pH, Ionic Strength, and Temperature Dependences of Ionization Equilibria for the Carboxyl Groups in Turkey Ovomucoid Third Domain†

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ABSTRACT: Two-dimensional NMR spectroscopy has been used to monitor the pH dependences of proton chemical shifts for turkey ovomucoid third domain (OMTKY3). Sample pH was varied from 7.0 to 1.4 in order to determine the apparent pK_a values of all six carboxyl groups in OMTKY3. At 35 °C and in the presence of 10 mM KCl, the pK_a values for Asp 7, Glu 19, and Asp 27 (<2.6, 3.2, and <2.3, respectively) are more than 1 pH unit below those for model compounds. The pK_a values for Glu 10 (4.1) and Glu 43 (4.7) show more modest deviations from model compound data. The low pK_a for the α-carboxyl group of Cys 56 (<2.5) is attributable, at least in part, to acidification by the disulfide group. Fitting the data to a modified Hill equation [Markley, J. L. (1975) *Acc. Chem. Res. 8,* 70−80] reveals little evidence for interactions between the acidic groups; most Hill coefficients fall between 0.8 and 1.2, with outlying values usually obtained with data that describe incomplete transitions. Most of the very low pK_a values show increases in the presence of 1.0 M KCl but, with the exception of that for glutamate 19, remain well below model compound values. pH-dependent changes in amide proton chemical shifts permitted identification of hydrogen bonds involving the side chains of Asp 7, Glu 19, and Asp 27, which may partially explain the low pK_a values for these groups. These hydrogen bonds, two of which involve side chains that are well exposed to solvent, were previously identified in high-resolution X-ray studies of turkey ovomucoid third domain [Fujinaga, M., Sielecki, A. R., Read, R. J., Ardelt, W., Laskowski, M., Jr., & James, M. N. G. (1987) *J. Mol. Biol. 195,* 397−418]. Results of additional experiments performed at 15, 25, and 40 °C suggest that apparent ionization enthalpies for all carboxyl groups in OMTKY3 are about 0 ± 2 kcal/mol. In the accompanying paper [Swint, L., & Robertson, A. D. (1995) *Biochemistry 34,* 4724−4732], the pH dependence of OMTKY3 stability is described and compared to expectations based on the pK_a values described herein.

The identity of the major forces in protein folding has been known for over 30 years (Kauzmann, 1959). During this time, much discussion and experiment has been devoted to a more detailed understanding of the relative magnitudes of these forces and their manifestation at the level of individual amino acid residues (Matthews, 1987; Dill, 1990; Goldenberg, 1992; Pace, 1992; Shirley et al., 1992; Fersht & Serrano, 1993). To this end, much effort has been directed at understanding electrostatic interactions in proteins (Tanford, 1962; Matthew et al., 1985; Warshel & Åqvist, 1991; Yang & Honig, 1992). Particular interest in electrostatics stems from its importance in binding, catalysis, and protein stability and from the unique opportunity to experimentally quantify a major fundamental force in protein structure and function without recourse to chemical modification or amino acid substitutions: electrostatic interactions between ionizing groups in proteins can, in principle, be assessed through variation of pH and ionic strength (Yang & Honig, 1992). Moreover, by virtue of their relatively simple physics, electrostatic interactions are more amenable to cycles of experiment and calculation than other forces in protein folding (Allewell & Oberoi, 1991), and indeed, electrostatics was the first of the principal noncovalent forces to be treated with theory (Linderstrøm-Lang, 1924).

Much attention over the years has been focused on calculation of electrostatic interactions in proteins (Linderstrøm-Lang, 1924; Kirkwood, 1934; Tanford & Kirkwood, 1957; Matthew et al., 1985; Sharp & Honig, 1990; Warshel & Åqvist, 1991). Comprehensive exprimental tests of the models are, however, limited to a few proteins in which the ionization behavior of a large fraction of the ionizing groups has been characterized under a consistent set of solution conditions [March et al., 1982; Kohda et al., 1991; Bartik et al., 1994; Oda et al., 1994; Szyperski et al., 1994; see Antosiewicz et al. (1994) for a summary of many experimental results]. The present study of ovomucoid third domain is prompted by the need for additional experimental data for proteins and by our aim to describe quantitatively the contributions of specific residues to the stability of ovomucoid third domain (Swint & Robertson, 1993, 1995).

