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Carboxyl pK_a Values, Ion Pairs, Hydrogen Bonding, and the pH Dependence of Folding the Hyperthermophile Proteins Sac7d and Sso7d

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Abstract

Sac7d and Sso7d are homologous, hyperthermophile proteins with a high density of charged surface residues and potential ion pairs. To determine the relative importance of specific amino acid side chains in defining the stability and function of these Archaeal chromatin proteins, pK_a s were measured for all of the acidic residues in both proteins using ^{13}C NMR chemical shifts. The stability of Sso7d enabled titrations to pH 1 under low salt conditions. Two aspartate residues in Sso7d (D16 and D35) and a single glutamate residue (G54) showed significantly perturbed pK_a values in low salt, indicating that the observed pH dependence of stability was primarily due to these three residues. The pH dependence of backbone amide NMR resonances demonstrated that perturbation of all three pK_a s was primarily the result of side chain-backbone amide hydrogen bonds. Titration data at higher salt for both Sso7d and Sac7d were consistent with this interpretation. Few of the significantly perturbed acidic pK_a s in Sac7d and Sso7d could be attributed to primarily ion pair or electrostatic interactions. A smaller perturbation of E48 (E47 in Sac7d) was ascribed to an ion pair interaction that may be important in defining the DNA binding surface. The small number (3) of significantly altered pK_a values was in good agreement with a linkage analysis of the temperature, pH, and salt dependence of folding. The linkage of the ionization of two or more side chains to protein folding leads to apparent cooperativity in the pH dependence of folding, although each group titrates independently with a Hill coefficient near unity. These results demonstrate that the acid pH dependence of protein stability in these hyperthermophile proteins is due to independent titration of acidic residues with pK_a s perturbed primarily by hydrogen bonding of the side chain to the backbone. This work demonstrates the need for caution in using structural data alone to argue the importance of ion pairs in stabilizing hyperthermophile proteins.

Keywords

Sulfolobus; chromatin; electrostatics; ion pairs; hydrogen bonding

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Introduction

Electrostatic interactions and ion pairs can potentially play an important role in defining protein structure, stability, and activity.¹⁻⁹ Experimental evidence for the importance of electrostatic effects in proteins can be provided by site-directed mutagenesis and the dependence of folding and stability on pH and salt concentration. Direct evidence is provided by the demonstration of perturbed pK_a s of the charged groups involved in the interactions.^{10,11} Measurement of perturbed pK_a s has the advantage of defining the relative importance of specific interactions involving ionizable groups. The database of pK_a s of charged groups in proteins has increased significantly in recent years so that an understanding of the relationship between structure, electrostatics, hydrogen bonding and pK_a s is beginning to emerge.¹² In addition to experimental data, extensive computational efforts have been devoted to addressing the contribution of electrostatics, hydrogen bonding, and pK_a s to protein structure and function.^{7,13-19}

Salt bridges have been often cited as an important determinant for the enhanced stability of thermophile proteins.^{3,4,20-31} Both theoretical^{6,7} and experimental^{32,33} evidence has indicated that ion pairs can be destabilizing due to the energy of desolvation. However, in a number of cases experimental evidence indicates that ion pairs or salt bridges can be stabilizing^{10,34,35} and computations have supported these results³⁶, especially at high temperature^{37,38} under conditions of optimal bond geometry.³⁹ Salt bridges have been substituted into a few mesophile proteins with some increase in stability, but little thermodynamic data has been reported to quantitatively demonstrate the importance of an electrostatic interaction in a thermophile protein.^{21,40-42} It is interesting to note that in many thermophiles such as *Pyrococcus* and *Methanothermus*, the intracellular salt concentration is 1 M or greater, and the stability of many hyperthermophile proteins is increased with high salt.^{43,44}

A characterization of the perturbations of ionizable side chains requires an understanding of the pK_a s in the folded native protein, as well as those expected in exposed residues in the unfolded proteins. Model compounds indicate that exposed carboxyls and those in the unfolded state should have pK_a s of about 4.0 for aspartate and 4.4 for glutamate.¹² There is significant evidence that the pK_a s of titrating groups in a number of *unfolded* proteins may differ from that expected from model compounds.^{11,45-52} In some proteins measures of the pK_a s in unfolded protein have indicated that this difference can be negligible.⁵³ In *folded* proteins, the average value for the pK_a of aspartate is 3.4 (± 1.0) and for glutamate about 4.1 (± 0.8). The smaller perturbation of glutamate pK_a s from that observed in model compounds has been attributed to fewer hydrogen bonds to glutamate side chains than for aspartate, and also the lower electrostatic potential observed at the more exposed glutamate carboxyls than seen typically for aspartate. However, the influence of long range electrostatic effects in proteins is most likely small as demonstrated by the lack of a significant correlation of pK_a s with the pI of the protein.¹² Interestingly, the dependence of pK_a s on calculated electrostatic potential is only about 10 to 20% of that expected. In fact, the scatter in the data collected to date is such that there is little evidence for a correlation between acid side chain pK_a and calculated electrostatic potential. However, this does not mean that electrostatic potential does not play a role in protein stability. Long range effects may be weak, but they are present, and in some structures they can accumulate to make a significant contribution.⁵⁴ A number of workers have clearly demonstrated that protein stability can be modified by design with changes in electrostatic potential.⁵⁵⁻⁶¹

Sac7d and Sso7d are homologous DNA binding proteins from the hyperthermophiles *Sulfolobus acidocaldarius* and *S. solfataricus*, respectively (Figure 1). They are especially interesting because they are small (7 kD, pI 9.7) with a high density of potential ion pairs (0.2/

residue, compared to a more typical value of 0.04/residue⁶²). The stabilities of Sac7d and Sso7d are some of the best characterized of any protein.^{63,64} This is the result of the fact that not only are these thermophile proteins with T_m values that approach 100 °C under physiological conditions, but they are also highly charged and basic proteins that retain a native fold as low as pH 0 (i.e. 1 M HCl). Both proteins are well-behaved for biophysical studies over an unusually wide range of experimental conditions (viz. pH 0 to 8, 0.0 to 0.3 M KCl, and 0 to 100 °C), which has enabled a detailed linkage analysis of the effects of pH and salt (anion binding) on stability.^{63,65}

The linkage of protonation and anion binding to protein folding may be described by the following simple model with one protonation reaction and one anion binding reaction linked to folding:

The intrinsic unfolding reaction ($N \leftrightarrow U$) is linked to protonation with potentially different pK_a s for the native (N) and unfolded (U) species. In addition, anion binding is linked to folding with binding explicitly indicated to both N and NH and the pK_a for the protonation reaction may be affected by anion binding (i.e. K_c may not be equal to K_n). Binding of L to U is assumed to not occur. Nonlinear least squares fitting of the stability data for Sac7d and Sso7d to such a model required an expansion to include at least two protonation and two ion binding reactions linked to folding.^{64,65} The fitted pK_a s for the carboxyls linked to folding were near 4.5 for the unfolded (4.7 for Sac7d, and 4.4 for Sso7d), and near 0 for the native species (0.0 for Sac7d, and 0.1 for Sso7d).⁶⁴ This is the minimal model that can explain the stability data, and the possibility of additional protonation and anion binding sites linked to folding has not been excluded. One of the goals of this work was to determine the accuracy of the ionization part of the linkage model by using NMR to localize the perturbed residues and the magnitudes of the perturbed pK_a s.

