Abnormal SDS-PAGE migration of cytosolic proteins can identify domains and mechanisms that control surfactant binding

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Abstract: The amino acid substitution or post-translational modification of a cytosolic protein can cause unpredictable changes to its electrophoretic mobility during SDS-PAGE. This type of “gel shifting” has perplexed biochemists and biologists for decades. We identify a mechanism for “gel shifting” that predominates among a set of ALS (amyotrophic lateral sclerosis) mutant hSOD1 (superoxide dismutase) proteins, post-translationally modified hSOD1 proteins, and homologous SOD1 proteins from different organisms. By first comparing how 39 amino acid substitutions throughout hSOD1 affected SDS-PAGE migration, we found that substitutions that caused gel shifting occurred within a single polyacidic domain (residues ~80–101), and were nonisoelectric. Substitutions that decreased the net negative charge of domain 80–101 increased migration; only one substitution increased net negative charge and slowed migration. Capillary electrophoresis, circular dichroism, and size exclusion chromatography demonstrated that amino acid substitutions increase migration during SDS-PAGE by promoting the binding of three to four additional SDS molecules, without significantly altering the secondary structure or Stokes radius of hSOD1-SDS complexes. The high negative charge of domain 80–101 is required for SOD1 gel shifting: neutralizing the polyacidic domain (via chimeric mouse-human SOD1 fusion proteins) inhibited amino acid substitutions from causing gel shifting. These results demonstrate that the pattern of gel shifting for mutant cytosolic proteins can be used to: (i) identify domains in the primary structure that control interactions between denatured cytosolic proteins and SDS and (ii) identify a predominant chemical mechanism for the interaction (e.g., hydrophobic vs. electrostatic).

Keywords: lysine acetylation; amyotrophic lateral sclerosis; superoxide dismutase; post-translational modification; electrophoretic mobility; surfactant; lipid; gel shifting; phosphorylation

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Introduction

The interaction between proteins and surfactants occur throughout biology and biotechnology,^{1,2} but remain poorly understood.^{3,4} One surfactant in particular, sodium dodecyl sulfate (SDS), is especially important because of its use as a model lipid to study membrane proteins, and the misfolding of cytosolic proteins,^{5–7} and because of its historic and

evolving^{8,9} use in protein electrophoresis. Nevertheless, many fundamental properties of protein-SDS complexes remain unestablished. For example, the morphologies of SDS-protein complexes that form above the critical micelle concentration (CMC) are unclear, although recent data suggests a mixture of “bead on a string” and “pearl necklace” configurations.¹⁰ Furthermore, there is no set of chemical principles available to explain how SDS binds to polypeptides with a stoichiometry that is largely irrespective of primary structure (i.e., ~1 SDS molecule per 2 amino acids, at [SDS] > CMC).

This weak understanding of protein-SDS interactions has riddled the research literature with examples of cytosolic proteins that migrate during SDS polyacrylamide electrophoresis (SDS-PAGE) at rates that are inconsistent with their molecular mass. For example, proteins that differ in sequence by a single amino acid are frequently observed to migrate during SDS-PAGE as if their molecular weights varied by thousands of Daltons.^{11–14} This anomaly is referred to as “gel shifting” (not to be confused with the gel shifting assay for protein-oligonucleotide binding). Gel shifting can also be observed among practically isobaric proteins from different organisms (e.g., Cu, Zn superoxide dismutase, from mouse and human; Δ mass = 0.009 kDa).¹⁵ The post-translational modification¹⁶ of identical residues in different domains can also result in dissimilar rates of migration (e.g., the phosphorylation of Ser63/73 in the oncoprotein c-jun will cause gel shifting; phosphorylation of Ser243/249 does not affect migration).¹⁷ We hypothesize that many of these electrophoretic anomalies describe—at least to some degree—the biochemical factors that drive a type of molecular recognition between surfactants and denatured cytosolic proteins.

The gel shifting of homologous proteins is routinely explained (but rarely investigated) by one or more of five hypotheses: (i) differences in the 2° structure or (ii) Stokes radius of the protein-surfactant complex, (iii) differences in the intrinsic net charge of the protein, or (iv) number of bound dodecyl sulfate, and (v) post-translational modification, including proteolysis.^{12,13,18–20} The only systematic investigation into the gel shifting of mutant proteins (that we can find in the literature) has recently shown that single amino acid substitutions in the α -helical transmembrane hairpin domain from CFTR (cystic fibrosis transmembrane conductance regulator) cause gel shifting by changing SDS stoichiometry and α -helical content.²¹ Unlike integral membrane proteins, cytosolic proteins do not typically regain native 2° or 3° structure after boiling in Laemmli’s buffer. The chemical mechanism(s) of gel shifting—that is, the relative contribution of electrostatic, hydrophobic, or conformational effects in surfactant binding—will likely be different for cytosolic proteins compared to integral membrane proteins.

In this article, we identify a chemical mechanism of gel shifting that is predominant among a large set of single amino acid variants of human Cu, Zn superoxide dismutase (hSOD1).²² Superoxide dismutase is a well-characterized cytosolic protein with 153 residues (15.8 kDa). The native SOD1 polypeptide consists of an 8 stranded β -barrel that can dimerize upon formation of an intramolecular disulfide bond in each subunit, and upon the binding of Cu and Zn to each subunit.²² The mutant hSOD1 proteins in this study cause familial amyotrophic lateral sclerosis (ALS) and many of these proteins—but not all—have been previously observed to migrate differently than wild-type (WT) hSOD1 during SDS-PAGE (e.g., the G85R substitution increases the migration of hSOD1, however, the G37R substitution does not alter migration).^{11,18,23–30}

We demonstrate that ALS-linked amino acid substitutions in hSOD1 increase migration during SDS-PAGE by altering the electrostatic properties of a specific, polyacidic domain (residues 80–101). Electrostatic perturbations to domain 80–101 alter the number of SDS molecules that bind to hSOD1, without significantly changing the gross 2° structure or Stokes radius of the hSOD1-SDS complex. The ability of domain 80–101 to cause gel shifting is dependent upon its high net negative charge. Lowering the negative charge of domain ~80–101 by 2–3 units (via chimeric human-mouse SOD1 proteins) inhibited ALS mutations from causing gel shifting.

