t_{agg} is correlated with shorter mean survival time. The clinical data for patients with Gly-93 mutations is limited to a small number of patients (43–48), precluding detailed conclusions for each mutant. The available data suggest that disease duration may be longer for G93D, although it is not clearly shorter for G93R and G93V (most destabilized). Additional studies are needed to explore this further.

**Thermal Unfolding of Holo-pseudo-WT SOD Is Reversible**—Holo-SOD unfolds at a much higher temperature than apo-SOD (Fig. 4A). The holoprotein shows an exotherm at temperatures above the unfolding endotherm, likely caused at least in part by irreversible protein aggregation. The reversibility of unfolding is not as high for holo- as for apo-SODs, presumably because of irreversible processes such as aggregation and chemical modification that are enhanced at higher temperatures (49). Holo-SOD unfolding is 52% reversible when scanning to the end of the unfolding endotherm. Full reversibility is only apparent when scanning through the first ~50% of the transition. Scanning through ~75% of the endotherm results in ~85% reversibility. Increasing the cooling rate for scans increases the reversibility, suggesting that some of the observed irreversibility is related to the amount of time that the protein is exposed to high temperatures. Consequently, only the first ~70% of holo-endotherms were used for data fitting. Reversible thermodynamic analysis has often also proven to be applicable in other systems showing some irreversibility (26, 50).

**Holo-pseudo-WT SOD Thermal Unfolding Fits a Dimer 2-State (N_2 ↔ 2U) Model**—Previous DSC studies on holo-SOD used 2-component, 2-state monomer fits to account for the asymmetric nature of the thermograms (2, 3). The two components were attributed to the existence of two different oxidation states of copper, Cu^{2+} and Cu^{+} (1, 3). We find, however, that unfolding holo-SOD in 20 mM HEPES, pH 7.8, in the presence of the mild reducing agent, sodium isoascorbate (ratio of 2 mol of isoascorbate per mol of copper), has no significant effect on the DSC data (data not shown). Similar molar ratios of isoascorbate were

**FIGURE 3.** DSC data for apo-Gly-93 mutants. A, typical thermograms (solid lines) with dimer 2-state (N_2 ↔ 2U) model fits (dashed lines). Scans and corresponding fits are offset for clarity. B, free energy plots for apo-mutants (solid lines) compared with pseudo-WT (dashed line). The free energy plots are the average plots generated from the fitted parameters according to Equations 5–8, assuming a constant ΔC_p = 3.3 kcal mol⁻¹ °C⁻¹. The mutants are destabilized relative to pseudo-WT because their curves are shifted to lower temperatures. The intersection of the horizontal line, −RTlnP (for 0.50 mg ml⁻¹), with the free energy plots gives predicted t_m values for each mutant. Predicted t_m values are in good agreement with fitted t_m values (within ±0.41 °C for the pseudo-WT, ±0.41 °C for G93A, ±0.42 °C for G93S, ±0.12 °C for G93V, ±0.38 °C for G93D, and ±0.28 °C for G93T on average). The average of the t_m values, 49.4 °C, is shown as the vertical line.
TABLE 2
Thermodynamic parameters of apo-mutant SODs