The NMR solution structures of Sac7d⁶⁶ and Sso7d⁶⁷ indicate a number of possible surface residues that may be responsible for the linkage indicated by the stability data. D36 in Sac7d (E36 in Sso7d) would appear to be positioned to form an ion pair network that involves K13 as well as E11 and links the β -sheet to the β -ribbon. Another potentially important interaction may involve the N-terminus which lies between D16 and E53 (E54 in Sso7d) (Figure 1B). Asp35 may be involved in ion pairs with K19 and K21. Finally, we note that E47 (E48 in Sso7d) is positioned to form an ion pair with R25 in both proteins.

The goal of this work was to provide experimental evidence for specific acidic residues that are important in the folding of Sso7d and Sac7d. This has been accomplished by measuring the pK_a s for all of the acidic residues in both proteins using a triple resonance 2D ^1H , ^{13}C NMR experiment⁵³ that permits direct observation of the side chain carboxyl chemical shifts. These results are compared with titration data obtained with 2D ^1H , ^1H TOCSY NMR as well as ^1H , ^{15}N HSQC NMR. The later data provides direct observation of the effect of pH titrations

on the backbone amides, and demonstrates that many of the most significantly perturbed acid side chains are hydrogen bonded to the backbone.

RESULTS

pK_a s for all of the aspartic and glutamic acid side chains in Sac7d and Sso7d were obtained from the pH dependence of ^{13}C and ^1H NMR chemical shifts. Triple resonance 2D NMR spectra⁵³ correlating the chemical shift of the acid side chain carboxyl carbon with a neighboring backbone NH ($^{13}\text{C}_i\text{-}^{15}\text{N}_{i+1}\text{H}$) permitted determination of the ^{13}C chemical shifts of the acid carbonyl carbons from pH 1 to 7 (Figure 2). pK_a s of the acidic residues of Sso7d (three aspartates and six glutamates) in the absence of salt were obtained (Table I) by nonlinear regression of the pH dependence of the carbon chemical shift (Materials and Methods). The measured pK_a s were compared to those expected for an exposed, unperturbed side chain to define those residues with significantly perturbed pK_a s. The pK_a for an exposed aspartate from model peptide data was assumed to be 4.0, and that for glutamate was 4.4¹².

Two aspartates showed a pronounced perturbation in Sso7d: D16 with a pK_a of approximately 2.11 ± 0.01 and D35 with a pK_a of 2.16 ± 0.01 ($\Delta pK_a = pK_a^{\text{obs}} - pK_a^{\text{model}}$ values of -1.9 and -1.8 , respectively). The perturbation of a third aspartate (D50) was nearly half that observed for the others, with a pK_a of 3.01 ($\Delta pK_a = -1.0$). Of the six glutamates, E54 was the most perturbed with a pK_a of 3.01 ($\Delta pK_a = -1.4$). E48 was somewhat less perturbed with a pK_a of 3.45 ($\Delta pK_a = -1.0$). The apparent Hill coefficients for all the side chains were nearly 1 (mean = 0.91; standard deviation = 0.03), indicating that the side chains titrated independently.

The ^{13}C titration data for Sso7d were compared to the pH dependence of acid side chain ^1H chemical shifts (i.e. geminal C_βH_2 protons of aspartic acid, and the $\text{C}_\gamma\text{H}_2$ protons of glutamic acid) monitored using 2D ^1H , ^1H TOCSY NMR spectra (Figure 3). In general, pK_a s determined using ^1H NMR chemical shifts were in qualitative agreement with those determined by ^{13}C NMR. The two geminal protons in each side chain, however, often showed different pK_a s for the adjacent titrating group. This indicated that the ^1H chemical shifts in the same residue were affected not only by the ionization of the adjacent carboxyl, but also were affected to different extents by titrations of one or more neighboring residues. In most cases the difference between the two pK_a s was on the order of 0.1, but in two cases the discrepancy was larger: 0.65 for E36, and 0.41 for E60. In a number of cases, the pK_a obtained from the ^{13}C NMR chemical shift was essentially an average of the two values indicated by the ^1H shifts (e.g. E12, D35, E36, and E60). However, in nearly just as many other cases the ^{13}C -derived value fell outside the range obtained from the ^1H data (e.g. E11, D16, E48, D50). Because the ^{13}C chemical shifts reflect the electron density in the titrating group, and the ^1H data often provided two different pK_a values for each side chain, the pK_a s obtained from ^{13}C data were assumed to be more accurate. Similar conclusions have been reported elsewhere.^{12,68}

All of the pK_a s for Sso7d were dependent on salt concentration. An increase in KCl concentration from 0.0 to 0.3 M resulted in an average increase in pK_a by 0.5 units (standard deviation of 0.07) (Table 2). The salt sensitivity of the most perturbed pK_a s noted above (i.e. D16, D35, E54) was similar to that of the less perturbed pK_a s. The Hill coefficients were insensitive to salt, and remained slightly less than 1.0 (mean = 0.89; standard deviation = 0.02). The magnitude of the difference in pK_a s indicated by the ^1H NMR chemical shifts of the geminal protons of many side chains was also observed at 0.3 M KCl, and even increased in some residues (e.g. E36).

The lower stability of Sac7d relative to Sso7d resulted in significant unfolding of Sac7d at lower pH, with about 75% of the protein unfolded at pH 2.⁶⁵ It was therefore not possible to obtain accurate pK_a s of native (folded) Sac7d in low salt. However, acid-induced unfolding of

Sac7d was negligible in the presence of 0.3 M KCl, so that meaningful pK_a s were readily obtained at higher salt. The pK_a s of all the acid side chains of Sac7d (5 aspartates and 7 glutamates) in 0.3 M KCl are given in Table 3. Comparison of the Sac7d and Sso7d pK_a s at the higher salt concentration indicates some similarities as well as some significant differences. The acid side chains titrate similarly in both the amino terminal β -ribbon and the first 3_{10} helix, with the most significantly perturbed aspartate being D16 in both. Similarly, comparison of homologous residues in the C-terminal 3_{10} helix and α -helix indicate the presence of similar interactions, with D49 in Sac7d having the same pK_a as D50 in Sso7d, and E53 being essentially identical to E54 in Sso7d. The Hill coefficients observed for the titrating acid side chains in Sac7d are similar to those observed for Sso7d, with an average of 0.87 (standard deviation=0.06).