Results

To assess the gel shifting of a diverse set of ALS mutant hSOD1 proteins, under identical electrophoretic conditions, we analyzed 27 different ALS-linked amino acid variants of hSOD1 with SDS-PAGE and anti-SOD1 Western blotting [Fig. 1(A)]. In addition, we analyzed the published SDS-PAGE results of 12 other ALS mutant hSOD1 proteins that are not included in our set [Fig. 1(B)]. Together, these 39 ALS-linked amino acid substitutions are scattered throughout the entire 153 amino acid sequence of hSOD1 and represent every ALS mutant hSOD1 that has been previously analyzed with SDS-PAGE.^{11,18,23–26,28–30}

Thirty of the 39 ALS-linked amino acid substitutions do not alter hSOD1 migration during SDS-PAGE [Fig. 1(B)]; eight substitutions increase migration by ~2–3 kDa: H80R, G85R, D90A, G93R, E100G, E100K, D101G, and D101N; only one substitution, G93D, decreases migration (by ~2–3 kDa). Remarkably, several ALS-linked amino acid substitutions occurring at one locus will cause gel shifting (e.g., G93D, G85R, and E100K); however, the identical substitution at a different location will not affect migration (e.g., G41D, G37R, and E21K).

The remarkable pattern of gel shifting in Figure 1(B) illustrates that ALS amino acid substitutions to

Figure 1. Gel shifting of ALS variant hSOD1 during SDS-PAGE. **A:** SDS-PAGE and anti-SOD1 Western blotting of ALS mutant hSOD1. Black = no change in migration towards positive electrode; red = increase; green: decrease. Untransformed (UT) cells and cells expressing WT hSOD1 were loaded as controls. Asterisk denotes smearing artifact of Western blotting. Image represents composite of three Western blots (borders indicated by vertical dashed lines). **B:** Summary of migration of 39 ALS variants of hSOD1 with SDS-PAGE from this study, and published reports. Substitutions that decrease the net negative charge of hSOD1 ($\Delta Z = +$) are highlighted in red; substitutions that increase the net negative charge ($\Delta Z = -$) are green; isoelectric substitutions ($\Delta Z = 0$) are black. **C:** Substitutions that cause gel shifting (indicated with red dashed lines) are clustered in a polyacidic domain (approximately residues 80–101) which has a high local net negative charge. **D:** Comparison of location of gel shifting domain with native 2° structure and number of known ALS amino acid substitutions at each residue in hSOD1.

hSOD1 will alter its migration during SDS-PAGE only if the substitution meets two criteria: (i) the substitution is not isoelectric, and (ii) the substitution occurs approximately within residues 80–101. There are no isoelectric ALS amino acid substitutions, within or outside domain 80–101, that significantly alter migration, and there are no nonisoelectric substitutions outside domain 80–101 that significantly alter migration. Furthermore, substitutions that increase the net negative charge of domain 80–101 uniformly decrease migration; substitutions that decrease the net negative charge of domain 80–101 uniformly increase migration. This simple electrostatic pattern suggests that ALS mutations cause gel shifting—that is, change the structure and/or stoichiometry of hSOD1-SDS complexes—by changing the local electrostatic environment of residues 80–101 in SDS-denatured hSOD1.

Because the biophysical and biochemical properties of ALS mutant hSOD1 proteins have been characterized in detail,^{31,32} several possible causes of gel shifting can be immediately excluded, including post-translational modification or incomplete unfolding in SDS. A detailed discussion of these points can

be found in Supporting Information. Briefly, it is unlikely that ALS-linked amino acid substitutions cause gel shifting by changing the degree of unfolding of the hSOD1 polypeptide in SDS. The disulfide reduced, apo-hSOD1 protein is marginally stable ($T_m = 42^\circ\text{C}^{31}$; $\Delta G_f = 2 \text{ kcal/mol}^{33}$) and will remain unfolded after boiling and reduction in Laemmli's buffer. Moreover, several of the substitutions that alter migration such as D101N, D90A, and E100K do not alter the native structure, thermostability, rate of hydrogen-deuterium exchange, or metalation of hSOD1 (and have thus been described as “cryptic”³⁴). Several mutations that do alter the structure and lower the conformational stability of native hSOD1 (e.g., A4V and G93A³¹), do not alter migration during SDS-PAGE [Fig. 1(A,B)].

We hypothesized that domain 80–101 might be partially resistant to associating with SDS because this domain is negatively charged, and lacks aromatic residues. For example, we hypothesize that substitutions that decrease the net negative charge (Z) of domain 80–101 will lower the electrostatic repulsion between SDS and hSOD1, and promote the binding of additional SDS (which would increase

migration towards the positive electrode). In contrast, the G93D substitution is, we presume, inhibiting the binding of SDS to hSOD1 by increasing the local net negative charge of domain 80–101.

