Kinetic Stability of Cu/Zn Superoxide Dismutase Is Dependent on Its Metal Ligands: Implications for ALS†

Sandra M. Lynch, Sarah A. Boswell,‡ and Wilfredo Colón*

Department of Chemical and Biological Engineering and Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, 110 Eighth Street, Troy, New York 12180

Received June 7, 2004; Revised Manuscript Received September 17, 2004

ABSTRACT: Over 100 mutants of the enzyme Cu/Zn superoxide dismutase (SOD) have been implicated in the neurodegenerative disease familial amyotrophic lateral sclerosis (FALS). Growing evidence suggests that the aggregation of SOD mutants may play a causative role in FALS and that aberrant copper chemistry, decreased thermodynamic stability, and decreased affinity for metals may contribute independently or synergistically to this process. Since the loss of the copper and zinc ions significantly decreases the thermodynamic stability of SOD, it is expected that this would also decrease its kinetic stability, thereby facilitating partial or global unfolding transitions that may lead to misfolding and aggregation. Here we used wild-type (WT) SOD and five FALS-related mutants (G37R, H46R, G85R, D90A, and L144F) to show that the metals contribute significantly to the kinetic stability of the protein, with demetalated (apo) SOD showing acid-induced unfolding rates about 60-fold greater than the metalated (holo) protein. However, the unfolding rates of SOD WT and mutants were similar to each other in both the holo and apo states, indicating that regardless of the effect of mutation on thermodynamic stability, the kinetic barrier toward SOD unfolding is dependent on the presence of metals. Thus, these results suggest that pathogenic SOD mutations that do not significantly alter the stability of the protein may still lead to SOD aggregation by compromising its ability to bind or retain its metals and thereby decrease its kinetic stability. Furthermore, the mutant-like decrease in the kinetic stability of apo WT SOD raises the possibility that the loss of metals in WT SOD may be involved in nonfamilial forms of ALS.

Amyotrophic lateral sclerosis (ALS) is a disease that characteristically destroys the motor neuron system within a few years of onset, yet the reason for its development remains a mystery (*1*). Familial ALS (FALS)[1] accounts for about 10% of all ALS cases, and in 20% of these, point mutations of the antioxidant enzyme Cu/Zn superoxide dismutase (SOD) are implicated in the disease (*2*). SOD is a dimeric protein with each monomer binding one copper and one zinc ion. The copper ion is bound in the catalytic site and is necessary for the enzymatic function, while the zinc ion is believed to play a structural role by stabilizing the protein (*3*). FALS pathology is characterized by the presence of neuronal protein aggregates containing SOD and other fibrillar material (*4*). This is a characteristic typical of other neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and the spongiform encephalopathies (*5*, *6*).

Aggregates typically form when a protein becomes misfolded and is not degraded or redirected to the proper fold (*7*). Many proteins with a propensity for misfolding and aggregation overcome this potentially toxic mishap by possessing high thermodynamic and/or kinetic stability (*8*). The latter term describes proteins that are trapped in their native state by an unusually high free energy barrier to unfolding (*9*). SOD has been characterized as having a high thermodynamic stability (*10*, *11*) that is intimately related to its degree of metalation (*11*−*13*) and the highly complementary hydrophobic interactions between subunits (*14*). Therefore, mutations at the sites of metal coordination and at the dimer interface result in loss of thermodynamic stability, leading to SOD proteins more prone to aggregation (*11*, *15*). However, several SOD mutations related to FALS appear to have a modest effect on stability (*12*) and, therefore, may facilitate its toxic effect by other means. One possibility is that, in certain cases, SOD mutations may predispose the protein to aggregation by somehow decreasing its kinetic stability and not via a direct thermodynamic effect. This idea is consistent with the compelling argument that thermodynamic stability alone may not always protect proteins that are susceptible to irreversible aggregation arising from partially denatured states (*8*). Therefore, the presence of a high kinetic energy barrier to unfolding may serve to trap some proteins in their native conformation and thereby protect them against misfolding and aggregation.

In this work, we investigate the kinetic stability of WT SOD and five FALS-related SOD mutants (G37R, H46R, G85R, D90A, and L144F), in both the metal-deficient (apo) and metalated (holo) forms. By comparing the acid-induced unfolding kinetics at physiological temperature, we deter-

† This work was supported by a NIH grant (R01NS42915) to W.C.

* Address correspondence to this author. E-mail: colonw@rpi.edu. Phone: (518) 276-2515. Fax: (518) 276-4887.

‡ Current address: Ordway Research Institute, 150 New Scotland Ave., Albany, NY 12208.

[1] Abbreviations: apo, metal deficient; FALS, familial amyotrophic lateral sclerosis; GdmCl, guanidinium chloride; HCl, hydrochloric acid; holo, metal loaded; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SOD, Cu/Zn superoxide dismutase; TS, transtion state; WT, wild type.

