NMR determination of pK_a values in α -synuclein

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Abstract: The intrinsically unfolded protein α -synuclein has an N-terminal domain with seven imperfect KTKEGV sequence repeats and a C-terminal domain with a large proportion of acidic residues. We characterized pK_a values for all 26 sites in the protein that ionize below pH 7 using 2D ^1H - ^{15}N HSQC and 3D C(CO)NH NMR experiments. The N-terminal domain shows systematically lowered pK_a values, suggesting weak electrostatic interactions between acidic and basic residues in the KTKEGV repeats. By contrast, the C-terminal domain shows elevated pK_a values due to electrostatic repulsion between like charges. The effects are smaller but persist at physiological salt concentrations. For α -synuclein in the membrane-like environment of sodium dodecylsulfate (SDS) micelles, we characterized the pK_a of His50, a residue of particular interest since it is flanked within one turn of the α -helix structure by the Parkinson's disease-linked mutants E46K and A53T. The pK_a of His50 is raised by 1.4 pH units in the micelle-bound state. Titrations of His50 in the micelle-bound states of the E46K and A53T mutants show that the pK_a shift is primarily due to interactions between the histidine and the sulfate groups of SDS, with electrostatic interactions between His50 and Glu46 playing a much smaller role. Our results indicate that the pK_a values of uncomplexed α -synuclein differ significantly from random coil model peptides even though the protein is intrinsically unfolded. Due to the long-range nature of electrostatic interactions, charged residues in the α -synuclein sequence may help nucleate the folding of the protein into an α -helical structure and confer protection from misfolding.

Keywords: ionization constant; fibrillogenesis; denatured state; membrane proteins; amyloid; protein folding; synucleinopathies

Introduction

Alpha-synuclein (αS) is a 14.5 kDa protein expressed predominantly at the presynaptic terminals of brain neurons.^{1,2} The physiological function of the protein remains unknown¹ although a role in synaptic vesicle recycling has been suggested.^{3,4} Misfolding of αS leads to the formation of fibrillar cytoplasmic aggregates^{5,6} called Lewy bodies, which are a defining characteristic of Parkinson's disease.^{7–10} Because the

number of Lewy bodies is often poorly correlated with the severity of symptoms, controversy surrounds the issue of whether fibrils or smaller soluble oligomers are responsible for the neurotoxicity of misfolded αS .¹¹ Regardless of the mechanism of neurotoxicity, genetic evidence establishes a link between the αS gene and Parkinson's disease. Although 90–95% cases of Parkinson's disease cases are sporadic,¹² the autosomal-dominant familial mutations A30P, E46K, A53T, as well as the triplication of the wild-type αS gene lead to early onset of the disease.^{1,7,13,14}

The amino acid sequence of αS can be subdivided into three domains with unusual distributions of charged residues [Fig. 1(A)]. The first 90 residues of αS contain seven imperfect repeats of the amino acid sequence xKTKEGVxxxx,¹⁵ which are important for

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Figure 1. Sequence of α S and structure of the protein bound to SDS micelles. (A) The three domains of the α S amino acid sequence and the locations of the seven xKTKEGVxxx repeats.¹⁵ (B) Structure of α S bound to SDS micelles (PDB code: 1XQ8),¹⁶ showing the side-chains of residues that are positively (blue) or negatively charged (red) at neutral pH. (C) Expanded view of the micelle-bound α S structure showing the location of His50 in relation to Glu46 and Ala53 (the sites of the familial Parkinson's mutations E46K and A53T). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the induction of α -helical structure in α S¹⁷ and for binding to membranes containing negatively charged lipids that the protein prefers.^{16–18} Residues 61–95 of α S correspond to the hydrophobic “non-amyloid- β component” (NAC), the most aggregation-prone part of the protein.^{19–24} The name NAC, derives from the occurrence of this segment as a second protein component of the extracellular amyloid- β plaques found in patients with Alzheimer's disease.²⁵ The mechanism by which the NAC fragment of the intracellular α S is cleaved and comes to be associated with extracellular amyloid- β plaques is unknown. The last two KTKEGV repeats are in the NAC segment,¹⁵ however, due to their imperfect nature only two charged residues Lys80 and Glu83 occur in the hydrophobic region between residues 62 and 95. The last 40 amino acids of α S contain 15 acidic residues, giving the C-terminal tail of the protein a negatively charged character at physiological pH.

Monomeric α S is intrinsically unfolded with a fractional population of at most 10–20% α -helix structure.^{22,26} The first $\sim$ 90 residues of the protein fold into an α -helical structure in the membrane-like environments provided by micelles, vesicles, or bilayers that contain a large fraction of negatively charged lipids.^{21,27–30} By contrast, the negatively charged C-terminal tail remains disordered when α S binds to lipid assemblies.^{16,27,28,31} The highest-resolution structure available for α S in a membrane environment is for the protein bound to sodium dodecyl-sulfate (SDS) micelles.¹⁶ SDS forms relatively small micelles that are amenable to direct high-resolution NMR studies.^{16,31} When bound to SDS micelles, the

amphipathic N-terminal domain of α S forms two long α -helices that lie on the surface of the micelle. Under other conditions the protein folds into an uninterrupted α -helix.^{29,32,33} The interconversion between a broken and uninterrupted α -helix has been shown to have a subtle dependence on micelle or vesicle size,^{29,32} the ratio of protein to micelles,³³ as well as the aggregation state of the protein.³⁴ The structure of α S bound to SDS micelles¹⁶ is shown in Figure 1(B). The view of the structure is opposite to the face of the protein that interacts with the micelles. Charged residues are shown in blue (positive) and red (negative). It is important to note that the structure of α S bound to SDS micelles was determined with perdeuterated protein samples using NMR experiments that look at the protein backbone.¹⁶ As such, there is no information on the conformations of side-chains in the α S structure; these are shown in random orientations in Figure 1(B,C). In an α -helix, residues separated by three or four positions in the sequence have the potential to form ion-pairs.³⁵ Consideration of the α S sequence and α -helix structure suggests that residues in the amphipathic domain of the protein are poised to form electrostatic interactions between the acidic and basic amino acids pairs Asp2-Lys6, Lys10-Glu13, Glu20-Lys23, Glu28-Lys32, Lys32-Glu35, Lys42-Glu46, Glu46-His50, Glu57-Lys60, Lys58-Glu61, and Lys80-Glu83. By contrast, the negatively charged residues in the C-terminal tail should be subject to electrostatic repulsion due to a high density of like charges.