In order to survey the local net charge of domains in the 1° structure of hSOD1 (i.e., “unfolded” SOD1), we used Eq. (1) to approximate the formal local net charge of each residue (Z_{local}^F) by averaging its formal charge at pH 8.0 (Z_X^F) with the formal charge of its three nearest neighbors on the C- and N-terminal flanks of the 1° structure ($Z_{X-3}^F \dots Z_{X+3}^F$).

$$Z_{\text{local}, X}^F = [Z_{X-3}^F + Z_{X-2}^F + Z_{X-1}^F + Z_X^F + Z_{X+1}^F \dots Z_{X+3}^F] / 7 \quad (1)$$

The number of flanking residues was limited to three in Eq. (1) because this distance is on the order of magnitude of the Debye radius (κ^{-1}) that we calculated in 25 mM Tris buffer, 3.5 mM SDS (κ^{-1} Å 1.8 nm). According to Eq. (1), most amino acids in “unfolded” hSOD1 have a local net positive or negative charge between $\sim +0.4$ and -0.4 formal units [Fig. 1(C)]; only $\sim 29\%$ of the sequence is neutral. Domain 80–101 contains the highest magnitude and longest sequence of net negative charge in the 1° structure of hSOD1, and is also low in Ile, Leu, and void of Phe, Trp, Tyr that bind the n-alkyl chain of SDS.⁴ The location of domain 80–101 in the 1° structure of hSOD1 was also compared with (i) a secondary structure map of native SOD1 [Fig. 1(D)] and (ii) a plot of the number of ALS-linked amino acid substitutions, deletions or insertions that occur at each residue (out of ~ 160 known mutations).

To determine whether ALS mutations cause gel shifting by (i) altering the number of SDS molecules that bind SOD1, and/or (ii) altering the structure or shape of SDS-hSOD1 complexes, we analyzed ALS mutant and WT hSOD1-SDS complexes with capillary electrophoresis (CE), circular dichroism (CD) spectroscopy, and size exclusion chromatography.

Capillary electrophoresis⁴ has been previously shown to be capable of detecting the binding of a single molecule of dodecyl sulfate to proteins.³⁵ This type of experiment is performed with a bare fused silica capillary (without any gel), and does not involve the same molecular sieving that occurs in polyacrylamide electrophoresis. The binding of anionic dodecyl sulfate to a negatively charged protein (such as hSOD1 at pH > 6.0) increases the CE mobility of the protein.³⁵ The electrophoretic mobility of a protein [Eq. (2)] can be expressed as a function of the net charge (Z) and molecular weight (M); C_p and α are constants.

$$\mu = C_p \cdot \frac{Z}{(M_{\text{SOD1}})^\alpha} \quad (2)$$

The capillary electrophoretic mobilities of WT, G85R, D90A, and G93R apo-hSOD1 were measured in 0.1%

SDS after boiling and disulfide reduction in Laemmli's buffer and compared to the mobility of the same native apo-hSOD1 in the absence of SDS [Fig. 2(A)]. The CE analysis was performed in triplicate, and an overlay of replicate electropherograms for each protein can be found in Supporting Information Figure S1.

The CE mobilities of G93R, G85R, and D90A apo-hSOD1 were less than WT apo-hSOD1 in the absence of SDS [as expected, because each protein has a lower net negative charge than WT apo-hSOD1; Fig. 2(A)]. After disulfide reduction and boiling in SDS, however, the electrophoretic mobility of all three SDS saturated ALS mutant SOD1 proteins is greater than SDS-saturated WT apo-hSOD1, which suggests that ALS mutant SOD1 binds more SDS than WT apo-hSOD1 (at $>$ CMC).

The difference in the number of SDS molecules bound to denatured WT and ALS variant apo-hSOD1 can be estimated with Eq. (3) [a modified form of Eq. (2)]. We cannot, however, use Eq. (3) to approximate the absolute number of SDS molecules bound to denatured hSOD1 because the shape and mass of the protein changes as the globular homodimer unfolds in SDS. In Eq. (3), M_{DS^-} = mass of dodecyl sulfate; M_{SOD1} = mass of apo-hSOD1 monomer; Z_0 = measured net charge of each apo-hSOD1 monomer ($Z = -7.5$ for WT; -6.0 for G85R; -6.0 for D90A; -5.6 for G93R); n = the number of SDS bound to hSOD1 (approximated to be 77), and ΔZ = change in Z from binding SDS (approximated as -0.9^{35}).

$$\mu = C_p \cdot \frac{Z_0 + (n) \cdot \Delta Z}{(M_{\text{SOD1}} + n \cdot M_{\text{DS}^-})^\alpha} \quad (3)$$

This calculation suggested that the G85R, D90A, and G93R substitutions promote the binding of 3.7 ± 0.4 , 3.4 ± 0.4 , and 3.2 ± 0.4 molecules of SDS (respectively) to apo-hSOD1 polypeptide at $[\text{SDS}] >$ CMC [Fig. 2(D)].