10.1021/bi048831v CCC: $27.50
Published on Web 12/03/2004

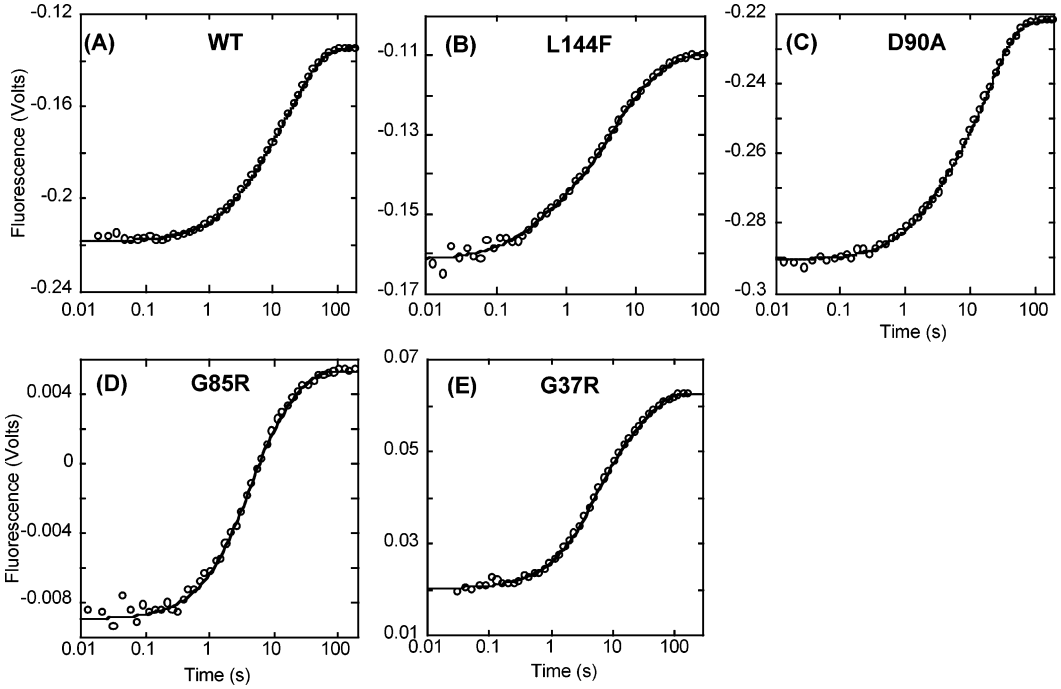

FIGURE 1: Acid-induced unfolding kinetics of WT, L144F, D90A, G85R, and G37R holo SOD. Open circles represent the data, and the solid line is the fit to a sum of exponentials as described in Materials and Methods.

mined the important role of the metals in the kinetic stability of SOD. Our results suggest that some FALS-related SOD mutations that do not significantly affect the thermodynamic stability of the protein may still enhance the misfolding and aggregation propensity of SOD by compromising its ability to bind or retain its metals and thereby decreasing its kinetic stability.

MATERIALS AND METHODS

SOD Expression and Purification. SOD was expressed in *Escherichia coli* (*16*) and purified as previously described (*17*), with the following modifications. After harvesting, cells were centrifuged for 9 min at 8000 rpm, resuspended in 2.5 mM sodium phosphate buffer (PB) (pH 7.4), and frozen overnight at −80 °C. After cell lysis by sonication, the cells were subjected to a series of ammonium sulfate precipitations, and the SOD present in the 60−85% (w/w) fraction was selected for further column purification. Column purification was done on a phenyl-sepharose hydrophobic column eluting with a gradient of 2−0 M ammonium sulfate in 150 mM NaCl/50 mM PB (pH 7.0), followed by a diethylaminoethyl (DE52) anion-exchange column eluting with a 2.5−200 mM PB gradient (pH 7.4). SDS−PAGE was used for fraction analysis and to check the purity of the SOD samples. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Preparation of Apo and Holo SOD Samples. Because SOD mutants expressed in *E. coli* do not have reliable metal binding, in part due to the lack of the copper chaperone for SOD, we did not use the protein as isolated for our experiments. To prepare apo SOD, the protein was fully demetalated on a cation-exchange carboxymethyl- (CM-) cellulose column using a method similar to that described by Sutter et al. (*18*), using 30 mM instead of 50 mM acetate buffer. Holo SOD was prepared by loading the metals onto apo SOD as previously described (*19*). In this protocol, the zinc is added first to allow correct binding to its site, followed by the addition of copper, which binds to the correct site within the zinc-containing SOD. This is particularly important for FALS-related SOD mutants, which have been shown to possess reduced copper ion binding specificity (*20*). The correct loading of metals was verified by determining the activity of SOD using an assay (Oxford Biomedical Research) that is based on a spectroscopic method developed by Nebot et al. (*21*). The extent of metal content was determined using a Hitachi Z-8200 polarized zeeman atomic absorption spectrophotometer (Hitachi Instruments, Tokyo, Japan).