Because charges could be important in the folding and misfolding of α -synuclein, we determined

pK_a values for all sites in the protein that ionize below pH 7. A number of studies have shown that pK_a values of unfolded proteins deviate from random coil model compounds.^{36–40} The unusual distribution of charged residues in α S provides a stringent test of the assumption that pK_a values of residues in unfolded proteins approximate those of random coils. The unfolded state is an important reference state for the theoretical analysis of the roles of charges in protein structure stabilization.^{37,40,41} Accurate pK_a values for unfolded α S will help model the effects of charges on the structures of membrane-bound and fibrillar α S. In addition to extensive studies on the free protein, we obtained pH titration data for His50 in α S bound to SDS micelles. His50 is a residue of particular interest because it is part of a high-affinity binding-site for Cu^{2+} , a metal which has been implicated in Parkinson's disease and that markedly increases the fibrillization rates of α S.⁴² Moreover, in the α S structure, His50 is within one turn of α -helix of the sites of the two familial Parkinson's disease mutations E46K and A53T [Fig. 1(C)] that could perturb the electrostatic environment of His50.

Results

pK_a values for α S in the absence of salt

Ionization constants for aspartate and glutamate residues in α S were obtained from 3D C(CO)NH experiments recorded at different pH values. The links the amide of a given residue with all the aliphatic ^{13}C resonances of the preceding residue. By correlating spin systems with the well dispersed nitrogen of the following amino acid in the sequence, the C(CO)NH experiment⁴³ circumvents the spectral overlap typically encountered for Asp and Glu side-chains, which is particularly severe for unfolded proteins like α S. For α S we found that the intensity of Asp C β correlations were stronger than those of Asp C α , the Glu C γ correlations were stronger than Glu C α , while Glu C β crosspeaks were usually not observed.⁴⁴ Fortunately, the strongest crosspeaks were from the aliphatic carbons closest to the titrating carboxyl groups of the acidic residues. Representative data for Glu28 and Glu114 showing superimpositions of 2D slices from the 3D C(CO)NH experiments obtained at pH values between 7.4 and 1.5 are shown in Figure 2(A). The pH dependence of the C γ chemical shifts of Glu28 and Glu114 in the absence of salt is shown in Figure 2(B). The glutamate C γ carbons shift from ~ 37 ppm at neutral pH, to ~ 33 ppm at acidic pH. For the aspartate C β carbons the corresponding shift is between ~ 42 and ~ 38 ppm. The C α resonances are also sensitive to the titration of the carboxyl groups but the pH dependence of these shifts is much smaller, typically from ~ 57.0 to ~ 56.0 ppm for glutamate and ~ 54.5 to ~ 53.0 ppm for aspartate between neutral and acidic pH.

The 3D C(CO)NH experiment enabled us to obtain data for 21 of the 26 sites that ionize between neutral and acidic pH in α S. The sites that could not be characterized were His50, Glu61, Asp119, Glu137, and the α -carboxyl group at the C-terminus. The 3D C(CO)NH experiment was not applicable for the residue pairs D119-P120 and E137-P138 because proline lacks an amide proton with which to correlate the preceding spin system. Similarly, the α -carboxyl group is at the end of the sequence (Ala140), and the titration of the His50 cannot be detected in the C(CO)NH experiment due to its aromatic side-chain. For these sites pK_a values were obtained by monitoring the pH dependence of ^1H - ^{15}N correlations in 2D ^1H - ^{15}N HSQC spectra. In the case of Glu61, the E61(C γ)-Q62(HN) spin system occurs as a partially overlapped shoulder on the E114(C γ)-D115(HN) spin system throughout most of the pH transition. While we could obtain accurate data for the C γ resonance of Glu114 from the C(CO)NH experiments, the titration of Glu61 was more accurately determined from the ^{15}N resonance in 2D ^1H - ^{15}N HSQC experiments. Consistent with previous results on unfolded proteins,⁴⁵ we found that the ^{15}N shifts of ionizable residues in α S are dominated by the protonation of the residue's own side-chain.

The sequence of α S has seven instances of Glu-Gly pairs [Fig. 1(A)]. The E83(C γ)-G84(HN) and E131(C γ)-G132(HN) spin systems were well resolved so that unambiguous pK_a values were obtained for Glu83 and Glu131 from the 3D C(CO)NH experiments. The two correlations E35(C γ)-G36(HN) and E110(C γ)-G111(HN) overlap between pH 7.4 and 4.0 but separate at lower pH. We based our assignments on a slightly more downfield ^{15}N chemical shift of 110.9 for Gly111 compared to 110.5 for Gly36.²² The assignments are tentative, however, as indicated in Table I. The three correlations E13(C γ)-G14(HN), E46(C γ)-G47(HN), and E105(C γ)-G106(HN) were unresolved throughout the entire pH range in the absence of salt. We assigned a single pK_a value obtained from the pH titration of the unresolved correlation to all three residues Glu13, Glu46, and Glu105 but estimate that the individual values differ by at most 0.1 pH unit from the unresolved pK_a of 4.21.

pH titration parameters for α S are summarized in Table I. In the absence of salt, the pK_a values of the 18 Glu residues in α S are spread over 0.8 pH units while those of the 6 Asp residues are spread over 1.0 pH units. The ranges are large for an unfolded protein and comparable to those of ionizable residues on the surfaces of folded proteins.^{37,39} The pK_a values show a pronounced dependence on residue position in the amino acid sequence (Table I). Acidic residues within the first 100 amino acids of α S have lowered pK_a values indicating a lower affinity for protons (hydronium ions) such that they resist losing their negative charge. Sites within the last 40

residues have raised pK_a values indicating a higher affinity for protons, which would facilitate the neutralization of acidic residues in the C-terminal tail.

pK_a values for α S in the presence of salt

Deviations of pK_a values from random coil model compounds have been reported for other unfolded proteins^{36,37,39,40} and the differences were found to decrease with increasing salt concentration.³⁸ To obtain

data at a physiological salt concentration we characterized the pH titration of α S in the presence of 150 mM NaCl [Fig. 2(A,C)]. The analysis of the data were similar to those for α S in the absence of salt. We determined pK_a values for 20 of 26 sites from 3D C(CO)NH experiments. The pH titrations of His50, Asp119, Glu137 and the C-terminal α -carboxyl group are not amenable to C(CO)NH spectroscopy and were determined using 2D ^1H - ^{15}N HSQC spectra (Table I). The pK_a values for two additional residues (Asp2, Asp121) were determined from ^{15}N shifts in ^1H - ^{15}N HSQC spectra because of overlap in the C(CO)NH experiments. For α S in the presence of 150 mM NaCl we were able to resolve the pH titrations of all seven Glu(C γ)-Gly(HN) spin systems, although the assignments of E35 and E110 remain tentative and could be reversed (Table I).