A comparison of the pK_a s for homologous acidic residues in the vicinity of the turn between the second and third strands of the β -sheet (D35-K40 in Sso7d, D35-K39 in Sac7d) indicate a difference in the interactions of the acidic residues in this region of the two proteins. This is the location of the additional glycine in Sso7d that expands the size of the loop by one residue (Figure 1). The most significantly perturbed pK_a in the D35-D36 acid pair in Sac7d is that of D36, while in Sso7d the most perturbed in the homologous D35-E36 pair is D35. In fact, D36 in Sac7d has one of the most perturbed pK_a s of any residue in that protein, while D35 is one of the most perturbed in Sso7d. Interestingly, the side chains of these residues are positioned on opposite sides of the β -sheet.

Similar to what was observed for Sso7d at high and low salt, the pK_a s for Sac7d at 0.3 M KCl determined by ^1H chemical shifts of the side chain geminal protons were in many cases not the same, and differed from values determined by ^{13}C NMR chemical shifts of the carboxyl carbon. As observed for Sso7d, the pK_a s determined from the ^1H data tended to be larger than those defined by the ^{13}C NMR shifts for the same residue. The influence of neighboring titrating groups on the ^1H data typically increased the pK_a measured by ^1H NMR by about 0.1 units.

Interactions between the titrating groups and backbone were indicated by the effect of pH on the chemical shift of backbone amide protons of Sso7d (in 0 and 0.3 M KCl) and Sac7d (0.3 M KCl) monitored using ^1H , ^{15}N HSQC spectra (Table I-III). Chemical shifts for backbone amide protons significantly affected by pH in Sso7d (in the absence of KCl) are shown in Figure 4. A number of NH's that were affected by pH were associated with amino acid residues without titrating side chains (e.g. S18), indicating either indirect inductive effects or hydrogen bonding with a titrating side chain. Some of the largest changes in NH chemical shifts with decreasing pH were upfield (e.g. S18, G37, G39, and E54; red curves in Figure 4), indicating hydrogen bonding of the amide NH to an acid side chain.⁶⁹ The magnitude and sign of the change in chemical shift in Sso7d was essentially independent of salt concentration (see the numbers in parentheses in the right hand columns of Tables I and II). Similar changes were observed in Sac7d, with large upfield changes with decreasing pH occurring for the NH of S18 and E53 ($\Delta\delta = -0.92$ and -0.79 , respectively). Interestingly, one of the largest downfield shifts with decreasing pH was observed for the NH of N37 in Sac7d ($\Delta\delta = +0.54$).

DISCUSSION

The goal of this work was to define those residues that are primarily responsible for the pH dependence of the stability of Sac7d and Sso7d, and to determine the structural basis for the linkage of the ionization of these residues to protein folding. The pH dependence of protein stability arises from the perturbation of the pK_a s of one or more amino acids due to the structure of the protein.^{9,13,14,70,71} In the acid pH region, this requires the perturbation of acid pK_a s in the folded state compared to those in the unfolded, denatured chain.⁷²

The titration behavior of the acid side chains of Sso7d and Sac7d indicates that in both proteins a small subset of the acid side chains play a significant role in defining the pH dependence of stability. The correlation of the pK_a s of many of these groups with the pH dependence of backbone NH chemical shifts indicates that the majority of these are perturbed due to hydrogen bonding to the backbone (see below). Although it has been recognized for some time that perturbed pK_a s of carboxylic acid side chains in proteins, especially in enzyme active sites, can result from effects other than Coulombic interactions,^{7,73-78} most attempts to explain perturbed carboxyl pK_a s and their role in defining protein stability have stressed electrostatic interactions (i.e. ion pairs).^{9,13,14,37,79-81} The importance of hydrogen bonding in influencing the pK_a s of proteins and protein stability has been most clearly described by Robertson, Jensen, Pace and Scholtz.^{18,47,78,82-84} pH titrations of ovomucoid third domain showed that the titrating groups with the most shifted pK_a s were involved in hydrogen bonding.^{47,82} Recent work has indicated that hydrogen bonding to backbone amide NH's is especially important in perturbing side chain pK_a s.^{12,83} A combination of quantum mechanics and electrostatic calculations has demonstrated that hydrogen bonding is expected to play a larger role in perturbing pK_a s than electrostatic interactions.¹⁸

Hydrogen bonding of an acidic side chain to an NH leads to an upfield shift of the amide protons by as much as 1 ppm with decreasing pH and protonation of the acid.⁶⁹ A correlation has been made between the magnitude of the shift of the NH with the magnitude of the perturbation of the pK_a relative to model compounds.⁸² In contrast, intrinsic through bond effects due to a neighboring titrating group lead to only small (0.2 ppm) downfield shifts of the NH with decreasing pH. Similarly, side chain protons of Asp and Glu shift *downfield* by approximately 0.2 ppm with protonation of the side chain.⁸⁵

The pK_a s of two of the acidic residues of Sso7d are perturbed by more than 1.5 units (D16 and D35) with pK_a s of 2.11 and 2.16. The structure of Sso7d indicates that the acid side chain of D16 could potentially form a hydrogen bond with the NH of S18 to cap the N-terminus of the 3_{10} helix made by T17-S18-K19, although the carboxyl group in both the NMR and crystal structures is turned away from the NH. Hydrogen bond formation is confirmed by the significant upfield shift of S18 with decreasing pH with an apparent pK_a in good agreement with that observed for the side chain of D16. Similar effects are observed in Sso7d at higher salt, with the pK_a of the side chain increasing at 2.62, and the apparent pK_a of S18 NH matching it at 2.7. The magnitude of the upfield shift with decreasing pH observed for the S18 NH is not affected by salt, indicating that the pH linkage is due to formation of a hydrogen bond with the titrating group. The data on Sac7d at higher salt are consistent with this. We interpret all of the data as indicating that in both proteins the most significant carboxyl pK_a perturbation is the result of hydrogen bonding of the side chain to a backbone NH.

The second most perturbed acid side chain in Sso7d in the absence of salt is D35, with a pK_a of 2.16. Inspection of the structure shows that the side chain is pointed into the turn between the second and third strands of the β -sheet. The NH of G37 is directed towards the D35 acid side chain, and titrates significantly upfield with decreasing pH with a pK_a in good agreement with that of D35. Similar effects were observed in 0.3 M KCl, with both D35 and the NH showing increased pK_a s near 2.70, and little change is observed in the magnitude of the titration shift due to salt.

Interestingly, the D35 pK_a in Sac7d is much less perturbed than in Sso7d, presumably due to the smaller loop between the second and third strands of the β -sheet. Hydrogen bonding of the D35 carboxyl to the G38 NH is indicated by the significant upfield shift of that NH with decreasing pH, and this is confirmed by the near identity of the pK_a s for the D35 carboxyl (3.42) and the apparent pK_a of G38 NH (3.41). The reason for the much smaller perturbation of the D35 side chain pK_a than in Sso7d is not clear. Notably, the NH of N37 is in close

proximity to the H-bond between D35 and the G38 NH, which may explain the unusually large downfield shift of that NH with pH.