Kinetic Experiments. Kinetic unfolding experiments were performed using an Applied Photophysics SX.18MV-R stopped-flow instrument (Surrey, U.K.) with a dead time of 0.8 ms. All experiments were done using a 10 mm pathlength cell and mixing at a 1:10 ratio of protein stock:buffer, resulting in 11-fold dilution of the protein sample. Initial SOD samples were 0.6−1.65 mg/mL in 20 mM PB, pH 7.4, and kept on ice until use. The baseline buffer used was 10 mM PB, pH 7.4, and the denaturant buffer was a standard 0.01 M HCl (pH 2.0) stock solution. Upon mixing, the samples were diluted to 0.05−0.15 mg/mL, pH 2.1. Excitation of the single tryptophan in each SOD monomer was performed at 280 nm, and fluorescence emission was monitored using a 305 nm cutoff filter and 0.4 mm slit width to prevent photobleaching. The temperature was maintained at 37 °C.

Data Analysis. Analysis of kinetic data was performed using KaleidaGraph version 3.5 (Synergy Software) to best fit the data to the appropriate first-order sum of exponentials:

$$A(t) = \sum_{i=1}^{n} A_i \exp^{-k_i t} + A_\infty \qquad (1)$$

where $A(t)$ is the observed kinetic trace, A_i is the amplitude

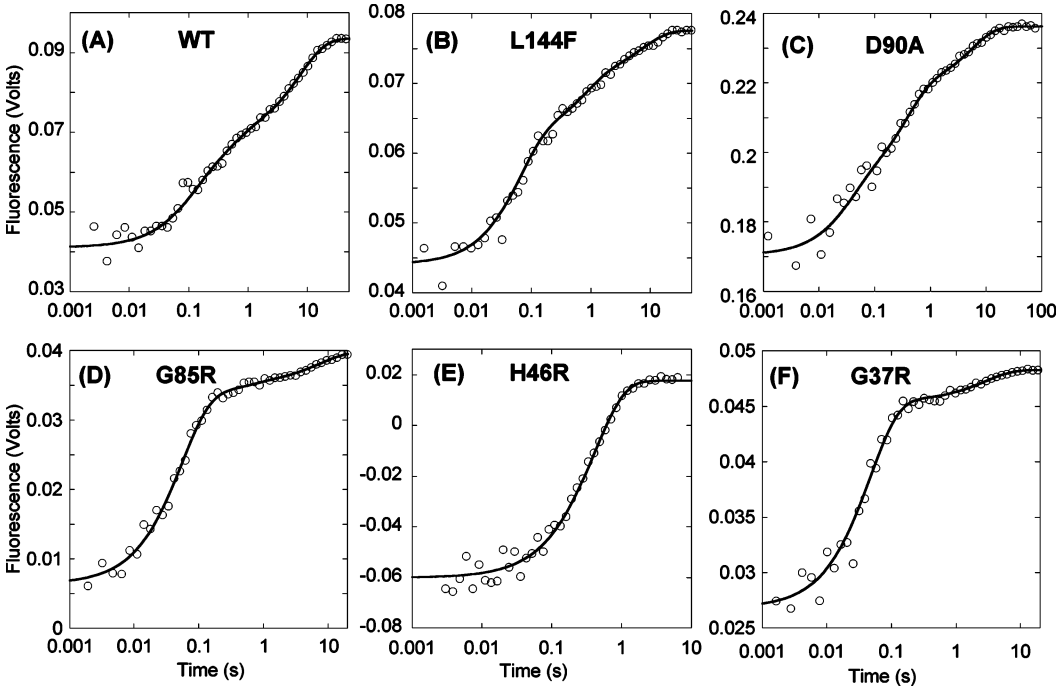

FIGURE 2: Acid-induced unfolding kinetics of WT, L144F, D90A, G85R, H46R, and G37R apo SOD. Open circles represent the data, and the solid line is the fit to a sum of exponentials as described in Materials and Methods.

associated with rate k_i, and A_∞ is the baseline signal of the completely reacted solution. The fluorescence kinetic profiles were fit to the simplest model that best described the data, which range from single to triple exponential fits.

The change in activation energy on mutation is given by the equation:

$$\Delta\Delta G_u^{\ddagger} = RT \ln(k_m/k) \quad (2)$$

where $\Delta\Delta G_u^{\ddagger}$ represents the change in activation energy, R is the gas constant, and T is the temperature in kelvin. The rate constants for the WT and mutant SOD are denoted by k and k_m, respectively.

RESULTS

Unfolding Kinetics of Holo and Apo SOD. The kinetics of protein unfolding measured under destabilizing conditions, such as low pH or high concentrations of chemical denaturants, provides direct information about the kinetic stability of a protein. To determine the effect of metals on the kinetic stability of WT SOD and mutants implicated in FALS, we determined the kinetics of holo and apo SOD unfolding induced by a rapid decrease in pH from 7.4 to 2.1. Figures 1 and 2 show representative time-resolved unfolding traces of the holo and apo SOD proteins, respectively. The metal content of the SOD samples used to generate these representative data and the corresponding activity of the holo proteins are shown in Table 1. The very high content of copper and zinc (85−100%) and the high SOD activity for the various holo SOD proteins indicate that the metals were mostly bound to their correct binding sites. H46R SOD binds very little metal in the native state because the metal binding site is compromised by the mutation (*11*), and therefore, the unfolding kinetics of the holo protein could not be studied with this mutant.