| Apo-SOD   | $t_m^a$ | $t_{mii}^b$ | $\Delta G(t_{mii})^c$ | $\Delta G(25^\circ C)^d$ | $\Delta H^e$ | $\Delta S^f$ | $\Delta G(t_{mii})^g$ |
|-----------|---------|-------------|------------------------|---------------------------|-------------|------------|------------------------|
|           | °C      | °C          | kcal mol$^{-1}$         | kcal mol$^{-1}$           | kcal mol$^{-1}$ | kcal mol$^{-1}$ K$^{-1}$ | kcal mol$^{-1}$         |
| Pseudo-WT | 59.0    | 52.3 ± 0.3  | 11.3 ± 0.8              | 11.2 ± 0.8                | 17.3 ± 2.4   | 15.8 ± 2.4  | NA                     |
| G93S      | 50.7    | 41.7 ± 1.2  | 7.7 ± 0.1               | 7.7 ± 0.1                 | 15.3 ± 0.4   | 14.4 ± 0.4  | +18.6 ± 0.06           |
| G93R      | 48.9    | 42.7 ± 1.4  | 6.9 ± 0.1               | 6.9 ± 0.1                 | 13.5 ± 0.7   | 12.7 ± 1.0  | +4.4 ± 0.02            |
| G93A      | 48.5    | 42.7 ± 1.1  | 6.7 ± 0.2               | 6.6 ± 0.3                 | 13.9 ± 0.6   | 13.2 ± 0.6  | +13.5 ± 0.06           |
| G93V      | 46.2    | 45.2 ± 0.3  | 5.6 ± 0.2               | 5.5 ± 0.2                 | 13.6 ± 0.3   | 13.0 ± 0.3  | +22.0 ± 0.08           |
| Pseudo-WT | 43.1    | 38.2 ± 1.1  | 4.3 ± 0.1               | 4.2 ± 0.1                 | 12.2 ± 0.2   | 11.6 ± 0.3  | +18.2 ± 0.08           |
| G93S      | 50.7    | 41.7 ± 1.2  | 7.7 ± 0.1               | 7.7 ± 0.1                 | 15.3 ± 0.4   | 14.4 ± 0.4  | +18.6 ± 0.06           |
| G93R      | 48.9    | 42.7 ± 1.4  | 6.9 ± 0.1               | 6.9 ± 0.1                 | 13.5 ± 0.7   | 12.7 ± 1.0  | +4.4 ± 0.02            |
| G93A      | 48.5    | 42.7 ± 1.1  | 6.7 ± 0.2               | 6.6 ± 0.3                 | 13.9 ± 0.6   | 13.2 ± 0.6  | +13.5 ± 0.06           |
| G93V      | 46.2    | 45.2 ± 0.3  | 5.6 ± 0.2               | 5.5 ± 0.2                 | 13.6 ± 0.3   | 13.0 ± 0.3  | +22.0 ± 0.08           |
| Pseudo-WT | 43.1    | 38.2 ± 1.1  | 4.3 ± 0.1               | 4.2 ± 0.1                 | 12.2 ± 0.2   | 11.6 ± 0.3  | +18.2 ± 0.08           |

$^a_t_m$ is at 0.50 mg ml$^{-1}$ from Fig. 3B.
$^b_t_{mii}$ is the average of two measurements.
$^c\Delta G$ extrapolated to the $t_m$ using a temperature-independent $\Delta C_p$ (see “Results”). Italicized values are extrapolated using a temperature-dependent $\Delta C_p$ (see “Discussion”).
$^d\Delta G$ extrapolated to 25 °C using a temperature-independent $\Delta C_p$ (see “Results”). Italicized values are extrapolated using a temperature-dependent $\Delta C_p$ (see “Discussion”).
$^e\Delta \Delta H = \Delta H_{m突} - \Delta H_{野生}$ Values are calculated using the temperature-independent $\Delta C_p$.
$^f\Delta \Delta S = \Delta S_{m突} - \Delta S_{野生}$. Values are calculated using the temperature-independent $\Delta C_p$.
$^g\Delta \Delta G = \Delta G_{m突} - \Delta G_{野生}$. A negative value indicates that the mutant SOD is destabilized relative to the pseudo-WT.

employed in NMR experiments, where the metal is fully reduced to Cu$^{+}$, but there is no reduction of the conserved intrasubunit disulfide bond (22). The oxidation state of the metal appears to have a minimal effect on stability. Fig. 4B shows a representative 2-component, 2-state fit for holo-pseudo-WT data. The fit accounts for the data very well; however, the average of the $\Delta H_{m突}/\Delta H_{cal}$ ratios for the two components is 2.01 after normalization per mol of monomer, suggesting that the cooperative unfolding unit is in fact a dimer.

Because holo-SOD monomers also associate with very high affinity ($K_d < 10^{-10}$ M (27)), it is again reasonable that there is no significant dimer dissociation prior to unfolding. The dimer 2-state ($N_2 = 2U$) model fits the holo-pseudo-WT data very well, as illustrated in Fig. 4C. Also, $t_m$ increases with increasing protein concentration as expected for a dimer, and the average $\Delta H_{m突}/\Delta H_{cal}$ ratio is essentially 1 (Table 3), supporting the applicability of the model. A plot of $\ln P$ versus $1/t_m$ (Equation 4) also gives a molecularity of $\sim 2$.