Circular dichroism (CD) was used to study the 2° structures of D90A, G85R, G93R, and WT apo-hSOD1 after thermal unfolding and disulfide reduction in SDS [Fig. 2(C)]. The binding of SDS to cytosolic proteins (at $>$ CMC) induces non-native α -helicity.^{3,4,35,36} CD spectroscopy is sensitive enough to detect these types of SDS-stabilized α -helices in cytosolic proteins.³⁵ The CD spectra of native apo-hSOD1 proteins [Fig. 2(B)] are characteristic of a globular β -barrel protein lacking α -helices; the boiling of WT and mutant apo-hSOD1 in SDS and TCEP, however, produced CD spectra with dual troughs at 208 and 222 nm [Fig. 2(C)] that are indicative of denatured proteins with SDS-stabilized α -helices.^{4,21,35} The similar CD spectra [Fig. 2(C)] of SDS denatured-D90A, G85R, G93R and WT apo-hSOD1 indicates that each protein has a similar overall 2° structure. The gel shifting of ALS mutant

Figure 2. ALS amino acid substitutions that increase migration during SDS-PAGE promote the binding of ~3–4 SDS molecules without altering the gross 2° structure of SDS-saturated hSOD1. **A:** Capillary electropherograms of G93R, G85R, D90A, and WT apo-hSOD1 under native (black trace) and SDS-denatured conditions (red trace). Peak at $\mu = 0$ in all electropherograms is neutral marker, dimethylformamide (DMF). The intense broad peak and shoulder at $\mu = 0$ –5 in red electropherograms is β -mercaptoethanol. The G85R, D90A, and G93R substitutions (which cause gel shifting) result in the binding of ~3–4 additional SDS molecules per hSOD1 polypeptide after boiling in SDS-PAGE buffer. Note: the native G93R apo-SOD1 protein migrated as a doublet during CE (the smaller peak with a lower mobility has not yet been identified but is not due to the binding of a metal ion). **B–C:** Circular dichroism spectroscopy of WT, G85R, D90A, and G93R apo-hSOD1 proteins under (B) native conditions and (C) after boiling and disulfide reduction in SDS/TCEP. **D:** Table showing: (i) summary of changes in SDS stoichiometry (“ Δ SDS”); standard deviation of three measurements listed in parentheses), and (ii) the abundance of secondary structure (% α -helix, β -sheet, random coil) in each SDS-denatured protein (standard deviation from at least 3 measurements listed in parentheses).

hSOD1 is therefore not caused by a change in the gross 2° structure of hSOD1-SDS. Analysis of CD spectra with a deconvolution program³⁷ suggested a minor increase in α -helicity (2–3% of residues) for each mutant protein, compared to WT hSOD1, however the increase in α -helicity is within the margin of experimental error [Fig. 2(D)].

The binding of additional SDS to ALS mutant hSOD1 will accelerate migration during SDS-PAGE, however, SDS stoichiometry might only partly contribute to gel shifting during SDS-PAGE. We hypothesized that ALS amino acid substitutions might also decrease the Stokes radius of hSOD1-SDS complexes (e.g., by promoting the burial of hSOD1 into the hydrophobic interior of an SDS micelle). The Stokes radii of WT and ALS mutant

hSOD1-micelle complexes were compared by analyzing WT and D90A apo-hSOD1-SDS complexes with two different types of size exclusion (SE) chromatography: (i) a G-100 Sephadex gravimetric column, and (ii) a high resolution GF-250 HPLC column. We were particularly interested in studying D90A apo-hSOD1 because its structure, activity, and free energy of folding are indistinguishable from WT hSOD1, and the two proteins appear to only differ (biophysically) by their surface charge.³¹ This similarity enables us to study the mechanism by which electrostatic perturbations in SOD1 affect its interaction with surfactants, and migration during SDS-PAGE.

Both WT and D90A proteins were boiled in Laemmli sample buffer, cooled, mixed together and

Figure 3. ALS-linked amino acid substitutions that cause gel shifting do not significantly alter Stokes radius of hSOD1-SDS complexes. A: SDS-PAGE of fractions of SDS-denatured D90A and WT apo-hSOD1 after boiling in Laemmli buffer and simultaneous analysis with G-100 size exclusion chromatography. B: Analysis of same SDS-denatured D90A and WT apo-hSOD1 with high resolution SE-HPLC. C: Plot of intensity of hSOD1 in fractions from G-100 SE column demonstrates that D90A and WT hSOD1-SDS complexes co-elute. D: Illustration of gel shifting of D90A and WT apo-hSOD1 during SDS-PAGE.

loaded (as a mixture) onto the same SE column that was equilibrated and operated with 0.1% SDS (pH 8.05). The elution of each protein was measured by analyzing each fraction with SDS-PAGE [Fig. 3(A,B)]. D90A and WT apo-hSOD1-SDS complexes have identical profiles of elution and thus appear to have similar Stokes radii [Fig. 3(C)].

To test the hypothesis that the high negative charge (acidity) of domain 80–101 is required for gel shifting, we sought to determine if lowering the net negative charge of domain 80–101 by several units would prevent ALS mutations from causing gel shifting.¹⁵ We expressed and analyzed different chimeric forms of mouse SOD1 (mSOD1) and hSOD1 with SDS-PAGE and anti-SOD1 Western blotting. Although the 153 amino acid sequences of mouse and human SOD1 share 86% homology (Fig. 4), there are large electrostatic differences at residues 80–102 in mSOD1 and hSOD1; domain 80–102 in mSOD1 is less negatively charged than domain 80–102 in hSOD1 by 3 formal units [Fig. 4(A)]. In contrast, residues 103–153 in mouse and human SOD1 are completely isoelectric [Fig. 4(A)]. Thus, the local net negative charge of domain 80–101 in human SOD1 can be reduced by 2 formal units (and domain 80–102 by 3 units) by generating a human-mouse chimeric protein that is singly fused at residue 80 [Fig. 4(B)].

The fusion of the N-terminal half of mSOD1 (residues 1–80) to the C-terminal half of hSOD1 (residues 81–153) resulted in a chimeric SOD1 polypeptide (denoted m^N/h^C WT) that migrated at a similar rate as hSOD1, but slower than mSOD1 [Fig. 4(C)]. In contrast, chimeric SOD1 with residues 1–80 from hSOD1 and residues 81–153 from mSOD1 (h^N/m^C WT) migrated faster than hSOD1 and at a rate that is similar to mSOD1 [Fig. 4(C)].