Table 1: Metal Content of Samples Used in Representative Unfolding Kinetics[a]

	% Cu	% Zn	SOD activity (units/mg)
apo SOD			
WT	0	0	
L144F	4.4	0	
D90A	2.5	2	
G85R	0	6	
H46R	0	0	
G37R	0	3	
holo SOD			
WT	88	100	4145
L144F	85	89	2801
D90A	88	100	5478
G85R	98	92	4098
G37R	85	100	3125

[a] See Figures 1 and 2.

The unfolding kinetic traces of holo SOD were usually fit to a double exponential equation (Figure 1). All holo SOD variants exhibited a slow phase with a rate constant of 0.03−0.06 s^{-1} (Table 2) and also exhibited a second unfolding phase with a rate constant of 0.2−0.5 s^{-1}. L144F and G37R holo SOD showed a faster rate (2−4 s^{-1}) but with a much smaller and inconsistent amplitude. In contrast to holo SOD, the unfolding kinetics of the apo SOD variants lacked the 0.04−0.06 s^{-1} rate constant and exhibited (except for H46R SOD) a much faster unfolding event with a rate constant of 15−25 s^{-1} (Table 2). In addition to this faster phase, most apo SOD variants revealed an intermediate unfolding phase with a rate constant of about 3 s^{-1}, which was basically absent in holo SOD. Finally, the kinetics of apo SOD unfolding revealed a phase with a rate constant of 0.2−0.5 s^{-1} that is similar to one of the phases seen in the holo SOD data (Table 2). Unlike the consistent rate constant observed for holo and apo SOD, there was a significantvariation in the amplitude of the various phases for reasons that are not clear at this time. Thus, a quick comparison of the holo and

Table 2: Rate Constants and Amplitudes of Unfolding Kinetics[a]

	k_1 (s^{-1})	amp k_1 (%)	k_2 (s^{-1})	amp k_2 (%)	k_3 (s^{-1})	amp k_3 (%)
apo						
WT	17.0 ± 2.4	23 ± 16	3.1 ± 1.2	49 ± 22	0.32 ± 0.16	28 ± 19
L144F	15.2 ± 2.9	34 ± 20	2.7 ± 0.7	46 ± 14	0.42 ± 0.19	19 ± 10
D90A	26.0 ± 2.4	33 ± 2	3.0 ± 0.2	41 ± 3	0.33 ± 0.08	26 ± 4
G85R	25.6 ± 6.5	78 ± 4	4.6 ± 2.6	14 ± 7	0.24 ± 0.10	8 ± 4
H46R			2.8 ± 0.4	100		
G37R	25.6 ± 5.8	76 ± 12			0.52 ± 0.20	24 ± 12
holo						
WT			0.30 ± 0.03	30 ± 7	0.04 ± 0.01	70 ± 7
L144F	1.9 ± 0.2	11 ± 9	0.31 ± 0.14	58 ± 14	0.06 ± 0.01	31 ± 17
D90A			0.50 ± 0.14	23 ± 2	0.05 ± 0.01	77 ± 2
G85R			0.23 ± 0.12	40 ± 18	0.04 ± 0.02	60 ± 18
G37R	4.2 ± 0.9	29 ± 35	0.27 ± 0.12	37 ± 25	0.03 ± 0.02	34 ± 10

[a] Standard deviation was determined from the average of three or more independent experiments.

apo SOD unfolding rates (Table 2) suggests that the slow 0.03−0.06 s^{-1} rate is associated with the presence of metals, that the 0.3 s^{-1} rate is related to a metal-independent conformational event, and that the faster rates (15−25 and ≈ 3 s^{-1}) are linked to the absence of metals.

DISCUSSION

Complex Unfolding Mechanism of Apo and Holo SOD. The presence of two to three unfolding kinetic phases for holo and apo SOD indicates that, regardless of the presence of metals, the unfolding mechanism of SOD is not a simple concerted transition from native dimer to unfolded monomer. In principle, the complex unfolding kinetics of holo and apo SOD could be due to sequential or parallel conformational events. Examples of sequential events include conformational changes within the dimer followed by dimer dissociation and subsequent monomer unfolding. Since metal ligands are lost by pH 2.1, conformational unfolding events for holo SOD may be coupled to loss of metal binding. Parallel unfolding events are likely to arise from heterogeneous SOD molecules that differ in their metal content. This is consistent with differential scanning calorimetry studies where multiple endotherms were observed for partially metalated species, with each peak reflecting the unfolding of a different species (*11*). We have been very careful to determine the metal content of each sample and have repeated experiments using proteins from different preparations and stock solutions. The metal content shown in Table 1 is representative of the levels present in apo and holo SOD prepared using established protocols (*18*, *19*). The presence of high activity (Table 1) also indicates that the metals are mostly in the correct site in holo SOD. Therefore, we believe that the holo and apo samples used in this study are largely homogeneous, although some heterogeneity is likely to exist, especially for the holo proteins, where copper content is typically about 85−90%. Therefore, the observed multiexponential unfolding kinetics of SOD largely arises from a series of sequential conformational events. This is consistent with similar results of holo and apo SOD unfolding using guanidine hydrochloride-induced denaturation (*13*).