The most surprising difference between the titration behavior of residues in Sac7d and Sso7d is the more perturbed pK_a of D36 side chain in Sac7d ($\Delta pK = -0.9$), compared to the essentially unperturbed E36 in Sso7d ($\Delta pK = -0.1$). D36 lies in the loop between strands 1 and 2 of the β -sheet, and the side chain is pointed away from the loop towards the N-terminal β -ribbon. There is no evidence of hydrogen bonding of the D36 side chain to any backbone NH based on the titration data. Potential candidates for interactions include an ion pair with K39, hydrogen bonding to N37, and hydrogen bonding with the hydroxyl of Y34 (~ 4 Å).

The third most perturbed pK_a in Sso7d is the side chain of E54, with a pK_a of 3.06 (ΔpK_a of -1.4). This correlates well with the apparent titration of the backbone amide NH of E54, which titrates upfield by over 1 ppm with decreasing pH and an apparent pK_a of 3.06. The titration behavior of both the side chain and NH is similar at higher salt, with both pK_a s increasing to 3.5, and the NH titrating upfield with decreasing pH by nearly 1 ppm. We conclude that the side chain of E54 is perturbed from that observed in model oligopeptides due to hydrogen bonding to its own backbone NH and a capping of the helix.

The other two most perturbed pK_a s in Sso7d are those of E48 and D50, both of which are in the 3_{10} helix before the C-terminal α -helix. Of the two, D50 has the greatest effect on the pH dependence of stability because of its lower intrinsic pK_a . The perturbation of the pK_a of D50 can be explained by hydrogen bonding to the backbone NH of S47, and the upfield shift of S47 NH with decreasing pH (Figure 4) with a similar apparent pK_a supports this assignment. A similar interaction (D49 side chain to S46 NH) is indicated by the data on Sac7d. The magnitude of the pK_a shift and the size of the upfield shift with decreasing pH are both smaller than observed for the other side chains affected by backbone hydrogen bonding. We conclude that the strength or population of this hydrogen bond is significantly less than the other three described above.

E48 appears to be the only amino acid side chain in Sso7d that is perturbed significantly due to ion pairing. E48 lies in close proximity to R25 which extends from the three-stranded β -sheet, on the opposite side of the face that inserts into the DNA. The perturbed pK_a of E48 in Sso7d would indicate the presence of an ion pair with R25 that may be important in stabilizing the structure of the intercalating loop. The data from Sac7d support a similar interaction.

Comparison of pK_a s to values calculated with PROPKA

A comparison of the pK_a s reported here to values calculated using PROPKA⁸⁴ leads to a number of interesting observations. (A tabulation of PROPKA pK_a s calculated using the coordinates of various NMR and crystal structures is provided in Supplementary Material.) PROPKA employs empirical parameters that have been derived from a set of “training” proteins with well-defined structures and pK_a s. PROPKA is used here to determine if the magnitude of the observed pK_a perturbations are consistent with this set of proteins. The calculated pK_a s are sensitive to side chain positions, and it is interesting to determine which of a number of structures of Sac7d and Sso7d are consistent with the observed pK_a s of specific acid residues. The influence of salt is not included in PROPKA. We use the pK_a s from the higher salt data since it allows a comparison of both Sac7d and Sso7d.

The majority of the structures of Sac7d (both crystal and solution) show hydrogen bonding of the D16 side chain to the NH of S18, and a significant perturbation of D16 is predicted by PROPKA for these structures with a magnitude in good agreement with that observed here (mean = 2.12, standard deviation = 1.0, 9 structures; see Table in Supplementary Material). In contrast, the NMR structure and one of the crystal structures of Sso7d does not show hydrogen

bonding of the D16 side chain to the backbone, and the calculated pK_a s for these structures are unperturbed. Two of the Sso7d crystal structures show significant hydrogen bonding, and the predicted pK_a s are significantly perturbed (1.23 and 2.20). The calculations therefore support the argument that the perturbation of D16 is due to hydrogen bonding to the backbone. The NMR structure of Sso7d (1sso), and also one of the crystal structures (1bnz), does not appear to accurately represent the D16 side chain position in solution.

The second most perturbed pK_a in Sso7d is that of D35. The NMR titration data indicates that the D35 side chain is hydrogen bonded to the NH's of both G37 and G39 in the turn between the second and third strands of the β -sheet. The PROPKA predicted perturbation for D35 is consistent with a hydrogen bond to the NH of G37 in only one of three crystal structures. Most importantly, none of the crystal structures or the NMR solution structure show the NH of G39 to be close enough to hydrogen bond to the D35 side chain. The NMR titration data indicates that the structure of Sso7d in the vicinity of the turn differs from that given for both the crystal and NMR solution structures in the PDB.

The perturbation of E54 in Sso7d is consistent with the PROPKA prediction (mean = 3.58; standard deviation = 0.78) using the Sso7d crystal structures where the E54 side chain is hydrogen bonded to its own backbone NH, but it is not consistent with the predicted value using the NMR structure (where the side chain is not hydrogen bonded to the backbone). The side chain of the homologous E53 in Sac7d is not hydrogen bonded to the backbone in most of the crystal structures and the NMR structure, and the calculated pK_a s based on these structures are inconsistent with the experimental value. There is no question that the side chain of E53 in Sac7d (and E54 in Sso7d) is hydrogen bonded to the E53 NH (E54 NH in Sso7d) based on the titration of the NH. We conclude that the E54 side chain position in both the NMR and crystal structures of Sac7d (and E54 in the Sso7d NMR structure) does not accurately reflect the side chain position in solution.

Comparison to Previously Reported Sso7d pK_a s

The pK_a s obtained here for Sso7d differ from those previously reported.⁸⁶ As noted elsewhere,¹² pK_a s obtained from the pH dependence of carboxyl ^{13}C chemical shifts can differ substantially from those indicated by ^1H chemical shifts of the acid side chain protons. Differences often noted between the pK_a s obtained from geminal protons on the same residue clearly draw into question the reliability of the ^1H pK_a s. While in most cases the difference between the pK_a s reported here and those of Cosonni et al. can be attributed to differences in ^1H and ^{13}C data, in one case the difference cannot be easily explained, namely the unusually high pK_a reported for E36 (viz. 5.4). The assignments used here have been confirmed using multi-dimensional NMR spectra with triple-labeled protein and NMR backbone assignments.