The spread of pK_a values in the presence of 150 mM NaCl is smaller as exemplified by the representative titrations of residues E28 and E114 [cf. Fig. 2(B,C)]. The distribution of lowered pK_a values in the N-terminal domain and raised pK_a values in the C-terminal domain is less pronounced but persists when α S is studied in the presence of 150 mM NaCl (Table I).

Differences from random coil pK_a values

To analyze the pK_a data for α S we needed to obtain reference values for residues in random coil conformations. Random coil pK_a values are available in the literature^{46–48} but to obtain accurate values for Glu and Asp residues, which account for 24 of the 26 ionizable sites in this study, we carried out ^1H NMR pH titrations on the model peptides Ac-AADAA-NH₂ and Ac-AAEAA-NH₂ under the exact same conditions used for the pH titrations for α S. The two H β protons of the aspartate in the Ac-AADAA-NH₂ peptide, which gave the same pK_a within experimental uncertainty, were used to obtain an ionization constant for aspartate. The H γ protons of the glutamate in the Ac-AAEAA-NH₂, which have degenerate chemical shifts throughout the entire pH range studied, were used to obtain an ionization constant for glutamate. The

Figure 2. Representative pH titration data. (A) Superposed 2D strips from 3D C(CO)NH experiments used to determine pK_a values for Glu and Asp residues in the absence and presence of 150 mM NaCl. Contours are shown for residues Glu28 and Glu114 on a red (neutral) to blue (acidic) color ramp between pH 7.4 and pH 1.5. The individual pH values are shown in panels B and C. The width of the strips in the HN dimension is 0.1 ppm. The contours centered at ~ 38 ppm in the strips for Glu114 are from another spin system that appears in the same HN and N frequency range at low pH. (B) pH titration data for Glu28 and Glu114 in the absence of salt. (C) pH titration data for Glu28 and Glu114 in the presence of 150 mM NaCl. The curves are nonlinear least squares fits to the data to Eq. 1 that were used to obtain the titration parameters in Table I. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.interscience.wiley.com).]

Table I. pK_a Values of αS in the Absence and Presence of Salt^a

Site	No salt				150 mM NaCl				Resonance
	pK_a	n	δ_{low}	δ_{high}	pK_a	n	δ_{low}	δ_{high}	
Asp2	3.61 ± 0.05	1.30 ± 0.21	38.64 ± 0.09	41.99 ± 0.06	3.63 ± 0.04	1.00 ± 0.08	122.81 ± 0.05	124.92 ± 0.03	Cβ/N ^b
Glu13 ^c	4.21 ± 0.06	0.90 ± 0.08	33.11 ± 0.08	36.79 ± 0.08	4.32 ± 0.09	1.42 ± 0.35	32.85 ± 0.14	36.24 ± 0.16	Cγ
Glu20	4.07 ± 0.04	0.93 ± 0.06	33.10 ± 0.05	36.74 ± 0.04	4.24 ± 0.04	0.90 ± 0.08	32.77 ± 0.19	36.49 ± 0.18	Cγ
Glu28	4.20 ± 0.06	0.96 ± 0.09	33.16 ± 0.08	36.92 ± 0.08	4.27 ± 0.04	0.85 ± 0.06	32.77 ± 0.06	36.50 ± 0.06	Cγ
Glu35 ^d	4.17 ± 0.06	0.93 ± 0.08	33.11 ± 0.07	36.69 ± 0.07	4.08 ± 0.13	0.88 ± 0.20	30.42 ± 0.41	36.39 ± 0.29	Cγ
Glu46 ^c	4.21 ± 0.06	0.90 ± 0.08	33.11 ± 0.08	36.79 ± 0.08	4.31 ± 0.10	0.86 ± 0.15	32.99 ± 0.18	36.39 ± 0.13	Cγ
His50	6.78 ± 0.04	1.08 ± 0.08	123.11 ± 0.02	125.05 ± 0.06	6.52 ± 0.07	1.52 ± 0.37	123.45 ± 0.02	125.05 ± 0.05	N
Glu57	4.20 ± 0.05	0.94 ± 0.08	33.10 ± 0.07	36.87 ± 0.07	4.33 ± 0.09	0.76 ± 0.11	32.89 ± 0.13	36.49 ± 0.13	Cγ
Glu61	4.04 ± 0.27	0.86 ± 0.35	121.05 ± 0.07	121.70 ± 0.08	3.97 ± 0.06	1.12 ± 0.22	121.35 ± 0.04	122.07 ± 0.02	N
Glu83	4.35 ± 0.05	1.07 ± 0.10	33.14 ± 0.06	36.71 ± 0.07	4.33 ± 0.03	0.91 ± 0.05	32.70 ± 0.05	36.32 ± 0.05	Cγ
Asp98	3.68 ± 0.06	0.87 ± 0.11	38.23 ± 0.10	41.56 ± 0.07	4.00 ± 0.03	1.44 ± 0.16	38.60 ± 0.05	41.01 ± 0.04	Cβ
Glu104	4.39 ± 0.13	1.12 ± 0.26	33.13 ± 0.15	36.74 ± 0.17	4.51 ± 0.11	1.01 ± 0.17	33.02 ± 0.12	36.36 ± 0.14	Cγ
Glu105 ^e	4.21 ± 0.06	0.90 ± 0.08	33.10 ± 0.08	36.79 ± 0.08	4.49 ± 0.17	1.04 ± 0.35	33.85 ± 0.18	36.33 ± 0.20	Cγ
Glu110 ^d	4.50 ± 0.09	0.98 ± 0.19	34.34 ± 0.06	36.71 ± 0.08	4.31 ± 0.07	0.85 ± 0.09	32.65 ± 0.12	36.36 ± 0.10	Cγ
Glu114	4.70 ± 0.05	0.86 ± 0.08	33.09 ± 0.06	36.97 ± 0.09	4.49 ± 0.09	0.64 ± 0.08	32.59 ± 0.13	36.58 ± 0.14	Cγ
Asp115	4.15 ± 0.05	0.76 ± 0.06	38.18 ± 0.06	41.69 ± 0.05	4.18 ± 0.09	0.94 ± 0.16	38.10 ± 0.12	41.11 ± 0.11	Cβ
Asp119	4.02 ± 0.07	0.69 ± 0.06	122.85 ± 0.08	125.87 ± 0.06	3.99 ± 0.02	0.77 ± 0.03	123.27 ± 0.03	126.20 ± 0.02	N
Asp121	4.62 ± 0.06	0.97 ± 0.08	39.11 ± 0.03	41.43 ± 0.06	4.39 ± 0.03	0.78 ± 0.04	117.42 ± 0.03	119.36 ± 0.03	Cβ/N ^b
Glu123	4.87 ± 0.05	0.77 ± 0.06	33.16 ± 0.05	36.95 ± 0.08	4.69 ± 0.10	0.78 ± 0.12	32.69 ± 0.13	36.46 ± 0.16	Cγ
Glu126	4.89 ± 0.06	0.97 ± 0.18	32.53 ± 0.08	36.72 ± 0.14	4.55 ± 0.10	0.91 ± 0.16	32.03 ± 0.16	36.12 ± 0.19	Cγ
Glu130	4.53 ± 0.06	0.85 ± 0.09	33.09 ± 0.09	36.82 ± 0.09	4.40 ± 0.09	1.09 ± 0.22	33.05 ± 0.14	36.38 ± 0.15	Cγ
Glu131	4.58 ± 0.05	0.93 ± 0.09	33.26 ± 0.06	36.84 ± 0.07	4.71 ± 0.15	0.61 ± 0.12	32.80 ± 0.22	37.09 ± 0.27	Cγ
Glu135	4.16 ± 0.07	0.70 ± 0.06	38.18 ± 0.08	41.74 ± 0.08	4.05 ± 0.06	0.77 ± 0.07	37.88 ± 0.09	41.16 ± 0.07	Cβ
Glu137	4.82 ± 0.16	0.56 ± 0.11	123.36 ± 0.11	125.77 ± 0.17	4.42 ± 0.03	0.92 ± 0.05	123.88 ± 0.02	125.56 ± 0.02	N
Glu139	4.69 ± 0.04	0.97 ± 0.10	33.14 ± 0.06	36.99 ± 0.08	4.54 ± 0.06	0.84 ± 0.09	32.72 ± 0.09	36.62 ± 0.11	Cγ
α-CO ₂ ⁻	3.65 ± 0.03	1.10 ± 0.07	8.537 ± 0.006	8.081 ± 0.003	3.67 ± 0.03	1.01 ± 0.06	8.541 ± 0.007	8.087 ± 0.005	HN(A140)