Ovomucoid third domains are small (51−56 residues) serine proteinase inhibitors which are proving to be a fruitful system for the study of protein stability and structure (Swint & Robertson, 1993). Sequences from over 150 avian species have been determined, and approximately 400 sequences of homologous Kazal-type proteinase inhibitors are known as well (Laskowski et al., 1987, 1990; Apostol et al., 1993). Structures for a large number of third domains and homologous proteins have been determined by X-ray crystallography

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or NMR[1] spectroscopy (Bolognesi et al., 1982; Papamakos et al., 1982; Read et al., 1983; Bode et al., 1985; Williamson et al., 1985; Fujinaga et al., 1987; Hecht et al., 1991; Klaus & Schomburg, 1993; Liepinsh et al., 1994; Krezel et al., 1994). In addition, proton and carbon NMR assignments have been made for the turkey ovomucoid third domain (OMTKY3; Robertson et al., 1988, 1990), which is the subject of the present studies.

OMTKY3 is a stable monomeric protein at and above pH 1.4 (Swint & Robertson, 1993, 1995). Consequently, ionization equilibria can be monitored over a wide pH range, facilitating accurate measurement of pK_a values for the acidic groups in the native protein. These pK_a values provide new information about the solution conformation and inhibitory properties of ovomucoid third domains. Moreover, the free energy of unfolding, ΔG_u, is known for OMTKY3 over the range of pH values encompassed by the present study (Swint & Robertson, 1993, 1995). We are thus in a position to test models that describe (1) electrostatic interactions in native proteins and (2) the electrostatic contribution to ΔG_u (Swint & Robertson, 1995).

MATERIALS AND METHODS

Materials. Domestic turkey (*Maleagris gallopavo*) eggs were a gift from Theis Farms (New Haven, IA). Turkey ovomucoid third domain was purified as described previously (Bogard et al., 1980; Swint & Robertson, 1993). Deuterium oxide (99.9 atom %) and sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (99.8 atom %) were obtained from Cambridge Isotope Laboratories (Cambridge, MA). pH 4.00 and 7.00 standard solutions for calibration of pH electrodes were from VWR Scientific (West Chester, PA), and the pH 2.00 standard was from Fisher Scientific.

NMR Spectroscopy. 500-MHz NOESY and double quantum filtered (DQF) COSY data were obtained in phase-sensitive mode (Aue et al., 1976; Maçura & Ernst, 1980; States et al., 1982). Data were acquired on a Varian UNITY 500 spectrometer located in the College of Medicine at the University of Iowa and equipped with an ID500 probe from Nalorac Cryogenics Corporation (Martinez, CA). The spectrometer's variable temperature controller was calibrated using a Model BAT-12 digital thermometer from Physitemp Instruments (Clifton, NJ) equipped with a flexible thermocouple, which was immersed in 0.5 ml of water in a 5-mm NMR tube and lowered into the probe for temperature measurements. The carrier frequency was set on the water signal, the spectral width was 6000 Hz, and each transient consisted of 2048 time-domain data points. 2D data were acquired as 256−512 blocks, each of which consisted of 4−16 summed transients. The mixing time for the NOESY experiments was 150 ms. Solvent suppression was achieved by continuous-wave irradiation of the solvent resonance during the 1.0−1.8-s relaxation delay. All samples contained 0.5 ml of 5 mM OMTKY3.