The incorporation of the G85R substitution into chimeric m^N/h^C SOD1 caused gel shifting (as it does in WT hSOD1, Fig. 1), however, the G85R substitution in h^N/m^C SOD1—whose domain 80–102 is less negatively charged than either m^N/h^C SOD1 or WT hSOD1 by 3 formal units—did not cause gel shifting. This result suggests that the high negative charge of domain ~80–101 in hSOD1 contributes to the ability of this domain to cause gel shifting. These results also show that electrostatic differences near domain 80–102 in mSOD1 and hSOD1 account for why mSOD1 is uniformly observed by researchers to migrate faster than hSOD1 during SDS-PAGE.¹⁵

Protein charge ladders³⁸ of WT holo-hSOD1 (i.e., Cu_2Zn_2 -SOD1) were generated to study how large systematic increases in the negative charge of multiple domains in hSOD1 affect its (i) migration during SDS-PAGE, (ii) the number of SDS that bind hSOD1, and (iii) its 2° structure in micellar SDS.

Figure 4. Decreasing the net negative charge of domain ~80–101 in SOD1 prevents ALS mutations from causing gel shifting. **A:** Sequence homology (% a.a.) and formal charge homology (%Z) of mouse and human SOD1; nonconserved charged residues are labeled +, 0, or −. **B:** Four chimeric forms of human and mouse hSOD1, two of which contain the ALS-linked G85R amino acid substitution that causes gel shifting in hSOD1. The h^N/m^C chimeric WT SOD1 contains N-terminal half (residues 1–80) from hSOD1 and C-terminal half (residues 81–153) from mSOD1 (and vice versa for m^N/h^C chimeric WT SOD1). The ALS-linked G85R substitution caused gel shifting with m^N/h^C but not with h^N/m^C. **C:** SDS-PAGE Western blot of mWT, hWT, and chimeric m/hSOD1 proteins expressed in cultured HEK293-FT cells. Faint bands in C (denoted **) represent endogenous hWT SOD1 expressed by HEK cells; faint bands at high molecular weight (*) represent nonspecific binding of immunoglobulin or oligomeric SOD1.

Charge ladders were generated by successively acetylating (neutralizing) each of the 22 Lys-ε-NH₃⁺ in homodimeric hSOD1 (e.g., Lys-ε-NH₃⁺ → Lys-ε-NHCOCH₃). We are uncertain about the biological relevance of lysine acetylation as a post-translational modification to hSOD1. A proteomic study reported that Lys70 in hSOD1 does undergo acetylation in HeLa S3 cells, however, this report did not present MS/MS data or Mascot scores for the acetylated hSOD1 peptide,³⁹ and we do not know of any other reports of lysine acetylation in SOD1.^{40,41} Nevertheless, lysine-acetyl hSOD1 charge ladders are ideal tools for further testing the local electrostatic hypothesis because: (i) only a single lysine exists in the gel shifting domain (Lys91), i.e., the acetylation of most lysine in hSOD1 should therefore not contribute as greatly to gel shifting, (ii) multiple charge isomers can be generated in minutes (i.e., 22 charge isomers of dimeric hSOD1),³⁸ and (iii) lysine

acetylation represents an ideal control, because it increases the net negative charge of hSOD1 (opposite of most ALS mutations that cause gel shifting).

Different stoichiometric equivalents of acetic anhydride were added to aliquots of hSOD1, resulting in a mixture (a charge ladder) of variably acetylated hSOD1 (Figs. 5, 6, and Fig. S2, Supporting Information). The charge isomers or “rungs” of each protein charge ladder (denoted Ac(N)) are resolved by Native

Figure 5. Acetylation of multiple Lys-ε-NH₃⁺ in human SOD1 induces gel shifting during SDS-PAGE. **A:** Acetylation of WT hSOD1 (denoted Ac(~N)) decreases migration during SDS-PAGE (N refers to the number of acetylated lysine in dimeric hSOD1). Inset shows side by side analysis of unmodified hSOD1-Ac(0) and peracetylated hSOD1-Ac(22) with SDS-PAGE. **B:** Increased migration of charge ladders during Native-PAGE (0 mM SDS). **C,D:** Plot of the magnitude of gel shifting of hSOD1 (in terms of molecular weight or migration distance) as a function of acetylated lysine residues (Lys-NHAc). Error bars represent the standard deviation from seven separate experiments.

Figure 6. Systematic neutralization of multiple Lys- ϵ -NH $_3^+$ in human SOD1 decreases the number of bound SDS. A: Acetylation of native WT holo-hSOD1 increases capillary electrophoretic mobility in the absence of SDS (black), but reduces mobility after boiling and reduction in SDS (red). Peak at $\mu = 0$ is neutral marker, DMF). The intense broad peak and shoulder present at $\mu = 0$ –5 in red electropherograms is β -mercaptoethanol. B: Decrease in number of SDS molecules bound to hSOD1 (Δ SDS per monomer) plotted as a function of number of acetylated lysine per hSOD1 monomer. C: Plot of number of SDS molecules dissociated from acetylated hSOD1 (Δ SDS) as a function of the magnitude of gel shifting. Error bars represent standard deviation from three separate experiments.

PAGE [Fig. 5(B)] or CE [Fig. 6(A)], according to net charge, and also by mass spectrometry (Fig. S2, and Table S1, Supporting Information).