^a All values are for a temperature of 10°C.^b The pK_a values of Asp2 and Asp121 were determined from Cβ chemical shifts in the absence of salt and ¹⁵N chemical shifts in the presence of 150 mM salt.^c Glu13, Glu46 and Glu105 titrate as a single unresolved group and the three residues are assigned the same parameters in the absence of salt. The pH titration of the three residues can be resolved in the presence of 150 mM NaCl.^d The assignments for Glu35 and Glu110 are tentative due to spectral overlap and may be reversed.

random coil pK_a values we obtained for aspartate were 4.00 ± 0.02 in the absence of salt and 3.94 ± 0.02 in the presence of salt at 10°C . For glutamate, the pK_a values were 4.42 ± 0.02 in the absence of salt and 4.38 ± 0.02 in the presence of 150 mM NaCl at 10°C . Our values are somewhat higher than those determined using potentiometry of the same peptides at a temperature of 25°C in the presence of 100 mM NaCl ⁴⁸ (3.67 for Asp, 4.25 for Glu), although the recent revision⁴⁹ of these values (3.94 for Asp, 4.3 for Glu) bring them closer to those determined in this work. Our values are in very good agreement with those reported for model peptides at 25°C by potentiometry (4.0 for Asp, 4.4 Glu)⁴⁶ and NMR (3.84 for Asp, 4.32 for Glu).⁴⁷ For histidine, which occurs only once in αS at position 50, we used a published random coil pK_a of 6.54 ± 0.04 .^{46–48} For the α -carboxyl group at the C-terminus we used a random coil pK_a of 3.67 ± 0.03 .⁴⁸

Differences between random coil and αS pK_a values are shown in Figure 3(A) for the protein without salt and Figure 3(B) for the protein in the presence of 150 mM NaCl . Uncertainty bars were calculated from the propagation of standard errors from the least squares fits of the pH titration data for αS and the random coil peptides. Alternatively, if we consider that the primary source of error in the pH titrations is the accuracy of the pH measurements, which in our study is on the order of 0.05 to 0.1 pH units, the uncertainties in the pK_a differences are between 0.07 and 0.14 pH units. Acidic residues from the N-terminal amphipathic domain of αS show uniformly negative differences [Fig. 3(A)] with pK_a values lowered on average by 0.24 pH units from random coil values. In the C-terminal tail the differences are positive, with pK_a values of acidic residues raised on average by 0.24 pH units compared to the corresponding random coil values. The switch-point from positive to negative differences occurs between residues Glu105 and Glu110, just outside the amphipathic and NAC domains, at the start of the acidic C-terminal tail. In the presence of 150 mM NaCl [Fig. 3(B)] the differences between αS and random coil pK_a values are smaller but maintain the same pattern, with residues from the N-terminal domain showing negative differences (average = 0.10) and those from the C-terminus showing positive differences (average = 0.15). The switch-point in the sequence is harder to define but occurs near the demarcation of the amphipathic and acidic domains between residues D98 and E114.

The N- and C-terminal domains also show small differences in the parameter n , the Hill coefficient for the pH titration. The Hill coefficient (Eq. 1) describes the cooperativity of pH titrations.⁵⁰ Values of n less than 1 are associated with negative cooperativity, while n greater than 1 corresponds to positive cooperativity.^{50,51} The Hill coefficients for most sites in αS are very close to unity (Table I). Nevertheless, the

Figure 3. Differences between αS and random coil pK_a values ($pK_{a, \alpha\text{S}} - pK_{a, r.c.}$). (A) αS in the absence of salt. (B) αS in the presence of 150 mM NaCl . The pK_a values for αS are given in Table I. Random coil values were obtained from NMR titrations of the model peptides Ac-AAEEA-NH₂ and Ac-AADAA-NH₂ as described in the text. Random coil values for a histidine side-chain and the C-terminal α -carboxyl group were taken from published values.⁴⁸ For His50 the difference was multiplied by -1 since this group gains a positive charge, in contrast to the acidic groups that lose a negative charge at low pH. All data are at a temperature of 10°C .

average values for residues 1–105 are significantly larger than for residues 110–140: 0.98 versus 0.86 in the absence of salt (statistical significance, $P < 0.03$); 1.06 versus 0.84 ($P < 0.01$) in the presence of salt. The ionization of sites at the N-terminus thus appears to be more cooperative than at the C-terminus.

pH titration of His50 in αS bound to SDS micelles

When αS binds to an SDS micelle it forms an $\sim 32\text{ kDa}$ complex with an effective correlation time of

Figure 4. Representative data from the pH titration of α S bound to SDS micelles. (A) ^1H - ^{15}N HSQC spectra showing decreases in backbone ^1H - ^{15}N correlation intensities below pH ~ 5.2 . (B) ^{13}C -filtered aromatic spectra of α S in the presence of SDS showing the NMR signal of His50 C ϵ 1 proton (near 8.6 ppm). Samples contained 0.25 mM α S and 50 mM SDS in 90% H_2O /10% D_2O , and spectra were recorded at a temperature of 37°C.