Effect of salt on pK_a s

No dramatic differences were observed for the effect of salt on the pK_a s of the acidic side chains in Sso7d. All of the observed changes were very similar with an average ΔpK_a of 0.5 (± 0.07). This is consistent with the general observation that the pK_a s of carboxyl side chains increase with increasing salt concentration by about 0.3 to 0.5 pK units.^{46,48,82,87} The effect of salt on the pK_a s of titrating groups has been attributed to Debye-Huckel screening effects as well as stabilization of the charged form of the ionizing group by the salt.^{54,88,89} The magnitude and consistency of the change with salt indicates that the effect can be attributed to differences in stability of the charged side chain and not screening of electrostatic interactions.

pK_a s in the denatured state

The experimentally determined pK_a s of acid side chains in the native state along with the pH dependence of protein stability can be used to determine if the pK_a s of carboxyl groups in the

unfolded state are also perturbed relative to those of model compounds.^{11,46,48} The linkage of protonation to protein stability is defined by the following relationship for the pH dependence of protein stability^{2,48}:

$$\Delta\Delta G^\circ = RT \sum_i \ln \left(\frac{1 + 10^{(pH - pK_i^N)}}{1 + 10^{(7 - pK_i^N)}} \right) \left(\frac{1 + 10^{(7 - pK_i^D)}}{1 + 10^{(pH - pK_i^D)}} \right) \quad (1)$$

$\Delta\Delta G^\circ$ is the difference in free energy of folding at any pH relative to that at pH 7. The summation is over all titrating acid side chains, and pK_i^N and pK_i^D are the pK_a s of the titrating group in the native and denatured states, respectively. A plot of the predicted effect of pH on stability is shown in Figure 5 using the pK_a s reported here for Sso7d, along with model pK_a s of 4.0 for aspartate and 4.4 for glutamate for the denatured state. Comparison to the pH dependence of Sso7d stability indicates that there is no evidence to indicate that the pK_a s of the denatured state differ significantly from model compounds. Similar results are obtained for Sac7d. The denatured state pK_a s determine the pH at which the titration begins at high pH, and in both cases this occurs near pH 5.0. The deviation between the calculated and observed curves increases at lower pH because of the effect of chloride binding, which leads to an obvious increase in stability and folding below pH 2.0.^{64,65} This comparison demonstrates the importance of chloride binding below pH 4.0. A nonlinear least squares fitting of the stability data as a function of pH with a model that included linkage of chloride binding led to fitted values for the pK_a s in the unfolded state of 4.1 (± 0.1).⁶⁴ Since the most significantly perturbed side chains are two aspartates, this value is in good agreement with that expected for an exposed aspartate in the unfolded protein (the pK_a for a model aspartate is 4.0).

Cooperativity of folding and independence of linked titrating groups

It is interesting to note the apparent cooperativity of protein folding as a function of pH, yet the ionizations that are linked to folding occur independently. The folding of Sac7d decreases abruptly below pH 3, so that at pH 2 only 25% of the protein is folded (Figure 6A). The steep dependence of folding on pH requires the linkage of folding to at least two titrating groups.⁶⁵ The titration data presented here show that these groups titrate independently with Hill coefficients near unity.

The origin of this paradox can be clarified by comparing simulations of the effect of pH on stability with one and two titrating groups linked to folding. (Equations for the simulation are provided in the Appendix.) For the sake of clarity, simulations were performed with a pK_a of 1.0 in the native species, and 5 in the unfolded, with $K_{un} = 0.01$. The secondary effect of chloride binding that promotes refolding at lower pH was not included here since the emphasis is on the higher pH arm of the v-shaped stability curve (pH 2-4). Titrations of the single acidic group in the native and unfolded species are shown in Figure 6B, and the effect of pH on folding linked to this titration is shown in Figure 6C. The pH of the midpoint of the folding progress curve is dependent on the pK_a s of the native and unfolded species as well as the stability of the native protein. The folding curve does not reflect the titration of a single group. This is made clear by the pH dependence of ΔG of unfolding as defined by Equation A6, and shown by the upper curve (1) in Figure 7A when compared to the titration curves in Figure 6B. The sigmoidal drop in free energy with decreasing pH is identical to that defined by Eqn. 1 above.^{2,11,48} The curve approaches an upper limit near the pK_a of the acid group in the unfolded protein, and the lower limit (maximal destabilization) is attained near the pK_a of the titrating group in the native protein (Figure 7B).

We compare this to the effect of linkage of two independently titrating groups, with identical pK_a s (1.0 in the native species, and 5 in the unfolded protein), and $K_{un} = 0.01$. The groups are

assumed to titrate independently, and therefore the individual titration curves are identical to those observed for the case of a single titrating group linked to folding (Figure 6B). However, the pH dependence of folding shows a sharper transition than observed with a single titrating group (Figure 7B). The steepness of the pH dependence near the midpoint might be interpreted to indicate a cooperativity in proton binding, but this is not the case. The steepness of the dependence of folding on pH is simply due to the summation of two independent $\Delta\Delta G$ curves (Eqn. 1), and this leads to a steeper dependence of the free energy of unfolding on pH near the midpoint of the titration (Figure 6D).

Comparison of titration data to linkage model parameters

The titration data for Sac7d and Sso7d presented here define the specific residues that determine the acid pH dependence of folding for both proteins. We have previously fit the pH dependence of Sac7d and Sso7d stability data with a simple model which contained two acid groups with significantly perturbed pK_a s coupled to protein folding.^{64,65} The titration data demonstrate that there are three acid side chains in Sso7d with significantly perturbed pK_a s (D16, D35, and E54), with the two aspartates with the lowest pK_a s (D16 and D35) expected to have the largest effect on the pH dependence of stability. Two additional residues (E48 and D50 in Sso7d) show smaller perturbations that contribute to a smaller extent. The pK_a perturbations indicated by fitting the stability data to the minimal model were greater than any of those determined experimentally by NMR since the model was limited to the minimal number of groups required to fit the data. Simulation of the pH dependence of folding with the pK_a s of the two groups in the native protein set to 2.0 is shown in Figure 6A (red curve). The degree of unfolding at pH 2 is decreased from around 75% to 50% since the difference between the pK_a s in the folded and unfolded species is significantly less. An increase in the number of titrating groups linked to folding increases the steepness of the pH dependence of folding (Figure 7B). Thus the higher level of unfolding at pH 2 can occur by having a larger number of titrating groups linked to folding, without requiring unusually large perturbations in pK_a s.

Structural origin of linkage

The structural origin of the linkage of side chain ionizations to folding is largely the result of secondary structure formation and side chain-backbone hydrogen bonding: formation of α -helix and 3_{10} helices (D16, D50, E54 in Sso7d; D16, D50, and E53 in Sac7d), a turn (D35 in both Sso7d and Sac7d), and β -sheet (D36 in Sac7d). As noted by Forsyth and Robertson,⁹⁰ one of the surprising aspects of hydrogen bonds that lead to significant pK_a perturbations is their solvent exposure. However, it should be noted that the NH's of S18 and E53 (E54 in Sso7d) have reduced solvent exposure since they occur at the N-termini of helices. The C_{α} - C_{β} bond vector of these residues results in less than optimal interactions of the adjacent NHs with water. The positioning of side chains in the first turn of a helix is such that they preferentially form N-capping interactions with NHs.^{91,92} This may be one reason why the pK_a s of acid residues in the N-terminal turns of helices are typically less than the average in folded proteins.¹²

Ion pairing and the stability of hyperthermophile proteins

One of the most surprising aspect of this work is the relative lack of importance of ion pairs in stabilizing Sac7d and Sso7d, two highly charged hyperthermophile proteins. Molecular dynamics calculations indicated that electrostatic interactions, and in particular charge clusters, should play an important role in stabilizing Sac7d with increasing temperature.³⁸ These calculations however did not include the differences in pK_a s between folded and unfolded protein, and also the effect of hydrogen bonding on pK_a s. The ion pair interaction indicated to be important by the titrations described here (viz. E47 to R25 in Sac7d; and E48 to R25 in Sso7d)) was also noted by deBakker et al.³⁸ This interaction was shown to be destabilizing at

lower temperatures due to desolvation, but the calculations indicated that it should become less destabilizing (and possibly stabilizing) at higher temperature. The high stability of Sso7d and Sac7d should permit an experimental measure of the temperature dependence of this interaction.