The migration of WT hSOD1 during SDS-PAGE could be slowed by only ~ 2 kDa, even after successively acetylating all 22 Lys- ϵ -NH $_3^+$ in each hSOD1 dimer [Fig. 5(A)]. The magnitude and direction of gel shifting of peracetylated hSOD1 (Ac(~ 22)) is similar to that of the G93D substitution, which is the only ALS mutation in our data set that increases the net negative charge of hSOD1 (and decreased migration during SDS-PAGE). The increased migration of acetylated hSOD1 during Native-PAGE demonstrated that acetylation does not slow migration during SDS-PAGE by increasing molecular mass [Fig. 5(B)].

The number of acetylated lysine in each charge ladder (Ac($\sim N$)) is plotted in Figure 5 against the magnitude of gel shifting. Gel shifting was expressed in terms of molecular weight [Fig. 5(C), $\Delta MW_{app}/MW_{Ac(0)}$, %], and in terms of migration distance from the stacking gel [Fig. 5(D), $D_{Ac(\sim N)} - D_{Ac(0)}/D_{Ac(0)}$, %]. The modest decrease in migration, even after increasing the formal net negative charge of monomeric hSOD1 by 11 units, suggests that the acetylation of most lysine residues in hSOD1 does alter

migration during SDS-PAGE to the degree that electrostatic perturbations to domain 80–101 will alter migration.

Equation (4) [a modified form of Eq. (3)] was used to determine if acetylation decreased the number of SDS that bind to hSOD1. In Eq. (4), M_{Ac} equals the change in mass associated with acetylation (p); $M_{Ac} = 42$ Da, $Z_0 = -4.0$; $\Delta Z = -0.9$ for each bound SDS (n) and acetylated lysine.

$$\mu = C_p \cdot \frac{Z_0 + (p + n) \cdot \Delta Z}{(M_{SOD1} + p \cdot M_{Ac} + n \cdot M_{DS^-})^2} \quad (4)$$

In the absence of SDS, the higher rungs of the charge ladder had greater CE mobilities than lower rungs. After boiling in SDS and BME, however, the higher rungs of the charge ladder had smaller values of CE mobility than lower rungs [Fig. 6(A)]. The decrease in CE mobility of SDS-denatured hSOD1-Ac(N) suggests that lysine acetylation diminishes the number of SDS that bind hSOD1. As previously reported for other proteins, the rungs of a charge ladder cannot be resolved after denaturation and saturation with myriad SDS.^{42,43} CE analysis was performed in triplicate for each charge ladder and an overlay of replicate electropherograms can be

Figure 7. The acetylation of the single lysine in domain 80–101 of WT hSOD1 (Lys91) correlates with greatest magnitude of gel shifting. A–C: Nonrandom acetylation of Lys91 in hSOD1. A: Triply charged ion of peptide 87-Lys91(Ac)-115 detected in variably acetylated hSOD1. B: Doubly charged ion of peptide 80-Lys91(Ac)-115. C: Singly charged ion of peptide 7-Lys9-16; increase in signal intensity for acetylated Lys9 is greater than acetylated Lys91 at equal concentrations of acetic anhydride. D: Doubly charged ions of C-terminal peptide standard (residues 144–153) that does not contain lysine, and is cleaved at Arg143. E: Plot of the percent of acetylation for four different lysine in SOD1 as a function of the average degree of acetylation of all lysine residues (Ac(~N)) in SOD1. F: Magnitude of gel shifting of hSOD1 plotted as a function of percent acetylation of four lysine residues from multiple hSOD1 domains; error bars represent standard deviation from five different enzymatic digests.

found for each charge ladder in Supporting Information Figure S3.

We calculated [with Eq. (4)] that each acetylation inhibited the binding of ~3 SDS [see slope of plot in Fig. 6(B)]. This stoichiometry is substantial considering that 77 molecules of SDS are predicted to bind hSOD1 during SDS-PAGE. Previous studies report that acetylation of lysine can inhibit the binding of SDS molecules by a similar order of magnitude at $[SDS] > CMC$.⁴

The number of SDS bound to SOD1 decreases linearly with lysine acetylation (i.e., net negative charge) [Fig. 6(B)]. The magnitude of gel shifting does not, however, correlate linearly with either (i) acetylation [Fig. 5(B,C)] or (ii) SDS binding [Fig. 6(C)]. These nonlinear correlations demonstrate that changes in SDS stoichiometry are insufficient to entirely explain the magnitude of gel shifting of acetylated hSOD1 [Fig. 6(C)].

It is reasonable to hypothesize that the polynomial relationship between acetylation (%), and the magnitude of gel shifting [Fig. 5(C,D)] is caused by nonrandom acetylation of specific lysine residues—each of which slow migration by a different magnitude, or not at all. We determined the susceptibility of lysine residues to acetylation by proteolyzing different hSOD1 charge ladders with pepsin and trypsin, followed by sequencing with tandem mass spectrometry (MS/MS). With the charge ladders that

were studied (e.g., Ac(0), ~0.5 ~2, ~10, ~19, ~22), only four lysine residues could be reproducibly sequenced in both acetylated and nonacetylated states: K9, K36, K91, and K136. From monitoring the intensity of MS ions for acetylated and unacetylated peptides, we determined that acetic anhydride acetylates K9, K36, and K136 more preferentially than K91 [Fig. 7(A–D)]. In general, the acetylation of K91 correlated with greater gel shifting than the acetylation of K9, K36, and K136 [Fig. 7(F)]. This correlation provides additional support for the hypothesis that domain 80–101 electrostatically governs the interaction of hSOD1 with SDS micelles.