It should be noted that implicit in the dimer 2-state analysis is the assumption that metals remain bound upon unfolding. Metals that are released upon unfolding would further increase the asymmetry of the thermogram and further increase the protein concentration dependence of $t_m$ (26). In the absence of excess free metals, when fully metalated native protein unfolds and releases metals, the metals have effects analogous to additional subunits, i.e. the system behaves more like a hexamer. Including the effects of metal release upon unfolding resulted in significantly lower quality fits. Therefore, it appears that metals stay bound to thermally unfolded SODs. Further support for this is the extremely high affinity of native SOD for metals (dissociation constants of $\sim 4 \times 10^{-14}$ M for zinc and $6 \times 10^{-18}$ M for copper (51)) and the fact that cofactors that bind very tightly to other proteins in the native state remain associated in the thermally unfolded states (52). Note that as long as the SOD concentration remains higher than the dissociation constant of the metal for the unfolded state, the metals will remain bound and not further affect the protein concentration dependence of the DSC transitions (26, 52). Another important point is that the preparations of holo-SOD were very extensively dialyzed against solutions and water with concentrations of metals lower than the detection limit of ICP–AES (<1 ppb), and SOD samples are confirmed to be fully metalated by ICP–AES, activity measurements, and DSC. Thus, the DSC samples are well defined as having 1:1 protein subunit:metal ratios, and DSC of different protein batches give highly reproducible results.

Considering the very good fits of the data to the dimer 2-state model, the lack of effect of isoscorbate on holo protein, $\Delta H_{m突}/\Delta H_{cal}$ of the two-component fit compared with the 2-state dimer fit, and the tight dimeric nature and very strong metal binding of the holoprotein, the
2-state dimer fit without metal release was taken to be the appropriate model for the thermal unfolding of holo-SOD.

Holo-Gly-93 Mutant SODs Are Destabilized Relative to Pseudo-WT—The reversibility of unfolding for the holo-mutants was as high as or higher than that for pseudo-WT. Holo-G93V, which had the lowest melting temperature, exhibited the highest level of reversibility, again suggesting that irreversibility is related to high temperature-induced modifications of the protein. Fig. 5A shows DSC thermograms and dimer 2-state fits for all the mutant holoproteins; fitted parameters are summarized in Table 3. The $t_m$ values for all holo-mutants increased with increasing protein concentration as observed for pseudo-WT, consistent with a dimer 2-state transition. The fitted $C_p$ values again showed considerable variability, likely related to truncation of the data in the fits. On average, the holo-mutants showed $\Delta H_{st}/\Delta H_{cal}$ ratios of close to 1 (Table 3). This is in marked contrast with the results obtained for the apo-mutants, which clearly showed ratios greater than 1 for all scans (Table 1).

A Kirchoff analysis to determine $C_p$ by varying pH was also conducted for holo-pseudo-WT (Fig. 5B). This was limited in pH and temperature range, however, by loss of metal coordination at low and high pH. Kirchoff analysis was therefore also conducted by varying guanidinium chloride (GdmCl) concentration (Fig. 5B). The $C_p$ values obtained from the pH and GdmCl data are 2.75 and 3.11 kcal mol$^{-1}$C$^{-1}$, respectively, in reasonable agreement with the average fitted values (Table 3) and with an expected decrease in $C_p$ at increased temperature (e.g. relative to apo) (35). Because GdmCl binding can cause overestimation of $C_p$ (53), 2.75 kcal mol$^{-1}$C$^{-1}$ was taken as a reasonable value for $C_p$.

Values of $\Delta G$ as a function of temperature were calculated for all the holoproteins (Fig. 5C), as for apoproteins, using the dimer 2-state fitted parameters and temperature-independent $C_p$ set to 2.75 kcal mol$^{-1}$C$^{-1}$ in Equations 5–8. The validity of the calculations was checked by determining the temperatures where the plotted lines of Equation 9 intersect with the $\Delta G$ plots (Fig. 5C). Predicted $t_m$ values agreed well with the fitted values.

Table 4 lists the calculated $\Delta G$ and $\Delta A G$ values for the holo-proteins at $t_{avg}$ (87.2 °C) and extrapolated to 25 °C. All the holo-mutants are destabilized relative to pseudo-WT, with relative stabilities being similar to those of the apoproteins, although the extent of destabilization is generally decreased for holo-protein. $\Delta G$ values range from −3.0 to −5.4 kcal mol$^{-1}$ at the $t_{avg}$ for G93S and G93V, respectively. In contrast to the results obtained for apo-mutants, which were entropically destabilized (Table 2), the decreases in $\Delta G$ for the holo-mutants resulted from decreases in $\Delta H$. The decreases in $\Delta H$ for the holo-mutants were partially compensated by decreases in $\Delta S$.