NMR data were processed using either FELIX (version 2.00, Hare Research, Inc., Bothell, WA) installed on a Silicon Graphics 4D-35 Personal Iris workstation or Varian software installed on a SparcStation 2 workstation from Sun Microsystems. Data were zero-filled to 4096 × 4096 data points prior to apodization and Fourier transformation. DQF-COSY and NOESY data were apodized with unshifted sine bells and $2\pi/5$-shifted sine bells in both dimensions, respectively. The final digital resolution was 1.46 Hz/point in both dimensions, and estimated uncertainties in chemical shifts are 0.02 ppm in ω_2 and 0.05 ppm in ω_1.

pH Measurements. pH was measured with an Orion Research Model 611 pH meter equipped with a 3 mm o.d. Ingold combination electrode (Wilmad Glass Co., Buena, NJ). All calibrations and measurements were performed by first equilibrating the standards, samples, and electrode at the desired temperature in a VWR Model 1130 constant-temperature water bath. Two-point calibrations of the pH meter were performed using either pH 7.00 and 4.00 or pH 4.00 and 2.00 standard solutions; appropriate corrections were made for temperature dependences of the standard solutions. The estimated uncertainty in measurements of pH is 0.02 pH unit.

pH Dependences of Proton Chemical Shifts. Stock solutions consisting of approximately 5 mM OMTKY3 and either 10 mM or 1.0 M KCl were prepared by dissolving 75 mg of lyophilized protein in 2.5 ml of 90% H_2O/10% D_2O (v/v) containing the desired salt concentration and 0.5 mM TSP as an internal chemical shift standard. The final step in purification of OMTKY3 is gel-filtration chromatography in a 15 mM ammonium bicarbonate buffer; we assume that, in the absence of added salts, solutions prepared from lyophilized protein are isoionic. Ionic strength thus varies from 0.01 at neutral pH to about 0.05 at pH 1.4 in samples containing 10 mM KCl, while solutions containing 1.0 M KCl undergo negligible changes in ionic strength over the same range of pH.

Solutions containing freshly dissolved OMTKY3 were pH 7.3 to 7.6, close to the p*I* for this protein (Swint & Robertson, 1994). For the first NMR measurement, solutions were brought to pH 7.00 by addition of concentrated HCl. Subsequent pH adjustments were made with small volumes of concentrated HCl or KOH. For each measurement, the protein stock solution was first brought to within 0.03 pH unit of the desired pH and then used to rinse the NMR tube thoroughly. The rinse solution was returned to the stock solution, and the pH was measured again. If the pH was within 0.03 unit of the desired value, 0.5 ml of the stock was then transferred to the NMR tube and a spectrum was acquired. Otherwise, the pH was readjusted and the rinse process repeated. DQF-COSY data were acquired at 0.5 pH unit increments between pH 7.0 and 6.0 and at 0.25 pH unit increments between pH 6.0 and 1.5. The reversibility of the titrations was assessed by returning acidified samples used in the initial measurements to pH 7.0 in 1 pH unit increments, acquiring DQF-COSY data at each increment. Chemical shifts from these experiments were superimposable on those measured during acidification of the protein.

All chemical shift data have been corrected for the pH dependence of the TSP chemical shift using eq 1 (DeMarco, 1977; Bundi & Wüthrich, 1979b).

$$\delta_{corr} = \delta_{obs} - 0.019[1 + 10^{(5-pH)}]^{-1} \quad (1)$$

The FELIX software was used to construct databases of assigned peaks which were monitored as a function of pH.

[1] Abbreviations: $C_\alpha H$, $C_\beta H$, $C_\gamma H$, and $C_\epsilon H$, protons attached to the α-, β-, γ-, and ϵ-carbons, respectively, of an amino acid side chain; COSY, correlation spectroscopy; DQF-COSY, double quantum filtered correlation spectroscopy; NH, peptide amide proton; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; OMTKY3, turkey ovomucoid third domain; pH*, pH of D_2O solutions reported without correction for isotope effects; TSP, sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4.

Data files were transferred to Apple Macintosh IIsi computers for graphical display and curve fitting. Data were fit to both the Henderson−Hasselbalch equation (eq 2) and a modified Hill equation (equation 3; Markley, 1975) to determine an apparent ionization constant, pK:

$$\delta_{obs} = \delta_{A^-}[1 + 10^{(pK-pH)}]^{-1} + \delta_{AH}\{1 - [1 + 10^{(pK-pH)}]^{-1}\} \quad (2)$$

$$\delta_{obs} = \delta_{A^-}[1 + 10^{n(pK-pH)}]^{-1} + \delta_{AH}\{1 - [1 + 10^{n(pK-pH)}]^{-1}\} \quad (3)$$

where δ_{obs} is the observed chemical shift; δ_{A^-} and δ_{AH} are the chemical shifts of the unprotonated and protonated species, respectively; and n is the Hill coefficient. Nonlinear least squares fitting was performed using a version of the NonLin program (Johnson & Frasier, 1985; Johnson & Faunt, 1992) that has been modified by Robert J. Brenstein to run on Apple Macintosh computers (Robelko Software, Carbondale, IL).