Thus, although both of these highly charged hyperthermophile proteins contain a number of potential ion pair interactions that might be suspected to contribute to protein stability, the experimental data do not support this. These results demonstrate the dangers associated with attempting to use structural data to argue that an increase in putative ion pair interactions can explain increased stability in thermophile proteins.

MATERIALS AND METHODS

Materials

Inorganic salts and buffers were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant Sac7d and Sso7d were over-expressed in *E. coli* Rosetta (DE3)pLysS and purified by ion exchange chromatography as described elsewhere.^{64,93} ¹³C and ¹⁵N labeled proteins were obtained by expression in M9 minimal media supplemented with ¹⁵NH₄Cl (1 gm/L) and ¹³C₆-D-enriched glucose (4 gm/L) as the sole nitrogen and carbon sources. Protein concentrations were determined using an extinction coefficient at 280 nm of 1.1 mL mg⁻¹ cm⁻¹.⁹⁴

NMR

NMR spectra were collected on samples containing typically 2.5 mM protein in 0.7 ml 90% H₂O/10% D₂O at 30 °C using Varian INOVA 500 and 800 MHz NMR spectrometers. ¹H chemical shifts were referenced to internal DSS, and the ¹³C and ¹⁵N chemical shifts were calculated from the relative frequencies (Ξ)⁹⁵ of DSS (¹H, ¹³C) and liquid ammonia (¹⁵N). Backbone and side chain spectral peak assignments were made for Sso7d using ¹H, ¹⁵N HSQC⁹⁶, 3D NOESY-HSQC⁹⁷ and TOCSY-HSQC⁹⁸ spectra, and 3D HN(CO)CA,⁹⁹ HNCA,^{100,101} HNCACB,^{102,103} CBCA(CO)NH,¹⁰³ (H)CC(CO)NH-TOCSY,¹⁰⁴ HCCH-TOCSY,¹⁰⁵ and H(CC)(CO)NH-TOCSY¹⁰⁴ data. 3D COO(CGCBCA)(CO)NH spectra⁵³ were used to assign side-chain carboxyl carbons of the acidic amino acids of Sac7d and Sso7d based on the NH chemical shifts, and a 2D version correlating the carboxyl carbon to the NH proton of the next amino acid (¹³C_i-¹⁵N_{i+1}H) were used for titrations. Assignments have been deposited in the BioMagResBank (www.bmrb.wisc.edu; Sac7d : 5908; Sso7d: 5909).

Aspartate and glutamate side chain titrations were monitored using the carboxyl carbon chemical shifts of the side chains from 2D COO(CGCBCA)(CO)(N)H spectra.⁵³ Protein samples were typically 1 mM protein in 90% H₂O/10% D₂O at 30 °C. pH was adjusted with concentrated HCl and KOH and measured with a Radiometer glass electrode. Acid side chain titrations were also followed using side chain proton chemical shifts monitored with 2D ¹H, ¹H TOCSY spectra collected on samples in 90% H₂O/10% D₂O at 30 °C. ¹H, ¹H TOCSY spectra were also collected on Sac7d in 100% D₂O. The influence of titration of acid side chains on neighboring amide NH's was monitored by using ¹H, ¹⁵N HSQC spectra collected under the same conditions as used for the side chain titrations.

Apparent pK_as of titrating groups were obtained by nonlinear regression of the pH dependence of NMR chemical shifts to a modified Hill equation:

$$\delta_{obs} = \delta_{HA} \left(1 - \left(1 + 10^{n(pK_a - pH)} \right)^{-1} \right) + \delta_A \left(1 + 10^{n(pK_a - pH)} \right)^{-1} \quad (1)$$

where δ_{obs} is the observed chemical shift, δ_{HA} and δ_A are the shifts of the acid and anion forms of the side chain atoms, respectively, and n is the apparent Hill coefficient.⁹⁰ All data were fit using IGOR (Wavemetrics) and the errors in the fitted parameters were obtained from the covariance matrix with 95% confidence limits.

Carboxyl side chain pK_a s were predicted using the computer program PROPKA.⁸⁴ Calculations were performed using the coordinates for NMR and crystal structures of Sac7d and Sso7d (PDB ID codes are given in the Supplementary Material) and the web interface to the program at <http://propka.ki.ku.dk>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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APPENDIX

I. Linkage of a single titrating group to protein folding

The native protein, N, can unfold to U with an equilibrium constant K_{un} . The pK_a of a single titrating group is perturbed by folding such that in N the pK_a is $\log(K_n)$, where K_n is the association constant for proton binding to the carboxylate. In the unfolded state the pK_a of the group is $\log(K_u)$, where K_u is the proton association constant in the unfolded form.

The binding polynomial¹⁰⁶ as a function of pH is given by

$$Q(pH) = 1 + K_{un} + K_n \cdot [H] + K_{un} \cdot K_u \cdot [H] \quad A1.$$

and the pH dependence of the fractions of each of the species is:

$$\begin{aligned} f_N(pH) &= 1 / Q(pH) \\ f_{NH}(pH) &= K_n \cdot [H] / Q(pH) \\ f_U(pH) &= K_{un} / Q(pH) \\ f_{UH}(pH) &= K_{un} \cdot K_u \cdot [H] / Q(pH) \end{aligned} \quad A2.$$

Progress curves showing the dependence of folding (or unfolding) on pH can be obtained by summing the fractions of the two N (or U) species as follows:

$$\begin{aligned}\theta_N(pH) &= \frac{1 + K_n \cdot [H]}{Q(pH)} \\ \theta_U(pH) &= \frac{K_{un}(1 + K_u \cdot [H])}{Q(pH)}\end{aligned}\quad A3.$$

The titration curve of the side chain in the folded protein (as reflected by NMR chemical shifts) is given by the fraction of the native species in the protonated form:

$$\frac{f_{NH}(pH)}{f_N(pH) + f_{NH}(pH)} = \frac{K_n \cdot [H]}{1 + K_n \cdot [H]} \quad A4.$$