Metal binding causes $t_m$ to increase markedly (Tables 1 and 3 and supplemental Table SI), with somewhat larger increases in $t_m$ occurring for the less stable proteins. A similar observation was reported for A4V and G93A mutations made in the wild-type SOD background (54). The increase in $\Delta G$ of holo relative to apo is quite uniform for all the proteins, approximately +20 kcal mol$^{-1}$ at the average $t_m$ value for holo and apo of 68.3 °C. Thus, the Gly-93 mutations have little effect on metal binding. This is further supported by full metallation based on ICP-AES and activity measurements (data not shown).

**DISCUSSION**

**Dimer 2-State ($N_2 \leftrightarrow 2U$) Thermodynamic Analysis of SOD**—Although previous studies (mostly on partially purified or partially metallated proteins) have indicated that many fALS-associated mutations may decrease the stability of SOD, the thermodynamic effects of mutations had not been characterized, because of complications of irreversible denaturation and of partial metallation (55, 56). In this study, using highly homogeneous preparations of holo- and apo-SOD mutants in a pseudo-WT background containing no free cysteines, thermal unfolding is shown to be highly reversible and follow dimer 2-state equilibrium.

**Table 3**

Fitted 2-state dimer parameters for holo-SODs

| Holo-SOD | [Protein] | $t_m$ | $\Delta H_{st}$ | $\Delta H_{st}/\Delta H_{cal}$ | $\Delta C_p$ |
|----------|-----------|-------|----------------|-----------------------------|-------------|
| pseudo-WT | 6.30 | 94.2 ± 0.3 | 238.7 ± 20.2 | 0.85 | 3.76 |
| pseudo-WT | 1.87 | 93.2 ± 0.3 | 276.9 ± 20.5 | 1.02 | 7.12 |
| pseudo-WT | 1.00 | 92.5 ± 0.5 | 269.0 ± 30.5 | 1.05 | 5.85 |
| pseudo-WT | 1.00 | 93.2 ± 0.0 | 272.6 ± 4.0 | 0.90 | 9.81 |
| pseudo-WT | 1.00 | 93.1 ± 0.2 | 268.8 ± 10.6 | 1.01 | 1.47 |
| pseudo-WT | 0.50 | 91.6 ± 0.5 | 230.6 ± 24.3 | 1.04 | 5.41 |
| pseudo-WT | 0.50 | 92.4 ± 0.4 | 256.5 ± 22.8 | 1.10 | 4.53 |
| pseudo-WT | 0.20 | 91.6 ± 0.2 | 265.5 ± 15.1 | 1.15 | 4.16 |

* $\Delta H_{st}$ errors (±) were propagated as in Table 1.
* $\Delta C_p$ errors for individual fits could not be reliably calculated since they are based on uncertainties of five different variables.
Stability and Aggregation of fALS-associated Gly-93 Mutant SOD

The DSC data for holo-Gly-93 mutants. A. scans of holo-mutants compared with pseudo-WT. Samples were in 20 mM HEPES, pH 7.8. The data (solid lines) are well fit by a dimer 2-state (K2→2U) model (dashed lines). The scans and corresponding fits are offset for clarity. B, Kirchoff plots of ΔHm versus Tm for scans of holo-pseudo-WT as a function of pH (open circles; 0.5 mg ml−1 protein in 20 mM glycine or HEPES, pH 6.8–9.8) and GdmCl (closed circles; 0–2.4 M GdmCl, 0.5 mg ml−1 protein in 20 mM HEPES, pH 7.8). The pH and GdmCl data fit to straight lines (dashed and solid, respectively) have slopes corresponding to ΔCp values of 2.75 and 3.11 kcal mol−1 °C−1 and coefficients of correlation of r = 0.988 and r = 0.918, respectively. C, free energy plots for holo-mutants (solid lines) and pseudo-WT (dashed line). The free energy plots are the average plots generated from the fitted parameters according to Equations 5–8, assuming a constant pseudo-WT. Samples were in 20 mM HEPES, pH 7.8. The data (pseudo-WT) for G93D, and for the pseudo-WT, shown as the vertical line. The DSC data and the quantitative thermodynamic analysis are consistent with considerable literature data. It is reasonable to assume that the protein persists as a dimer until it thermally unfolds, considering the very tight association of the monomers for both apo- and holo-SOD (27). ΔG (25 °C) for apo-pseudo-WT (17.3 ± 2.4 kcal mol−1) as determined by using constant ΔCp agrees with chemical denaturation calculations for human apo-SOD. For example, a recent study estimated ΔG for apo-pseudo-WT as 15.8 kcal mol−1 based on urea denaturation experiments (57), and a kinetic study on apo-pseudo-WT reported a ΔG of ~19 kcal mol−1 (58). Chemical denaturation studies in our laboratory using GdmCl estimate ΔG at ~18 kcal mol−1 (data not shown). Because 25 °C is fairly far from the melting temperatures, ΔG(25 °C) was also calculated using a temperature-dependent ΔCp according to Ref. 59 (and see Equations 10 and 11),