Although a detailed structural description of the unfolding mechanism of SOD is beyond the scope of this work, a comparison of the holo and apo SOD data may allow us to gain some insight about the role of metals in this process. In the case of holo SOD, of the two phases present with rates of 0.2−0.5 and 0.03−0.06 s^{-1}, the latter is unique to holo SOD and therefore is associated with the presence of metals, while the former is also seen in apo SOD, perhaps due to an analogous metal-independent conformational event occurring in the unfolding of both species. In apo SOD, the slowest rate observed in holo SOD disappears and is usually replaced by the two faster rates (15−25 and ≈ 3 s^{-1}, Table 2). Since the metals directly stabilize the β barrel of SOD, we speculate that the unfolding of holo SOD may first involve the dissociation of the dimer (*22*−*24*), followed by monomer unfolding. However, in apo SOD the β barrel is destabilized, and the denaturation of the protein may involve partial unfolding of the β barrel (faster rates in Table 2) before dimer dissociation.

The unfolding kinetics of G37R and H46R SOD deviate a little from that observed for the other variants. We suspect that it may be due to some heterogeneity in the sample or conformational effects unique to these mutants. For example, the G37R mutation may have a different effect on the fluorescence of the single conformational probe, Trp 32, since they are only five residues apart. In the case of apo H46R SOD, which exhibits a single unfolding rate, it is not clear why the H46R mutation, which is destabilizing to the β barrel (*12*), does not exhibit the higher unfolding rates seen for other apo SOD variants. Further studies are needed to elucidate the mechanism of holo and apo SOD unfolding.

Kinetic Stability of SOD Is Dependent on Metal Binding. The importance of the metals for the thermodynamic stability of SOD has been well established. Using differential scanning calorimetry, Rodriguez et al. have reported that copper or zinc binding conferred a similar degree of incremental stabilization to SOD mutants, as well as to WT SOD (*11*). SOD samples metalated to a higher degree showed larger endotherms at higher transition temperatures than corresponding samples with a lower degree of metalation, thereby demonstrating the stabilizing effect of metal binding on SOD. Specific studies with H46R SOD showed that zinc binding produced an additional thermal transition with a temperature midpoint that was 16 °C higher than that seen for apo H46R SOD. Lindberg et al. (*12*) also demonstrated the stabilizing role of the metal ligands by showing that a set of mutants and WT SOD exhibited a significant decrease in thermodynamic stability in the apo state relative to the holo state. However, they reported that, for the "WT-like mutants" (mutations on the surface of the protein and not in binding or structural sites) such as D90A SOD, no significant destabilization occurred in the holo form, but destabilization

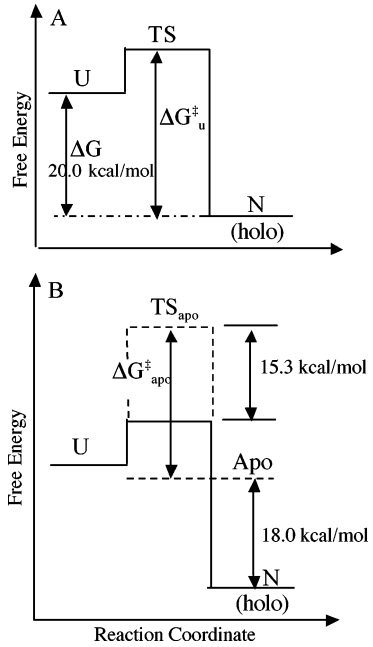

FIGURE 3: Energy diagram illustrating the relative free energies of the native and transition states of holo (A) and apo (B) SOD (U, unfolded state; N, native folded state; TS, transition state; TS_{apo}, transition state of apo state; $\Delta G_u^{\ddagger}$, activation energy for unfolding). The solid line (—) represents holo SOD, and the dashed line (- - -) represents the change in the free energy of the native and TS in apo SOD.

occurred in all the mutant apo forms relative to the WT apo protein. This suggests that metal binding can sometimes compensate for the destabilization of apo SOD due to mutations and further emphasizes the importance of the metals in the thermodynamic stability of SOD.