Finally, the pH dependence of protein stability can be found from the pH dependence of the effective equilibrium constant for unfolding, K_{un}^{eff} , given by:

$$K_{un}^{eff} = \frac{f_U(pH) + f_{UH}(pH)}{f_N(pH) + f_{NH}(pH)} \quad A5.$$

so that the free energy of unfolding as a function of pH is:

$$\Delta G_{un} = -RT \ln(K_{un}^{eff}) \quad A6.$$

II. Linkage of two titrating groups to the folding of a protein

The linkage of protein folding to the ionization of two titrating groups is described by the following scheme:

Two titrating groups have pK_a s of K_{1n} and K_{2n} in the native protein, and K_{1u} and K_{2u} in the unfolded species. We assume that the ionization state of one does not affect that of the other, i.e. they titrate independently. The groups can be protonated in either order to give NH or NH' in the native protein (UH and UH' in the unfolded), and then NHH' for the doubly protonated species (UHH' in the unfolded). The binding polynomial is given by:

$$\begin{aligned}Q(pH) &= 1 + K_{1n} \cdot [H] + K_{2n} \cdot [H] + K_{1n}K_{2n} \cdot [H]^2 + K_{un}(1 + K_{1u} \cdot [H] + K_{2u} \cdot [H] + K_{1u}K_{2u} \cdot [H]^2) \\ &= (1 + K_{1n} \cdot [H])(1 + K_{2n} \cdot [H]) + K_{un}(1 + K_{1u} \cdot [H])(1 + K_{2u} \cdot [H])\end{aligned}\quad A7.$$

and the pH dependence of the fractions of each species is:

$$\begin{aligned}
f_N(pH) &= 1 / Q(pH) \\
f_{NH}(pH) &= K_{1n} \cdot [H] / Q(pH) \\
f_{NH}'(pH) &= K_{2n} \cdot [H] / Q(pH) \\
f_{NHH}'(pH) &= K_{1n}K_{2n} \cdot [H]^2 / Q(pH) \\
f_U(pH) &= K_{un} / Q(pH) \\
f_{UH}(pH) &= K_{un}K_{1u} \cdot [H] / Q(pH) \\
f_{UH}'(pH) &= K_{un}K_{2u} \cdot [H] / Q(pH) \\
f_{UHH}'(pH) &= K_{un}K_{1u}K_{2u} \cdot [H]^2 / Q(pH)
\end{aligned} \tag{A8}$$

Progress curves showing the dependence of folding (or unfolding) on pH can be obtained from summing the fractions of the two N (or U) species:

$$\begin{aligned}
\theta_N(pH) &= \frac{1 + K_{n1} \cdot [H] + K_{n2} \cdot [H] + K_{n1}K_{n2} \cdot [H]^2}{Q(pH)} \\
\theta_U(pH) &= \frac{K_{un}(1 + K_{u1} \cdot [H] + K_{u2} \cdot [H] + K_{u1}K_{u2} \cdot [H]^2)}{Q(pH)}
\end{aligned} \tag{A9}$$

The titration curve of one of the side chains in the folded protein (as reflected by NMR chemical shifts) is given by the fraction of the native species in the protonated form:

$$\frac{f_{NH}(pH) + f_{NHH}'(pH)}{f_N(pH) + f_{NH}(pH) + f_{NH}'(pH) + f_{NHH}'(pH)} = \frac{K_{n1} \cdot [H] + K_{n1}K_{n2}[H]^2}{1 + K_{n1} \cdot [H] + K_{n2} \cdot [H] + K_{n1}K_{n2} \cdot [H]^2} \tag{A10}$$

Finally, the pH dependence of protein stability can be found from the pH dependence of the effective equilibrium constant for unfolding, K_{un}^{eff} , given by:

$$K_{un}^{eff} = \frac{f_U(pH) + f_{UH}(pH) + f_{UH}'(pH) + f_{UHH}'(pH)}{f_N(pH) + f_{NH}(pH) + f_{NH}'(pH) + f_{NHH}'(pH)} \tag{A11}$$

and the free energy of unfolding as a function of pH is $\Delta G_{un} = -RT \ln(K_{un}^{eff})$.

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Figure 1. Structures of Sac7d and Sso7d from crystal structures of protein:DNA complexes (Sac7d from 1azp, and Sso7d from 1bnz). For clarity, the DNA is not shown. The DNA binding surface is on the backside of the view shown here.^{107,108} Aspartate and glutamate side chains are shown in red, and lysine and arginines are shown in dark blue and light blue, respectively. The amino acid sequences of Sac7d and Sso7d are given at the bottom, with differences highlighted in red. Note that the insertion of G39 in Sso7d increases the numbering of Sso7d by one from G39 to the C-terminus.

Figure 2. pH titrations of the three aspartates (blue) and representative glutamate (red) side chains in Sso7d followed by ^{13}C NMR chemical shifts. Carboxyl carbon chemical shifts were obtained from 2D NMR spectra that correlated the carbonyl chemical shift to the NH of the following amino acid as described by Tollinger et al.⁵³ Smooth curves through the data are the result of nonlinear regression to obtain the pK_a s and apparent Hill coefficients reported in Table 1.

Figure 3.

Representative data showing the pH titration of acid side chains in Sso7d followed by ^1H NMR chemical shifts obtained from 2D ^1H , ^1H TOCSY spectra. The pH dependence of ^1H chemical shifts of geminal C_βH_2 protons of E48 and E60 are shown to demonstrate the differences in the magnitude of the chemical shift changes, Hill coefficients, and pK_a s observed for neighboring protons. The smooth curves through the data are the result of nonlinear least squares fitting to obtain the pK_a s and associated errors reported in Table 1.

Figure 4. pH dependence of chemical shifts of backbone amide NHs of Sso7d obtained from $^1\text{H}, ^{15}\text{N}$ HSQC spectra. NH's that shift upfield with decreasing pH due to hydrogen bonding to a titrating acidic side chain are shown in red, while those shifting in the opposite direction due to through bond and through space electrostatic interactions are shown in blue. Smooth curves through the data are the result of nonlinear regression to obtain the apparent pK_a s reported in Table 1.

Figure 5.

Comparison of the pH dependence of Sso7d stability determined experimentally (red) with that calculated (blue) using Equation 1 with the pK_a s reported here for the native protein, and model pK_a s for the titrating acid side chains in the denatured state (i.e. 4.0 for aspartate, and 4.4 for glutamate). The calculated stability curve has been displaced vertically by adding 7.14 kcal/mol so that the curves coincide at pH 7.

Figure 6.

Comparison of the pH dependence of the folding of Sac7d and Sso7d to simulations of the titration behavior of one and two titrating groups. A) The experimentally observed pH dependence of the folding of Sac7d and Sso7d as indicated by circular dichroism data at 25 ° C in the absence of salt (data from Clark et al.⁶⁴). The lower intrinsic stability of Sac7d makes it possible to observe the steep dependence of folding on pH below pH 3. The smooth curves (black) through the data are those obtained from fitting the data to a minimal linkage model with two protonation and two chloride binding reactions linked to folding. The effect of increasing the pK_a s of the two titrating groups in the native protein from the fitted value of 0.1 to 2.0 is shown by the red curve. B) Simulated titration curves for a single acid side chain in the native (blue, $pK_a = 1$) and unfolded (red, $pK_a = 5$) protein. C) Simulated dependence of protein folding (native in blue; unfolded in red) on pH due to linkage of folding to the ionization of a single residue with the pK_a s indicated in panel B. D) Simulated dependence of protein folding (native in blue; unfolded in red) on pH due to linkage of folding to the ionization of two residues with pK_a s of 1 and 5 in the native and unfolded protein, respectively.