The 2° structures of acetylated and unacetylated WT hSOD1 were also examined with CD spectroscopy after boiling in SDS/TCEP. The CD spectra of the variably acetylated hSOD1-SDS complexes indicate the presence of SDS-stabilized α -helices. Peracetylation did cause a minor decrease in absorbance between 200 and 240 nm ($\sim 1000 \text{ deg}\cdot\text{cm}^2 \text{ dmol}^{-1}$; Fig. S4, see Supporting Information). The deconvolution of CD spectra for each charge ladder yielded insignificant differences in secondary structure of each SDS-saturated charge ladder (Supporting Information Fig. S4). For example, the differences in the α -helicity of unacetylated and peracetylated proteins were only ~2% greater than the margin of experimental error. We conclude that the differences in the secondary structure of unacetylated and

peracetylated SOD1 are negligible after denaturation in SDS.

Discussion

ALS-linked amino acid substitutions to hSOD1 cause gel shifting by altering the electrostatic properties of a single polyacidic domain (residues ~80–101), which changes the number of SDS that bind to hSOD1, without changing the gross 2° structure or Stokes radius of the hSOD1-SDS complex. Thus, the polyacidic “gel shifting” domain of denatured hSOD1 can singularly mediate hSOD1-SDS interactions by a predominantly electrostatic mechanism.

The five amino acid substitutions at Gly93 (i.e., G93A, D, R, S, V) are notable because they demonstrate, at a single locus, that electrostatic perturbations to residues 80–101 are causing gel shifting—not small changes in hydrophobicity or polarity [Fig. 1(B)]. We do expect, however, that hydrophobic substitutions could cause gel shifting, if the changes in hydrophobicity are large enough to facilitate hydrophobic interactions with the alkyl chain of SDS (e.g., G,D→W,Y,F,L).

The pattern of gel shifting that we observe for mutant hSOD1 is different than the pattern observed by Rath *et al.* for mutant transmembrane hairpins from CFTR.²¹ For example, amino acid substitutions that either increase or decrease the net charge of the CFTR hairpin can have the same effect on migration (e.g., both V232K and V232D increase migration), which is opposite of the uniform electrostatic pattern observed with mutant hSOD1. Isoelectric amino acid substitutions to CFTR hairpins can also cause gel shifting (e.g., G228L and Q220W), however, nonisoelectric substitutions do not cause gel shifting in hSOD1. Although the gel shifting of mutant hSOD1 and CFTR are both caused by changes in surfactant binding, the chemical mechanism of altered surfactant binding is primarily electrostatic in cytosolic hSOD1 and is driven, presumably, by hydrophobic and/or conformational effects in the membrane protein CFTR.²¹

Our finding that the neutralization of 11 Lys- ϵ -NH₃⁺ throughout multiple domains in hSOD1 slowed SDS-PAGE migration by the same magnitude as the introduction of a single negative charge in domain 80–101 (i.e., G93D), suggests that the acetylation of lysine outside of domain 80–101 did not contribute as significantly to gel shifting as acetylation within domain 80–101. Likewise, the polynomial relationship between lysine acetylation and gel shifting demonstrated that changes in the net charge of the entire hSOD1 peptide do not cause gel shifting *per se*. For example, the acetylation of only ~2 lysine per dimer did not induce significant gel shifting [Fig. 5(A,C,D)], presumably because this ladder did not contain sufficient concentrations of acetylated Lys91. Rather, gel shifting only began to occur at

higher concentrations of acetic anhydride, coincidentally with the acetylation of Lys91 [Fig. 7(F)]. The slower reactivity of Lys91 with acetic anhydride compared with other lysine is surprising—considering its high solvent accessibility—and is worth briefly discussing from the standpoint of chemical biology.^{44,45} The carboxylic acids of Asp90 and Asp92 that flank Lys91 will likely raise the pK_a of Lys- ϵ -NH₃⁺, thus lowering its reactivity (i.e., ϵ -NH₃⁺ is less reactive with acetic anhydride than ϵ -NH₂⁴⁶). An approximation of the electrostatic surface potentials of holo-SOD1 (with solutions to the nonlinear Poisson Boltzmann equation, Supporting Information Fig. S5) suggested that Lys91 is indeed located in the most anionic region of native holo-hSOD1, and that Lys9, 36, and 139 reside in cationic regions (Supporting Information Fig. S5).

The electrostatic interactions that we observed between apo-hSOD1 and SDS micelles are not entirely orthogonal to the type of interactions that might occur *in vivo* between hSOD1 and anionic lipids (or possibly fatty acids).^{47–49} ALS amino acid substitutions have been shown to promote interactions between hSOD1 and anionic lipid membranes and fatty acids, and these interactions are hypothesized to induce misfolding and neurotoxicity.^{47–49} Our results suggest that ALS amino acid substitutions, such as D90A, can promote the interaction between disordered hSOD1 and anionic amphiphiles by a purely electrostatic mechanism.

Conclusions

This study has only begun to fully explain the gel shifting of ALS mutant hSOD1. For example, this study did not identify the location where additional SDS bind to ALS mutant hSOD1. We presume that binding occurs in domain 80–101. More than beginning to explain a routinely disregarded anomaly, this study has shown that decrypting the patterns of gel shifting for cytosolic proteins can be used to identify: (i) mutations or post-translational modifications that alter the molecular recognition of lipid-like molecules, (ii) the predominant chemical mechanism of the interaction in question (e.g., hydrophobic or electrostatic), and (iii) the domains that somehow control interactions between denatured proteins and surfactants.