Although it is known that the metals are required for the biological function and thermodynamic stability of SOD, it is not clear what role they may play in the kinetic stability of SOD and the physiological effect that this may have. A high kinetic stability could prevent misfolding of a protein on a given time scale, even in the presence of low thermodynamic stability (*8*). For example, a protein with low thermodynamic stability and a turnover rate of 1 day in its physiological environment may be protected from misfolding and aggregation if it has an unfolding half-life of 100 days under native conditions. Previous work on lysozyme has shown that when the kinetic barrier for unfolding is high enough, the overall irreversible misfolding of the protein can only go as fast as this rate-limiting step, which has a half-life of several weeks at room temperature (*8*). This is also illustrated in the case of transthyretin (TTR) and its related amyloid disease, where the precursor species leading to TTR amyloid fibril formation is a partially denatured monomeric intermediate. WT TTR had been characterized as possessing high kinetic stability (*25*). However, the significant decrease in the kinetic stability of TTR upon mutation, even when the thermodynamic stability is not affected, makes the amyloidogenic intermediate more accessible, resulting in TTR aggregation (*25*).

Free energy diagrams are a convenient way of illustrating the relationship between the thermodynamic and kinetic stability of proteins (Figure 3A). Kinetic stability is related to the height of the activation energy ($\Delta G_u^{\ddagger}$) for unfolding, with kinetically stable proteins having a large $\Delta G_u^{\ddagger}$ and, therefore, a very slow unfolding rate. From the rate constants shown in Table 2 it is clear that apo SOD is kinetically destabilized relative to the holo protein, with an increase of about 60-fold in the fastest unfolding rate. On the basis of eq 2, the decrease in activation energy for unfolding upon metal loss is only about 2.7 kcal/mol. In contrast, the thermodynamic stabilities (ΔG) of apo and holo WT SOD are 2.1 (*26*) and 20 (*27*) kcal/mol, respectively, resulting in a difference of ≈18 kcal/mol. The small decrease in the unfolding activation energy for apo SOD indicates that the transition state of SOD is destabilized by about 15 kcal/mol due to the loss of metals (Figure 3B). Thus, the transition state for SOD unfolding is very nativelike, and therefore, upon the loss of metals both the native state and the TS are significantly destabilized. It is worth pointing out that if the TS for SOD unfolding were destabilized to the same extent as the native state upon the loss of metals, then there would have been no change in kinetic stability (i.e., same unfolding rate) despite the dramatic decrease (18 kcal/mol) in thermodynamic stability. This seems to explain the interesting observation that the unfolding rates for WT SOD and the SOD mutants studied here are very similar to each other, in both the holo and apo states. Thus, it appears that most FALS-related SOD mutations affect the free energy of the native state and TS for unfolding to a similar extent, thereby resulting in a small or negligible effect on the kinetic stability of holo SOD relative to the WT protein.

Implications for Sporadic and Familial ALS. There is substantial evidence suggesting that the apo form of SOD is linked to its tendency to aggregate. Stathopulos et al. (*13*) demonstrated that SOD mutants with a lower melting point temperature (T_m), as is the case for apo SOD, have an enhanced tendency for aggregation (*13*). Also, it has been shown that apo SOD is taken into the mitochondria, a site of early damage in ALS, much more readily than the holo protein (*28*). An X-ray crystallization study of metal-deficient WT SOD has shown that the β barrel stacking arrangement is similar to that normally found in amyloid fibrils and is facilitated by intermolecular attractions of the disordered zinc-deficient loops of the apo subunits (*29*). An investigation of the crystal structures of A4V and I113T SOD mutants suggests a destabilization of the dimeric interface, leading to the increased propensity of these mutants to lose the metal ligands (*24*). Furthermore, it has been shown that WT and several SOD mutants in the apo form readily aggregate into pore-like structures as a result of copper-induced oxidative damage (*30*). Although the toxicity of these SOD species remains to de determined, membrane permeabilization by pore-like aggregates has been observed for other proteins associated with misfolding diseases (*6*, *31*). Thus, these results support the hypothesis that apo SOD may be the critical species leading to the formation of SOD aggregates found in ALS patients.

This study has shown a decrease in kinetic stability of WT and mutant apo SOD compared to the holo protein *in vitro*, thereby resulting in a significant enhancement in unfolding rate. It is still not clear which metal ion is the major contributing factor to the kinetic stability of holo SOD, but preliminary experiments suggest that it is predominantly the zinc ion (unpublished results), consistent with the role of zinc on SOD stability. Considering the evidence from other studies showing that apo SOD has increased propensity

for misfolding and aggregation, our results suggest that even if there is no loss in the thermodynamic stability of SOD upon mutation, the decreased affinity for metals, a feature of many SOD mutants (*32*), or any event that enhances the propensity for apo SOD formation may be a factor in the development of the disease. For example, oxidative damage to SOD may lead to a decrease in metal affinity (*33*, *34*), resulting in the formation of apo SOD. Interestingly, our results also demonstrate that once the WT protein loses its metals, it has a similar decrease in kinetic stability as the FALS-related SOD mutants and a higher tendency to aggregate (*12*, *30*; unpublished results). Therefore, if apo SOD were to be the critical precursor to the formation of toxic SOD aggregates in FALS (*12*, *28*, *30*), then we speculate that the aberrant formation of apo WT SOD could potentially lead to sporadic ALS, thereby explaining one of the greatest mysteries of ALS: the molecular basis for the remarkable similarity between the familial and sporadic forms of the disease.