Figure 7.

Effect of varying pK_a s and increasing the number of titrating groups on the linkage of pH to protein folding. A) The effect of varying the difference in pK_a s for a single titrating group with different pK_a s in the native and unfolded protein. The pK_a in the unfolded protein is fixed at 5, while the pK_a in the native is varied from 1 to 4. Note that the slope of the curve does not change, only the beginning and end points shift. B) Simulated dependence of the free energy of unfolding on pH with one (1) and two (2) independently titrating groups linked to folding with pK_a s as indicated in Figure 6C and D, and an intrinsic unfolding equilibrium constant at high pH of 0.01.

Table 1

Titration parameters for Sso7d in the absence of KCl.

Residue	$^{13}\text{C}=\text{O}^a$	Δp^b	n^c	$-\text{C}^1\text{H}_2^d$		$^{15}\text{NH} (\Delta\delta)^e$
				H1	H2	
E11	3.78 ^f	-0.6	0.87	3.90	4.03	2.90 (+0.07)
E12	3.90	-0.5	0.87	3.86	4.19	-
D16	2.11	-1.9	0.94	2.17	2.18	2.00 (+0.12)
S18	-	-	-	-	-	2.05 (-0.74)
D35	2.16	-1.8	0.97	2.10	2.19	-
E36	4.33	-0.1	0.87	3.97	4.62	2.66 (-0.06)
G37	-	-	-	-	-	1.93 (-0.40)
G39	-	-	-	-	-	2.01 (-0.58)
S47	-	-	-	-	-	2.77 (-0.25)
E48	3.45	-1.0	0.91	3.69	3.88	-
D50	2.96	-1.0	0.93	3.19	3.23	2.67 (+0.15)
E54	3.01	-1.4	0.90	3.85	-	3.06 (-1.05)
E60	3.82	-0.6	0.90	3.77	4.18	-

^a Apparent acid side chain pK_{as} determined from the ^{13}C NMR chemical shifts of titrating carboxyl carbons. Values in bold indicate the most significantly perturbed pK_{as} .

^b Difference between the pK_{a} measured by ^{13}C NMR chemical shifts of the titrating carboxyl carbon and the pK_{a} expected for a solvent exposed side chain ($\text{pK}_{\text{a}}^{\text{obs}} - \text{pK}_{\text{a}}^{\text{avg}}$). The average pK_{a} for solvent exposed aspartate is 4.0, and that for glutamate is 4.4.¹²

^c Hill coefficient from ^{13}C NMR chemical shifts.

^d Apparent pK_{as} of titrating side chains from the pH dependence of chemical shifts of geminal ^1H 's adjacent to the titrating carboxyl.

^e Apparent pK_{as} for selected NH 's that had significant pH dependent chemical shifts. Numbers in parentheses are changes in chemical shift with pH ($\delta_{\text{acid}} - \delta_{\text{base}}$).

^f Errors in pK_{as} from fitting the ^{13}C NMR data to the Henderson-Hasselbalch equation were less than ± 0.01 (1 standard deviation). Errors in the associated n values were less than ± 0.04 . Errors in pK_{as} from the geminal H 's adjacent to the titrating carboxyl were typically less than ± 0.1 . Errors in pK_{as} reflected in the NH shifts were less than 0.03.

Table II

Titration parameters for Sso7d in 0.3 M KCl

Residue	¹³ C=O ^a	Δp□	Δ□□□ ^b	n	-C ¹ H ₂ -		-15NH (Δδ)
					H1	H2	
E11	4.17	-0.2	0.39	0.89	-	-	3.54 (+0.13)
E12	4.33	-0.1	0.43	0.89	-	-	-
D16	2.62	-1.4	0.51	0.89	2.45	2.50	2.72 (+0.14)
S18	-	-	-	-	-	-	2.70 (-0.70)
D35	2.67	-1.3	0.51	0.89	2.54	2.65	-
E36	4.89	+0.5	0.56	0.84	3.98	4.85	3.83 (-0.06)
G37	-	-	-	-	-	-	2.62 (-0.37)
G39	-	-	-	-	-	-	2.73 (-0.54)
S47	-	-	-	-	-	-	3.57 (-0.23)
E48	4.03	-0.4	0.58	0.90	4.00	4.23	-
D50	3.55	-0.4	0.59	0.90	3.49	3.62	3.47 (-0.23)
E54	3.56	-0.8	0.55	0.91	3.36	4.24	3.55 (-0.99)
E60	4.24	-0.2	0.42	0.91	4.36	4.42	-

^a See footnotes to Table I for explanation of column labels (except for column 4) and confidence limits.

^b Difference between the pK_as determined from the ¹³C NMR chemical shifts of the titrating carboxyl carbon measured in 0.3 M KCl and in 0.0 M KCl (pK_a^{0.3} - pK_a^{0.9})

Table III

Titration parameters for Sac7d in 0.3 M KCl

Residue	¹³ C=O ^a	ΔpK	n	-C ¹ H ₂ -		¹⁵ NH (Δδ)
				H1	H2	
E11	4.19	-0.2	0.92	4.17	4.36	3.97 (+0.09)
E12	4.41	0	0.83	4.28	4.46	-
E14	4.00	-0.4	0.75	4.18	4.26	-
D16	2.89	-1.1	0.81	3.00	3.06	2.54 (+0.25)
S18	-	-	-	-	-	2.96 (-0.92)
D35	3.42	-0.6	0.92	3.36	3.45	2.97 (+0.06)
D36	3.12	-0.9	0.89	3.39	3.50	3.50 (+0.07)
N37	-	-	-	-	-	3.15 (+0.54)
G38	-	-	-	-	-	3.41 (-0.70)
G41	-	-	-	-	-	3.66 (-0.22)
S46	-	-	-	-	-	3.46 (-0.22)
E47	4.21	-0.2	0.90	4.30	4.31	-
D49	3.55	-0.4	0.93	3.59	3.60	3.07 (+0.11)
E53	3.53	-0.9	0.87	3.56	3.70	3.62 (-0.79)
D56	3.35	-0.7	0.90	3.43	3.46	3.56 (-0.24)
E62	3.99	-0.4	0.88	4.03	4.18	4.10 (-0.12)
E64	4.23	-0.2	0.92	4.26	4.27	2.94 (-0.23)
K66	-	-	-	-	-	3.22 (+0.43)

^a See footnotes to Table I for information on column entries and confidence limits.