Experimental

Expression and/or purification of SOD1

Human WT, ALS mutant, and chimeric SOD1 were prepared and expressed in HEK293FT cells as previously described.¹¹ The migration of all 27 hSOD1 proteins, and chimeric mouse-human proteins during SDS-PAGE was visualized with anti-SOD1 Western blotting. For analysis with either CE, size exclusion chromatography, or CD spectroscopy, a

select group of ALS mutant hSOD1 (D90A, G93R, and G85R) and WT hSOD1 were expressed in yeast and purified as previously described.^{50,51}

Capillary electrophoresis

Capillary electrophoresis was performed using a Beckman Coulter P/ACE MDQ instrument. A bare fused silica capillary (50 cm) was used in all experiments. Solutions of hSOD1 (50 µg) were transferred from 10 mM phosphate buffer into either: (i) a buffer that is similar to SDS-PAGE running buffer (3.5 mM SDS, 25 mM Tris-base, 192 mM glycine, pH 8.05) or (ii) the same buffer lacking SDS. β-mercaptoethanol was added to samples containing SDS (the final concentration of β-mercaptoethanol was 0.7 M). Solutions were then boiled at 95°C for 5 min prior to analysis CE (samples that did not contain SDS were not boiled or reduced). Boiled samples were cooled on ice and re-equilibrated to room temperature prior to analysis. A neutral marker of electro-osmotic flow (dimethylformamide) was added to each sample. The running buffer for capillary electrophoresis was 25 mM Tris-base, 192 mM Glycine, pH 8.05 (with or without 3.5 mM SDS). We used the metal-free (apo) form of the WT and ALS mutant protein for comparing the number of bound SDS. Use of the apoprotein ensured that all SOD1 proteins had identical metalation (i.e., Cu₀Zn₀). The net charge of apo-hSOD1 proteins was determined using protein charge ladders as previously described.² All samples were injected at 0.5 psi for 5 s; electrophoresis was performed at 30 kV for 30 min at 20°C. CE analysis was repeated in triplicate on each sample.

Circular dichroism spectroscopy

The 2° structure of SDS-denatured hSOD1 was studied with CD spectroscopy under solvent conditions that mimic SDS-PAGE. Protein-SDS solutions were boiled (95°C for 5 min) in a reducing agent (TCEP) that does not interfere with CD spectroscopy. Solutions were cooled and briefly centrifuged prior to analysis. Spectra were collected on a JASCO J-815 spectrometer at 20°C at 15 µM hSOD1, 3.5 mM SDS, 25 mM Tris, 192 mM Glycine, 10 mM TCEP, pH 8.05. Solutions of native hSOD1 (without SDS or TCEP) were analyzed in 25 mM Tris, 192 mM glycine, pH 8.05 (without boiling). The spectrum generated for each sample was an average of 5 scans collected (0.5 nm intervals) on three replicate samples (i.e., an average of 15 scans).

Size-exclusion chromatography

Sephadex media (G-100, Sigma-Aldrich) was swelled in Tris-Glycine buffer (25 mM Tris-HCl, 192 mM Glycine, pH 8.05) for 24 h, packed into a 1 × 100 cm Econo-column (Bio-Rad) and equilibrated with two bed volumes of SDS-running buffer (25 mM Tris-HCl, 192 mM Glycine, 0.1% SDS). D90A and WT

apo-hSOD1 proteins were transferred into SDS-running buffer (25 mM Tris-HCl, 192 mM Glycine, 0.1% SDS) using a centrifugal filtration device, and the two proteins were then mixed together. Freshly dissolved dithiothreitol (1 M) was added to yield a final concentration of 100 mM DTT, 0.1 mg/mL hSOD1. The mixture was heated at 95°C for 5 min, cooled, and loaded onto the column; flow rate = ~200 µL/min. Fractions were analyzed with SDS-PAGE, stained with Coomassie blue; band intensity was quantified with ImageJ. Identical procedures were used for high resolution SE-HPLC experiments, except that a SE-HPLC column was used (Agilent Zorbax, Bio Series GF-250, 4.6 mm × 250 mm; flow rate = 250 µL/min).

Lysine-acetyl protein charge ladders

Systematic increases in the net negative charge of domains throughout the hSOD1 sequence were accomplished with lysine acetyl protein charge ladders.³⁸ Protein charge ladders of wild-type hSOD1 were prepared from the fully metalated holoenzyme (Cu₂Zn₂ hSOD1; purchased from Sigma) using acetic anhydride, as previously described.³⁸ Holo-SOD1 was used in the preparation of charge ladders because of its high conformational stability, and ability to remain folded after exhaustive modification of lysine residues. Detailed experimental procedures for mass spectrometric analysis of charge ladders are included in Supporting Information.

SDS-PAGE and native-PAGE

For SDS-PAGE, each set of acetylated hSOD1 proteins (10 µg of SOD1 in 10 mM potassium phosphate buffer, pH 7.4) was mixed with 8 µL of concentrated ("2X") Laemmli sample buffer (Bio-Rad) that contained β-mercaptoethanol. The samples were heated at 95°C for 5 min and then run in 15% polyacrylamide gel containing 3.5 mM SDS at 120 V for 150 min in SDS-Tris-Glycine buffer, pH 8.05, and stained with Coomassie blue. Solutions of hSOD1 were analyzed with Native-PAGE (10% polyacrylamide) without boiling or reduction; the running buffer consisted of 25 mM Tris, 192 mM Glycine, pH 8.05, and did not contain SDS or β-mercaptoethanol. 10 µg of hSOD1 in 10 mM potassium phosphate buffer, pH 7.4 was loaded into each lane.

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