REFERENCES

1. Hand, C. K., and Rouleau, G. A. (2002) Familial amyotrophic lateral sclerosis, *Muscle Nerve 25*, 135−159.
2. Cleveland, D. W., and Rothstein, J. D. (2001) From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS, *Nat. Rev. Neurosci. 2*, 806−819.
3. Forman, H. J., and Fridovich, I. (1973) On the stability of bovine superoxide dismutase. The effects of metals, *J. Biol. Chem. 248*, 2645−2649.
4. Julien, J. P. (2001) Amyotrophic lateral sclerosis. unfolding the toxicity of the misfolded, *Cell 104*, 581−591.
5. Kirkitadze, M. D., Bitan, G., and Teplow, D. B. (2002) Paradigm shifts in Alzheimer's disease and other neurodegenerative disorders: The emerging role of oligomeric assemblies, *J. Neurosci. Res. 69*, 567−577.
6. Caughey, B., and Lansbury, P. T. J. (2003) Protofibrils, pores, fibrils, and neurodegeneration: Separating the responsible protein aggregates from the innocent bystanders, *Annu. Rev. Neurosci. 26*, 267−298.
7. Dobson, C. M. (1999) Protein misfolding, evolution and disease, *Trends Biochem. Sci. 24*, 329−332.
8. Plaza del Pino, I. M., Ibarra-Molero, B., and Sanchez-Ruiz, J. M. (2000) Lower kinetic limit to protein thermal stability: a proposal regarding protein stability in vivo and its relation with misfolding diseases, *Proteins 40*, 58−70.
9. Baker, D., and Agard, D. A. (1994) Kinetics versus thermodynamics in protein folding, *Biochemistry 33*, 7505−7509.
10. Battistoni, A., Folcarelli, S., Cervoni, L., Polizio, F., and Desideri, A. (1998) Role of the dimeric structure in the Cu, Zn superoxide dismutase, *J. Biol. Chem. 273*, 5655−5661.
11. Rodriguez, J. A., Valentine, J. S., Eggers, D. K., Roe, J. A., Tiwari, A., Brown, R. H., Jr., and Hayward, L. J. (2002) Familial amyotrophic lateral sclerosis-associated mutations decrease the thermal stability of distinctly metallated species of human copper/zinc superoxide dismutase, *J. Biol. Chem. 277*, 15932−15937.
12. Lindberg, M. J., Tibell, L., and Oliveberg, M. (2002) Common denominator of Cu/Zn superoxide dismutase mutants associated with amyotrophic lateral sclerosis: Decreased stability of the apo state, *Proc. Natl. Acad. Sci. U.S.A. 99*, 16607−16612.
13. Stathopulos, P. B., Rumfeldt, J. A. O., Scholz, G. A., Irani, R. A., Frey, H. E., Hallewell, R. A., Lepock, J. R., and Meiering, E. M. (2003) Cu/Zn superoxide dismutase mutants associated with amyotrophic lateral sclerosis show enhanced formation of aggregates in vitro, *Proc. Natl. Acad. Sci. U.S.A. 100*, 7021−7026.
14. Forest, K. T., Langford, P. R., Kroll, J. S., and Getzoff, E. D. (2000) Cu, Zn superoxide dismutase structure from a microbial pathogen establishes a class with a conserved dimer interface, *J. Mol. Biol. 296*, 145−153.
15. Deng, H., Hentati, A., Tainer, J., Iqbal, Z., Cayabyab, A., Hung, W., Getzoff, E. D., Hu, P., Herzfeldt, B., Roos, R. P., Warner, C., Deng, G., Soriano, E., Smyth, C., Parge, H. E., Ahmed, A., Roses, A. D., Hallewell, R. A., Pericak-Vance, M. A., and Siddique, T. (1993) Amyotrophic lateral sclerosis and structural defects in Cu, Zn superoxide dismutase, *Science 261*, 1047−1051.
16. Crow, J. P., Sampson, J. B., Zhuang, Y., Thompson, J. A., and Beckman, J. S. (1997) Decreased zinc affinity of amyotrophic lateral sclerosis-associated superoxide dismutase mutants leads to enhanced catalysis of tyrosine nitration by peroxynitrite, *J. Neurochem. 69*, 1936−1944.
17. de Beus, M., Chung, J., and Colon, W. (2004) Modification of cysteine 111 in Cu/Zn superoxide dismutase results in altered spectroscopic and biophysical properties, *Protein Sci. 13*, 1347−1355.
18. Sutter, B., Bounds, P. L., and Koppenol, W. H. (2000) The preparation of apo-Cu,Zn superoxide dismutase by ion-exchange chromatography on iminodiacetic acid-sepharose, *Protein Expression Purif. 19*, 53−56.