\[
\Delta G(T) = \Delta H(T_m) + \int_{T_m}^{T} \Delta C_p \, dT - T \Delta S(T_m) - T \int_{T_m}^{T} \frac{\Delta C_p}{T} \, dT
\]

(Eq. 10)

where

\[
\Delta C_p(T) = 0.1614T^2 + 76.048T + 3747.8
\]

(Eq. 11)

is the predicted temperature-dependent ΔCp based on the primary amino acid sequence of SOD (Table 2). This decreases ΔG(25 °C) slightly, by ~0–1.5 kcal mol−1. Similarly, the value of ΔG(25 °C) for holo-pseudo-WT determined using constant ΔCp (Table 4; 35.8 ± 3.2 kcal mol−1) is in the same range as that determined by chemical denaturation in our laboratory (35.1 ± 1.2 kcal mol−1) (60). By using a temperature-dependent ΔCp, ΔG(25 °C) is again slightly decreased (32.0 ± 3.2 kcal mol−1; Table 4) and in good agreement with chemical denaturation.

Decreased Stability of fALS Mutant SODs—All the fALS mutations of Gly-93 are found to be significantly destabilizing for both apo- and holo-SOD, with relative stabilities being similar in apo and holo, but more pronounced in apo (approximately ~1.8 to ~3.5 kcal mol−1 monomer for apo and approximately ~1.6 to ~2.7 kcal mol−1 monomer for holo; see Tables 2 and 4). The mutations to alanine, serine, and arginine have roughly comparable effects, whereas aspartic acid is slightly more destabilizing, and valine is the most destabilizing. There is no other directly comparable stability data in the literature on Gly-93 mutants, except for G93A. Measurements of ΔΔG for apo-G93A using a monomeric SOD construct or dimeric pseudo-WT give values of ~2.4 and ~1.5 kcal mol−1 monomer, respectively (57), similar to the value obtained here (~2.3 kcal mol−1 monomer; see Table 2). The larger decrease in melting temperature and free energy of unfolding for apo-mutants may be particularly relevant in vivo, because aggregation-prone species may be more readily accessed from this less stable state.

The relative stabilities of the different mutants follow statistical preferences observed for residues at this position (i + 3) in type I turns (61). Glycine is the only prefered residue at this position, presumably because this residue can readily adopt the required left-handed helical backbone conformation, with minimal conflicting steric interactions involving side chain atoms, facilitating the return of the polypeptide chain to run antiparallel to its original direction. Valine residues are slightly statistically disfavored, likely because of the increased steric clash of the β-branched side chain. The differences in the statistical occurrences of valine, alanine, serine, aspartic acid, and arginine are not very pronounced, however, and this is consistent with comparable changes in stability for all these mutations. The very marked preference for glycine, with no other marked high or low preferences, suggests that any substitution at this position will tend to be destabilizing.

unfolding behavior. The similarity of the melting temperatures for holo-pseudo-WT and wild-type SOD (2) and for G93A mutants in the pseudo-WT (Tables 1 and 3) and wild-type background (54) indicates that the effects of the fALS mutations are not altered by mutating the free cysteines.

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It is interesting that all the Gly-93 mutations have similar effects; in all cases holo-SOD is enthalpically destabilized, whereas apo-SOD is entropically destabilized. Thus, the changes appear to be governed by the removal of the glycine, rather than by the specific characteristics of the substituted residues. Other studies have found that changes in thermodynamic stability caused by mutation tend to follow structurally based statistical preferences for amino acids; however, quantitative interpretation of changes in entropy and enthalpy upon mutation have proven very difficult to rationalize, because of complex effects that may occur in the native and in the denatured states for the protein and for the associated solvent (41). The same is true here. NMR studies have found that the G93A mutation in pseudo-WT causes a disruption in the hydrogen bonding network around the site of mutation and increases the mobility of the native holoprotein (62); this would lead to an increase in entropy of the native state, and hence decrease in $\Delta S$, giving a negative $\Delta \Delta S$. Increased mobility in the native state is likely to be associated with weakened stabilizing enthalpic interactions, and hence negative $\Delta H$. In addition, removal of a glycine is expected to decrease the entropy of destabilization at high temperatures (63), also contributing to a negative $\Delta \Delta S$. Negative $\Delta \Delta S$ and $\Delta H$ are in fact observed for the holo-mutants.