15.1 ns.¹⁶ The large size of the complex makes it technically difficult to characterize pK_a values using C(CO)NH spectroscopy due to short T2 relaxation times, although this may be possible to circumvent at high magnetic fields using TROSY-based experiments in conjunction with perdeuterated α S samples. Another complication as shown in Figure 4(A), is that ^1H - ^{15}N HSQC spectra of α S bound to SDS micelles deteriorate at low pH due to NMR signal broadening. This broadening is even more severe when the α S micelle complex is studied in the presence of 150 mM NaCl (not shown). The effects occur well above the pK_a of the sulfate group in SDS⁵² which is at about pH 1.5, so we can conclude that the changes are associated with protonation of Asp and Glu residues in α S, although the mechanism of NMR line broadening is unknown. The transition has a mid-point near pH 5.2, about 0.8 and 1.2 pH units above the random coil pK_a values of Glu and Asp, respectively. As we have described for amyloid-beta(1-40), the pK_a values of acidic residues are uniformly raised in proteins bound to SDS micelles, due to electrostatic repulsion between the negatively charged side-chains and negatively charged sulfate groups on the SDS micelles.⁵³

The aromatic C ϵ 1 proton of His50 can be seen at low pH presumably because of the high flexibility of this side-chain in the complex [Fig. 4(B)]. In the WT α S bound to SDS micelles, His50 titrates with a pK_a of 7.7, with the titration reaching a low-pH chemical shift plateau by pH 6.5 (Fig. 5) well before the broadening of crosspeaks in ^1H - ^{15}N HSQC spectra

below pH 5 [Fig. 4(A)]. The changes in the spectrum associated with the protonation of the C-terminal tail thus should not affect the pK_a value obtained for His 50. Under similar conditions (37°C, 99.96% D_2O) free α S has a pK_a of 6.3.⁵³ The pK_a of His50 is thus raised by ~ 1.4 pH units when WT α S binds to SDS micelles compared to the unbound state. The raised pK_a of His50 indicates that protonation is facilitated in the micelle bound state, either because the charged form of the histidine ion-pairs with Glu46 in the α -helical structure adopted by the micelle-bound protein [Fig. 1(C)] or because the positively charged histidine interacts with the negatively charged sulfate groups of SDS.

To distinguish between these possibilities we collected titration data for His50 in the Parkinson's disease-linked mutants E46K and A53T (Fig. 5). Glu46 is the only negatively charged residue positioned to form an ion pair with the charged form of His50 in the α -helical structure of micelle-bound α S. The E46K mutant, which replaces the glutamate with a positively charged lysine, gives a pK_a of 7.5 for His50 in micelle-bound α S (Fig. 5). The pK_a shift of -0.2 pH units from the WT is comparable to the shifts for residues positioned to form ion pairs in the N-terminal domain of uncomplexed α S (Table I, Fig. 3). For A53T the pK_a shift of -0.1 pH units is intermediate and close to the experimental uncertainty. With both mutants, the pK_a perturbations are much smaller than the difference of ~ 1.4 pH units between free and micelle-bound α S. We can thus safely conclude that the pK_a perturbation of His50 in micelle bound α S is dominated by the interaction of the histidine cation

Figure 5. pH titrations of the His50 Cε1 proton from α S bound to d_{25} -SDS micelles. The samples contained 99.96% D_2O solutions of 0.25 mM α S and 50 mM d_{25} -SDS (0.8 mM micelles). All data were collected at 37°C. The pH values obtained in D_2O were corrected for the deuterium isotope effect ($pH = pH_{\text{measured}} - 0.4$) as described in the methods. The parameters obtained from least-squares fits of the titration data were: WT, $pK_a = 7.66 \pm 0.01$, $n = 0.90 \pm 0.02$; E46K, $pK_a = 7.46 \pm 0.03$, $n = 0.89 \pm 0.06$; A53T, $pK_a = 7.57 \pm 0.02$, $n = 0.85 \pm 0.02$. For WT α S under the same conditions in the absence of SDS micelles,⁵³ we obtained $pK_a = 6.33 \pm 0.02$, $n = 0.99 \pm 0.04$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with the negatively charged sulfate groups on the surface of the micelle.

We looked for alternative membrane mimetics amenable to NMR studies of α S including micelles composed of the negatively charged lipid LPPG (1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-RAC-(1-glycerol)]), the neutral lipid LMPC (1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine) and the neutral detergent DPC (dodecyl phosphocholine). Besides SDS, only LPPG gave a complete set of 1H - ^{15}N HSQC resonances for α S but the NMR spectra were of poorer quality than with SDS micelles (A.T.A and Andrew Mehta, unpublished results). High-quality NMR spectra have been recently demonstrated⁵⁴ for α S complexed to micelles of the surfactant SLAS (sodium lauroyl sarcosinate) but like SDS and LPPG, the detergent is negatively charged and would be expected to perturb the pK_a values of α S. With neutral DPC micelles, we confirmed that α S binds only weakly,¹⁹ such that most of the 1H - ^{15}N HSQC correlations from the N-terminal domain of α S are lost due to fast hydrogen exchange through the free form of the protein, which is in dynamic equilibrium with the micelle bound form. Since α S requires negatively charged lipids to bind to membranes we explored the amenability of mixed DPC/SDS micelles for NMR studies. Starting from micelles composed of the neutral DPC detergent we

gradually added negatively charged SDS detergent. Above DPC:SDS molar ratios of 70:30 the 1H - ^{15}N HSQC spectra of α S were poor with most 1H - ^{15}N resonances lost due to fast solvent exchange. At a DPC:SDS molar ratio of 70:30 the 1H - ^{15}N HSQC spectrum was comparable to that of α S with pure SDS micelles¹⁶ but resonances were too broad for the more demanding C(CO)NH experiments used to determine pK_a values. The enhanced solvent-exchange broadening of backbone amide protons under these conditions suggests that α S binds only weakly to micelles with DPC:SDS molar ratios of 70:30, so that even if we were to determine pK_a values these would likely constitute a population weighted-average of the protein bound to micelles and a significant fraction of the free protein. Thus the characterization of pK_a values for α S in a membrane-like environment is limited by the requirement of a small micelle size amenable to direct NMR studies, coupled with the requirement of micelles containing a high proportion of negatively charged lipids or surfactants that affect the ionization constants of titratable groups from the protein.