19. Boissinot, M., Karnas, S., Lepock, J. R., Cabelli, D. E., Tainer, J. A., Getzoff, E. D., and Hallewell, R. A. (1997) Function of the Greek key connection analysed using circular permutants of superoxide dismutase, *EMBO J. 16*, 2171−2178.
20. Goto, J. J., Zhu, H., Sanchez, R. J., Nersissian, A., Gralla, E. B., Cabelli, D. E., and Valentine, J. S. (2000) Loss of in vitro metal ion binding specificity in mutant copper−zinc superoxide dismutases associated with familial amyotrophic lateral sclerosis, *J. Biol. Chem. 275*, 1007−1014.
21. Nebot, C., Moutet, M., Huet, P., Xu, J.-Z., Yadan, J.-C., and Chaudiere, J. (1993) Spectrophotometric assay of superoxide dismutase activity based on the activated autooxidation of a tetracyclic cathecol, *Anal. Biochem. 214*, 442−451.
22. Rakhit, R., Crows, J. P., Lepcock, J. R., Kondejewski, L. H., Cashman, N. R., and Chakrabarty, A. (2004) Monomeric Cu, Zn-superoxide dismutase is a common misfolding intermediate in the oxidation models of sporadic and Familial amyotrophic lateral sclerosis, *J. Biol. Chem. 279*, 15499−15504.
23. Ray, S. S., Nowak, R. J., Strokovich, K., Brown, R. H. J., Walz, T., and Lansbury, P. T. J. (2004) An intersubunit bond prevents in vitro aggregation of a superoxide dismutase-1 mutant linked to familial amyotrophic lateral sclerosis, *Biochemistry 43*, 4899−4905.
24. Hough, M. A., Grossmann, J. G., Antonyuk, S., Strange, R. W., Doucette, P. A., Rodriguez, J. A., Whitson, L. J., Hart, P. J., Hayward, L. J., Valentine, J. S., and Hasnain, S. S. (2004) Dimer destabilization in superoxide dismutase may result in disease-causing properties: Structures of motor neuron disease mutants, *Proc. Natl. Acad. Sci. U.S.A. 101*, 5976−5981.
25. Lai, Z., McCulloch, J., Lashuel, H. A., and Kelly, J. W. (1997) Guanidine hydrochloride-induced denaturation and refolding of transthyretin exhibits a marked hysteresis: equilibria with high kinetic barriers, *Biochemistry 36*, 10230−10239.
26. Assfalg, M., Banci, L., Bertini, I., Turano, P., and Vasos, P. R. (2003) Superoxide dismutase folding/unfolding pathway: role of the metal ions in modulating structural and dynamical features, *J. Mol. Biol. 330*, 145−158.
27. Stroppolo, M. E., Malvezzi-Campeggi, F., Mei, G., Rosato, N., and Desideri, A. (2000) Role of the tertiary and quaternary structures in the stability of dimeric copper, zinc superoxide dismutases, *Arch. Biochem. Biophys. 377*, 215−218.
28. Okado-Matsumoto, A., and Fridovich, I. (2002) Amyotrophic lateral sclerosis: a proposed mechanism, *Proc. Natl. Acad. Sci. U.S.A. 99*, 9010−9014.
29. Strange, R. W., Antonyuk, S., Hough, M. A., Doucette, P. A., Rodriguez, J. A., Hart, P. J., Hayward, L. J., Valentine, J. S., and Hasnain, S. S. (2003) The structure of holo and metal-deficient wild-type human Cu, Zn superoxide dismutase and its relevance to familial amyotrophic lateral sclerosis, *J. Mol. Biol. 328*, 877−891.
30. Chung, J., Yang, H., de Beus, M. D., Ryu, C. Y., Cho, K., and Colón, W. (2003) Cu/Zn superoxide dismutase can form pore-like structures, *Biochem. Biophys. Res. Commun. 312*, 873−876.

31. Kagan, B. L., Hirakura, Y., Azimov, R., Azimova, R., and Lin, M. C. (2002) The channel hypothesis of Alzheimer's disease: current status, *Peptides 23*, 1311−1315.
32. Beckman, J. S., Estevez, A. G., Crow, J. P., and Barbeito, L. (2001) Superoxide dismutase and the death of motoneurons in ALS, *Trends Neurosci. 24*, S15−20.
33. Valentine, J. S. (2002) Do oxidatively modified proteins cause ALS?, *Free Radical Biol. Med. 33*, 1314−1320.
34. Rakhit, R., Cunningham, P., Furtos-Matei, A., Dahan, S., Qi, X. F., Crow, J. P., Cashman, N. R., Kondejewski, L. H., and Chakrabartty, A. (2002) Oxidation-induced Misfolding and Aggregation of Superoxide Dismutase and Its Implications for Amyotrophic Lateral Sclerosis, *J. Biol. Chem. 277*, 47551−47556.

BI048831V