RESULTS

The salt concentrations used in these studies differ from that used to assign the ^{1}H-NMR spectrum of OMTKY3 (0.2 M KCl; Robertson et al., 1988), so DQF-COSY and NOESY data were used to reassign the spectrum at pH 4.0 in 10 mM KCl. Only minor differences from the original assignments were observed (data not shown), and the differences are consistent with the pH and ionic strength dependences described below. The choice of 10 mM added KCl for the low ionic strength experiments was a compromise dictated by (1) our desire to observe the largest possible perturbations of pK_a values in native OMTKY3, (2) the need to minimize or control for the effect of varying ionic strength on the measured pK_a values, and (3) practical constraints in preparing NMR samples. In principle, constant ionic strength could be achieved by preparing separate NMR samples for each pH, with the balance of added salt and titrant kept constant at a value determined by the lowest pH. In practice, this would require approximately 5 times the amount of protein used in the present study. Solutions containing 10 mM KCl will undergo an approximately 2-fold change in ionic strength when adjusted to pH 2 and about a 5-fold change when the pH is reduced to 1.4. Unfortunately, increasing ionic strength will sharpen transitions broadened by interactions between ionizing residues (Edsall & Wyman, 1958), which is one of the phenomena we would like to detect. Nevertheless, working with a low concentration of added salt increases the likelihood of observing pK_a values that differ significantly from those measured in model compounds.

OMTKY3 contains six carboxyl groups that are expected to titrate over the pH range employed in this study (Figure 1). The single histidine at position 52 of both the turkey and the chicken ovomucoid third domain has been the subject of previous NMR investigation (Ogino et al., 1982; Ortiz-Polo, 1985). The present study focuses on two types of proton resonances: those of aliphatic protons on ionizing side chains and those of backbone amide protons (NHs). In small peptides, C$_\beta$H and C$_\gamma$H resonances of aspartate and glutamate, respectively, shift upfield by approximately 0.2−0.3 ppm upon deprotonation of the carboxyl group (Bundi & Wüthrich, 1979a). NH resonances of acidic residues