Discussion

The pK_a values obtained for α S in this work show surprisingly large differences from random coil model peptides considering that the protein is intrinsically unfolded (Fig. 3). The results are consistent with previous findings that ionizable sites in unfolded proteins can have electrostatic environments that are poorly approximated by random coils.^{36,37,39,40,55} That α S adopts a highly unfolded structure in solution is supported by small chemical shift deviations from random coil values,^{22,23,28} averaged $^3J_{HNHa}$ coupling constants,²² fast dynamics as measured by ^{15}N relaxation,^{23,56} largely averaged RDC values at least for the N-terminal domain,^{23,56} and rapid exchange of backbone amide protons with solvent.²⁶ The long-range nature of electrostatic interactions allow weak charge-charge interactions to occur even though α S is highly unfolded, as evidenced by the lowered pK_a values of the N-terminus, the raised pK_a values of the C-terminus, and the salt dependence of the pK_a values. We do not imply that these electrostatic interactions are the type of ordered hydrogen-bonded salt-bridges observed in X-ray structures of folded proteins. Rather these “nascent ion pairs” occur because of the positioning of oppositely charged acidic and basic residues in the KTKEGV repeats in the amphipathic domain, and because of electrostatic repulsion ensuing from the high density of negative charges in the C-terminal tail of the protein. Indeed theoretical work predicts perturbed pK_a values for a Gaussian chain model of the protein staphylococcal nuclease due to the alternating arrangement of acidic and basic residues in the amino acid sequence, with these perturbations particularly strong for segments of the protein that form the α -helical structure in the native

protein.⁵⁷ Similarly, the present work demonstrates that electrostatic interactions governed by the placement of charged residues in the amino acid sequence can play a role in determining the properties of α S even though the protein is intrinsically unfolded.

Electrostatic repulsion between acidic groups in the C-terminal domain (residues 101-140) should have the effect of keeping this segment in extended, disordered conformations. Neutralization of the charges in the C-terminal tail by polycations⁵⁸ or low pH^{22-24,58} enhances the aggregation and fibrillization of α S. It has been recently shown that neutralization of the C-terminal tail at acidic pH causes a conformational transition that leads to transient interactions between this segment and the nonpolar NAC domain. This state of α S aggregates with faster rates than the state populated at neutral pH.²²⁻²⁴ The conformational transition to the low pH state of α S leads to small changes in backbone chemical shifts of ~ 0.3 ppm for the C α carbons of residues from the C-terminus^{22,23} but is unlikely to affect the pK_a measurements in the present study. First, the pH titrations of most residues in α S were followed by side-chain carbons which undergo much larger changes in chemical shifts with pH, on the order of 3 to 4 ppm (Table I, Fig. 2). The chemical shifts of side-chain carbons from acidic residues are affected by the protonation of carboxyl groups but are much less sensitive to any residual secondary structure in the protein. Second, the differences in pK_a values correspond to differences in the midpoints of the pH transitions. The limiting low- and high-pH chemical shift plateau values of side-chain ^{13}C resonances in α S are similar and show no dependence on residue position in the sequence (Table I). Deletion of the C-terminal tail by mutagenesis also enhances the rate of fibrillization.^{58,59} Functionally, the high density of negative charges may keep the C-terminal tail from associating with negatively charged lipids so that it is primed for recognition by its binding partners on the surfaces of membranes.

In contrast to the C-terminal tail, residues from the N-terminal domain show pK_a values that are uniformly lowered compared to random coil models (Fig. 3). This behavior indicates that acidic residues from the N-terminal domain resist losing their negative charges, becoming neutralized at larger hydronium ion concentrations (lower pH) than the corresponding amino acids from the C-terminal tail of α S, or random coil peptides. The lowered pK_a values suggest that the negatively charged acidic sites in the N-terminal domain form "nascent ion-pair interactions" with positively charged basic amino acids in the KTKEGV repeats. Although 150 mM NaCl screens these weak electrostatic interactions, they persist at a physiological salt concentration [Fig. 3(B)]. The observed average pK_a shifts of -0.10 (with salt) to -0.25 (no salt) pH units for acidic residues in the N-terminal domain correspond to stabi-

lizing $\Delta\Delta G_{\text{titr}}$ contributions of only -0.1 to -0.3 kcal/mol at 10°C . As such, the effects of individual ion pairs on the stability of α -helix structure in α S should be negligible. In analogy to other proteins, rather than stabilizing structure, electrostatic interactions between oppositely charged residues may be more important in nucleating the folding of α -helix structure.^{60,61} Once an ion pair is formed, it would allow localized folding which could then propagate α -helical structure through the rest of the N-terminal amphipathic domain. The slightly raised n values for the acidic residues in the N-terminal domain are consistent with cooperativity amongst the ionization reactions. Nucleation of α S folding could occur in the cytosol, with α -helical structures selected for membrane binding. Alternatively, the folding of α -helix structure may occur after the protein binds to the surfaces of lipid bilayers. In addition to facilitating folding of α S into an α -helix structure, nascent ion-pairs in the N-terminal domain may decrease the rates of misfolding of the protein into alternative toxic conformations. In this regard, it is interesting to note that reducing the number of KTKEGV repeats in α S increases the rate of fibrillization while increasing the number of repeats has the opposite effect.¹⁵

α S is primarily distributed in the cytosol.⁶² The membrane-bound protein which accounts for only $\sim 15\%$ of synuclein in the brain, however, is probably the functional form, and membranes may play role in α S aggregation.^{7,62} The highest-resolution structural data for α S in a membrane-like environment has come from NMR but the technique is limited by the sizes and types of complexes that can be studied. Although indirect NMR studies have recently provided information on the interactions of α S with lipid assemblies as large as vesicles,²¹ direct NMR studies which afford the highest resolution and which are necessary for pK_a measurements, require that the protein is tightly bound to lipid assemblies with sizes below ~ 50 kDa.¹⁸ Micelles composed of the detergent SDS meet the requirements of tight association and small complex size for detailed NMR studies.^{18,27,63} For the purposes of investigating pK_a values, SDS micelles pose the complication of presenting a high density of negative charges from the sulfate groups on the surface of the micelle. Studies with the A β (1-40) peptide show that SDS micelles strongly perturb the electrostatic properties of ionizable residues with both acidic and basic residues experiencing increases in pK_a values of 1 to 2 pH units.^{53,64} These effects are specific to proteins and peptides, since the pK_a of single amino acids are unperturbed in the presence of SDS micelles.⁶⁴ The pK_a shifts observed for proteins bound to SDS micelles, however, do not appear to depend on the context of the ionizable site within the protein structure or on the degree of interaction with the micelle.⁵³