One might expect that similar arguments would apply to the apoprotein, and so it is perplexing why in fact opposite effects are observed, i.e. positive $\Delta \Delta S$ and $\Delta H$. The explanation for this may be related to the very different temperatures where stability changes were measured for holo and apo, i.e. more than 30 °C lower for the apo. If $\Delta C_p$ is lower for the mutant proteins, as may be the case for apo (Table 4), then a change from entropy-driven destabilization at low temperatures to enthalpy-driven destabilization at high temperatures could occur (64). The metals may also significantly affect entropy and enthalpy changes. However, metal binding has similar effects on $t_m$ and $\Delta G$ in mutants and pseudo-WT (supplemental Table S1) and so the mutations do not substantially weaken metal binding. In other proteins, comparable mutations involving glycines resulted in similar changes in stability, because of either enthalpy or entropy changes (65–68). Further study is clearly still required to understand the molecular basis for changes in stability caused by apparently small changes such as residue substitution or metal binding. We note, however, that because Gly-93 is relatively rigid in SOD (6, 62), mutations may be expected to be more strongly destabilizing compared with mutations at more mobile positions (68).

Aggregation of APO-mutants—A very striking result is that all the scans of all the Gly-93 mutant apo-SODs exhibited $\Delta H_{\text{agg}}/\Delta H_{\text{cal}}$ ratios greater than 1, indicative of increased aggregation compared with pseudo-WT. We have also observed high $\Delta H_{\text{agg}}/\Delta H_{\text{cal}}$ ratios for various other FALS mutant apo-SOD, suggesting that this is a common characteristic for the mutant proteins. The deviations from unity in the ratios could be the result of high $\Delta H_{\text{agg}}$ values or low $\Delta H_{\text{cal}}$ values, or a combination of both; all cases indicate increased aggregation. Significant intermolecular cooperation involving more than 2 subunits during unfolding could cause abnormally high $\Delta H_{\text{agg}}$ (26, 69, 70). Alternatively, misfolded and/or aggregated protein may not contribute to $\Delta H_{\text{agg}}$ thus producing unusually small endothermic areas (71). The most destabilized mutants (G93V and G93D) tend to have the highest $\Delta H_{\text{agg}}/\Delta H_{\text{cal}}$ ratios and particularly low $\Delta H_{\text{cal}}$ values in a plot of $\Delta H_{\text{cal}}$ versus $t_m$ (Tables 1 and 2). This suggests that the least stable mutants may have the greatest propensity to aggregate.

One of the main hypotheses for disease mechanism in mutant SOD-associated FALS is increased toxic aggregation of mutant protein (8, 72). Previous studies have reported evidence that more destabilizing mutations have increased tendency to aggregate (modulated by effects on net charge of protein), and this may be associated with shorter disease duration (20, 57); however, not all FALS mutations destabilize the apoprotein (73). Thus, the factors underlying aggregation are not yet well understood, and other forms of SOD than apo may also have a role in aggregation in disease. In vitro measurements of aggregation can be problematic because aggregation may occur very slowly, as may also be the case in vivo. Conditions that decrease protein stability, chemically or by increased temperature, have been used to bring in vitro measurements of aggregation into an experimentally tractable time scale (20, 57). Here we establish a novel approach for measuring aggregation of mutant SOD, which appears to be reversible or constant with time, using DSC. It is intriguing that the aggregation may be reversible or constant upon resampling and that light scattering measurements as a function of temperature suggest that the aggregates are small, because evidence is mounting that small aggregates may be the toxic species in disease (8). Given that a general correlation between protein and FALS disease characteristics has still not been identified, it is important to further characterize the effects of mutations on holo- and apoprotein. In this study, we have established a calorimetric method for measuring the effects of mutations on both thermodynamics and aggregation of apo and holo-SOD. These types of measurements may ultimately contribute to understanding the molecular basis for ALS and to developing urgently needed ALS treatments.

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