When α S is bound to SDS micelles, the pK_a value of His50 is raised by 1.4 pH units compared to the free protein. The Parkinson's disease mutation E46K breaks the pattern of acidic residues alternating with basic residues with an $i, i+3$ or $i, i+4$ spacing in the N-terminal domain of α S [Fig. 1(A)]. To see if the raised pK_a value of His50 in α S complexed to SDS micelles is due to an electrostatic interaction within the protein or between the histidine and the micelle, we obtained titration data for His50 in the micelle-bound form of the E46K mutant. In the E46K mutant, the pK_a value of His 50 is lowered by 0.2 pH units from 7.7 to 7.5 but is still 1.2 pH units higher than the 6.3 pK_a of the histidine in natively unfolded WT α S at 37°C. Based on these observations we conclude that there is only a weak charge-coupling between Glu46 and His50. The large pK_a shift of His50 when α S binds to SDS micelles is due to ion pairing between the histidine cation and the anionic sulfate groups on the surface of the micelle. In a membrane where the density of negatively charged lipids is lower, the interaction between Glu46 and His50 may be stronger. Considering the sequence of α S [Fig. 1(A)] the possibility also exists that Glu46 could interact with Lys43 in addition or in lieu of His50. Lys43 occurs in the break between the two α -helices in the structure of α S bound to SDS micelles¹⁸ so that it is not positioned to interact with Glu46 [Fig. 1(B,C)] but the two residues could ion-pair when α S folds into an uninterrupted α -helix conformation.^{29,32}

Materials and Methods

Materials

The peptides Ac-AADAA-NH₂ and Ac-AAEAA-NH₂ used to obtain random coil pK_a values for Asp and Glu were prepared by solid-phase synthesis at >95% purity by NEO-Peptide (Cambridge, MA). Deuterated SDS (d₂₅-sodium dodecyl sulfate) was from Cambridge Isotopes (Andover, MA). The chemical shift standard 2,2-Dimethyl-2-silapentane-5-sulfonate (DSS) was from Sigma (St. Louis, MO).

Site-directed mutagenesis of α S

The E46K and A53T mutants were prepared starting from the *pT7-7/asyn-WT* vector⁶⁵ which was a gift from the Lansbury lab (Harvard Medical School). Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA). For E46K, the forward primer was 5'-CC AAA ACC AAG AAG GGA GTG GTG-3' and the reverse primer 5'-CAC CAC TCC CTT CTT GGT TTT GG-3'. For A53T, the forward primer was 5'-GTG CAT GGT GTG ACG ACA GTG GCT GAG-3' and the reverse primer 5'-CTC AGC CAC TGT CGT CAC ACC ATG CAC-3'. Site-directed mutagenesis was performed using the QuikChange protocol, utilizing PFU Turbo

Polymerase from Stratagene (La Jolla, CA). The PCR product was purified using digestion with the restriction endonuclease DpnI from New England Biolabs (Ipswich, MA) at 37°C, followed by transformation into Stratagene *E. coli* BL21 (DE3)-pLysS cells according to the manufacturer's instructions. Sequences of the α S clones were verified using Beckman Coulter CEQ 2000 Dye Terminator Cycle Sequencing at the UConn Biotechnology facility.

Preparation of α S NMR samples

Expression and purification of α S was carried out according to published methods.^{26,66} Cells were grown in M9 minimal medium supplemented with 1 g/L ¹⁵N ammonium chloride and/or 3 g/L ¹³C-glucose, at a temperature of 37°C and a shaker speed of 250 rpm. Ampicillin (100 μ g/mL) was used to select for the *amp* resistance marker in the *pT7-7/asyn-WT* vector, and 37 μ g/mL chloramphenicol was used to maintain the pLysS copy number. When the cells reached an OD₆₀₀ of 0.6, α S expression was induced by addition of IPTG to a final concentration of 1 mM, and the cells were incubated for an additional 4 h at 37°C. Cells were collected by sedimentation at 4°C for 30 min in a Sorvall GSA rotor operating at 3150 g. The cell pellet was resuspended in lysis buffer (10 mM Tris at pH 8.0, 1 mM EDTA, 1 mM PMSF, 1 mM DTT and a few crystals of RNase, DNase, and lysozyme) and was sonicated at 70% (pulse 45 sec, delay 15 sec) for 10 minutes on a Fisher Scientific Sonic Dismembrator Model 500. After sonication, the cell lysate was boiled for 20 min and the cell debris was separated by sedimentation for 30 min at 28,300 g (Sorvall GSA rotor) and a temperature of 4°C. The supernatant was boiled again for 20 min, taking advantage of the heat stability of α S to achieve partial purification from other proteins in the lysate,^{67,68} and the precipitate was removed by sedimentation for 30 min at 12,800 rpm and 4°C. The resulting supernatant was subject to ammonium sulfate precipitation (361 g/L) for 1 h while stirring at 4°C, followed by collection of the pellet containing α S by sedimentation for 30 min at 12,800 rpm, 4°C. The ammonium sulfate precipitate was resuspended in 20 mM Na₂HPO₄, pH 7.4 and dialyzed overnight against the same buffer, followed by application on an Amersham Q-Sepharose Fast Flow anion exchange column. A linear 1 M NaCl gradient was used to elute α S. SDS-PAGE was used to identify column fractions containing α S, the fractions were pooled and dialyzed for 24 h against 20 mM Na₂HPO₄, pH 7.4. The α S purified in this way was determined to be at least 90% homogeneous by 15% tricine gel electrophoresis and electrospray ionization (ESI) mass spectroscopy. Final yields of purified α S were about 50 mg/L of *E. coli* culture. Before use, purified α S samples were stored at -80°C in 20 mM Na₂HPO₄, pH 7.4 containing 0.02% NaN₃.

pH titrations

The pH titrations of free α S (Figs. 2 and 3), α S in the presence of SDS micelles (Fig. 4), and random coil model peptides were performed on samples dissolved in 90% H_2O /10% D_2O . All α S and peptide samples used in this study were initially in 20 mM sodium phosphate buffer at pH 7.4. The sample pH was adjusted by adding aliquots of 1 M HCl and NaOH stock solutions. For comparisons of the titration of His50 in WT α S, E46K- α S and A53T- α S in the presence of d_{25} -SDS micelles (Fig. 5) samples were dissolved in 99.96% D_2O and pH adjustments were made using 1 M stock solutions of DCl and NaOD.

Three-point electrode calibrations (pH 4, 7, 10) were done before each pH titration. Solution pH values were measured before and after each experiment with typical differences between measurements of 0.05 to 0.1 pH units. The average of the pH measurements before and after each NMR experiment was taken as the solution pH. We found that the following protocol reduces fluctuations in pH measurements before and after NMR experiments. The pH after the NMR experiment is measured by transferring the sample from the NMR tube to an Eppendorf tube. For the next pH value, acid or base are added to adjust the pH, the sample is stirred on a Vortex mixer, transferred to the NMR tube, and then back to the Eppendorf for the pH measurement before the NMR experiment. This minimizes pH fluctuations from traces of the sample before the pH adjustment that remain in the NMR tube. The same NMR tube, Eppendorf tube, and pipette for transferring samples were used throughout the entire pH titration. NMR samples were contained in 5 mm (Wilmad 535-pp) tubes, with a larger initial volume (0.75–1.0 mL) than the recommended 0.6 mL, to make up for sample losses due to pipetting and pH measurements during the titration.

For pH measurements in aqueous solution we used a Mettler Toledo (Columbus, OH) Inlab Micro pH glass electrode. For solutions containing SDS we used a PH46-SS probe metal electrode from IQ Scientific (Loveland, CO), since trace amounts of KCl from glass electrodes precipitate SDS. Glass electrodes are known to be self-correcting for the deuterium isotope effect, since the effect of D_2O on pH is equal and opposite in sign to the effect of D_2O at the pH electrode.⁶⁴ We established, however, that this is not the case for the PH46-SS probe metal electrode used in these studies. First, we established that under identical conditions the pH titration of His50 measured on a ^{13}C , ^{15}N -labeled α S sample in 90% H_2O /10% D_2O using 1D ^{13}C -edited spectra gave a pK_a value of 7.7 compared to the value of 8.1 obtained measured using 1D ^1H -NMR of a natural abundance α S sample in 99.96% D_2O . Both titrations were followed with a metal electrode and the difference of 0.4 pH units corresponds to the deute-

rium isotope effect.⁶⁹ Second, we calibrated both the glass and metal electrodes with H_2O buffers and compared readings on the two electrodes for a series of buffers prepared in D_2O . The readings for D_2O solutions spanning the range pH 2–10 were consistently 0.2–0.4 pH units higher for the metal compared to the glass electrode. The only experiments done in D_2O in this work were the titrations of His50 in WT, E46K, and A53T α S complexed to SDS micelles (Fig. 5). Because unlike the glass electrode^{50,69} the metal electrode we used does not appear to compensate for the D_2O isotope effect, the following correction was applied to the D_2O data in Figure 5 measured with a metal electrode: $\text{pH} = \text{pH}_{\text{measured}} - 0.4$.

NMR spectroscopy

NMR experiments were done on a Varian 600 MHz instrument equipped with a cryogenic probe. The α S concentration for all experiments was 0.25 mM. DSS was used as an internal ^1H chemical shift reference standard for all experiments. ^{13}C and ^{15}N chemical shifts were referenced indirectly as described in the literature.⁷⁰ The pH titration of ^{13}C , ^{15}N -labeled α S in the absence of salt was characterized with twelve 3D C(CO)NH and 2D ^1H - ^{15}N HSQC experiments recorded between pH 7.4 and pH 1.5. Eleven experiments over the same pH range were used to determine pK_a values for α S in the presence of 150 mM NaCl. An additional four 2D ^1H - ^{15}N HSQC experiments were recorded up to pH 8.1 for each titration, to define the high pH chemical shift plateau of the His50 backbone ^{15}N resonance. Experiments on α S in the absence of micelles were done at 10°C to prevent loss of amide proton signals due to fast exchange with solvent.²⁶ Experiments with α S in the presence of SDS micelles were done at 37°C since lower temperatures reduce the quality of NMR spectra.²⁸

The peptides Ac-AADAA-NH₂ and Ac-AAEAA-NH₂, dissolved to 3 mM concentrations in 90% H_2O /10% D_2O were used to determine random coil pK_a values for Asp and Glu. The titration of the peptides in the absence of salt was followed using twelve 1D ^1H NMR spectra recorded between 6.7 and 1.7. For experiments in the presence of 150 mM NaCl ten spectra were obtained between pH 6.4 and 2.4. All peptide experiments were done at a temperature of 10°C for direct comparison with the α S experiments.

The titration of ^{13}C , ^{15}N -labeled α S in the presence of 50 mM SDS was characterized with 2D ^1H - ^{15}N HSQC and 1D ^{13}C -filtered aromatic spectra. Four representative pH points that show the deterioration of ^1H - ^{15}N HSQC spectra below ~pH 5.0 are shown in Figure 4. The titration of the His50 C α 1 proton in WT, E46K and A53T α S was followed by 1D ^1H NMR using protein samples at natural isotopic abundance in the presence of 50 mM d_{25} -SDS dissolved in 99.96% D_2O . For each of the

experiments at least twelve spectra were recorded between pH 10.0 and 4.6 (Fig. 5).

The following spectral parameters were used for NMR experiments. 1D ^1H NMR spectra were collected with spectral windows of 8000 Hz digitized into 2048 complex points. 2D ^1H - ^{15}N HSQC spectra were acquired with $^1\text{H} \times ^{15}\text{N}$ spectral windows of 6000×1380 Hz digitized into 1024×128 complex points. The total acquisition time for each spectrum was 10 min. Three dimensional C(CO)NH spectra were acquired with $^1\text{H} \times ^{13}\text{C} \times ^{15}\text{N}$ spectral windows of $6000 \times 8800 \times 1380$ Hz digitized into $1024 \times 64 \times 32$ complex points. The total acquisition time for each spectrum was 12 h.

NMR data analysis

Identification of NMR signals over the pH range studied, was facilitated by published ^1H , ^{15}N , and ^{13}C assignments for αS at pH 7,⁵⁴ pH 3,²² as well as data on the pH dependence of αS NMR spectra from our lab.²⁶ For 1D spectra, chemical shifts were obtained from the center of the peak envelope. For 2D and 3D spectra, 1D traces were extracted along the frequency of the nucleus of interest. The traces were phased as necessary, before measuring chemical shifts from the center of the peak envelope.

The pH dependence of chemical shifts was analyzed using nonlinear least squares fits of the data to the modified Henderson-Hasselbalch equation:^{50,71}

$$\delta = \delta_{\text{low}} - \frac{\delta_{\text{low}} - \delta_{\text{high}}}{1 + 10^{n(\text{pK}_a - \text{pH})}} \quad (1)$$

where the pK_a is the ionization constant, δ_{low} is the low pH chemical shift plateau, δ_{high} is the high pH chemical shift plateau, and n is the apparent Hill coefficient. The parameters obtained from the fits and their associated standard errors are given in Table I.

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