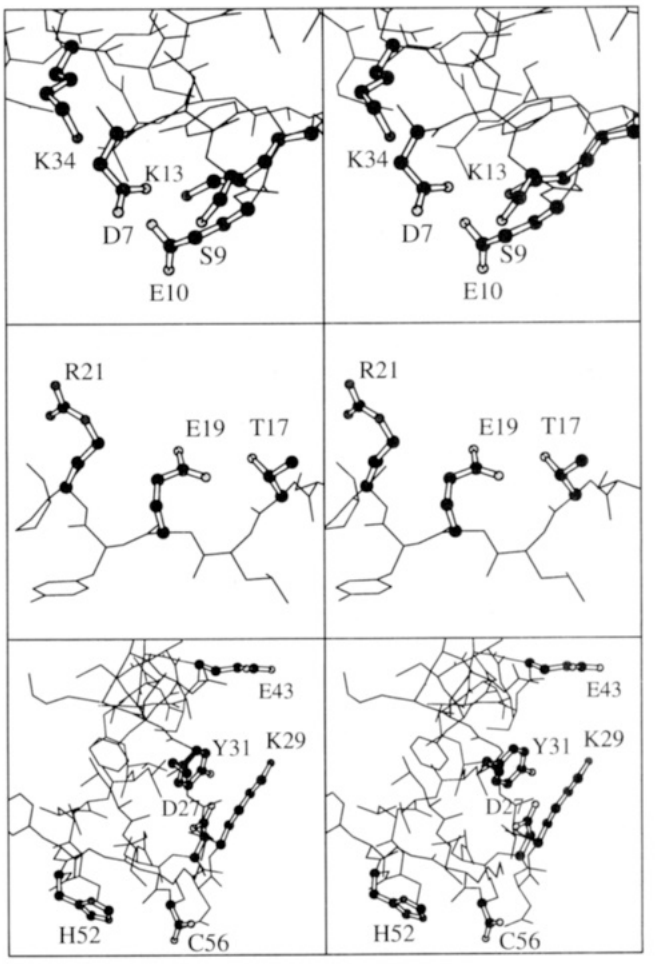

FIGURE 1: Stereo diagrams of turkey ovomucoid third domain, derived from the structure of a complex with *Staphylococcus griseus* protease B (file 3SGB in the Protein Data Bank; Read et al., 1983) generated with Molscript (Kraulis, 1991). Amino acids are labeled by sequence number and the one-letter code for amino acids. The ball-and-stick representation is used for residues discussed in the text, and the remainder of the structure is represented by thin lines. The three sets of panels illustrate the environment of (top) Asp 7 and Glu 10, (middle) Glu 19, and (bottom) Asp 27, Glu 43, and Cys 56. Side chains of cationic and hydrogen bond donor residues within 9 and 3.1 Å, respectively, of the carboxyl groups are also highlighted and labeled.

generally show small through-bond or inductive effects as side chains ionize, but chemical shifts change substantially when an NH is hydrogen bonded to an ionizing group (Bundi & Wüthrich, 1977, 1979b; Mayer et al., 1979; Ebina & Wüthrich, 1984; Szyperski et al., 1994). The pH dependence of more than 200 resonances in OMTKY3 was monitored from pH 7.0 to 1.4, and approximately 50 resonances showed chemical shift changes ≥0.1 ppm (Schaller, 1993). Of the 12 resonances with chemical shift changes ≥0.2 ppm, six arise from protons residing in the side chains of ionizing residues (Table 1), while another six are backbone NH resonances (Table 2). Only two additional NH resonances show chemical shift changes ≥0.1 ppm (Table 2).

Aliphatic Proton Resonances. Cross peaks involving the C$_\beta$H resonances of Asp 27 and the carboxy-terminal Cys 56 are readily resolved over the entire pH range employed in this study (Figure 2), as is the C$_\alpha$H/C$_\beta$H cross peak for Cys 56. The C$_\beta$H/C$_\beta$H′ cross peak of Asp 7 overlaps with other cross peaks at low pH (Figure 2a). pK_a values for Asp 7 are determined with greater confidence from the C$_\beta$H′ resonance, the better resolved of the methylene resonances. The C$_\beta$H/C$_\gamma$H cross peaks for Glu 10 and Glu 43 are well

Table 1: pH Dependence of OMTKY3 Aliphatic Proton Chemical Shifts in 10 mM KCl[a]

amino acid	T (°C)	proton	δ_{A^-} (ppm)	$\Delta\delta^b$ (ppm)	pH_{mid}[c]
Asp 7	15	$C_\beta H'$	2.96	0.26	2.63(0.07)
	25		2.95	0.24	2.67(0.06)
	35		2.94	0.26	2.54(0.17)
	40		2.93	0.25	2.72(0.09)
Glu 10	15	$C_\gamma H$	2.09	0.29	4.45(0.15)
	25		2.15	0.23	4.14(0.06)
	35		2.15	0.23	4.04(0.12)
	40		2.13	0.25	4.09(0.05)
Asp 27	15	$C_\beta H$	2.56	0.41	2.22(0.06)
	25		2.56	0.39	2.30(0.06)
	35		2.56	0.41	2.32(0.05)
	40		2.55	0.43	2.33(0.08)
	15	$C_\beta H'$	2.99	0.46	2.19(0.05)
	25		2.99	0.43	2.27(0.05)
	35		2.98	0.48	2.25(0.08)
	40		2.98	0.48	2.33(0.05)
Glu 43	15	$C_\gamma H$	2.42	0.20	4.68(0.09)
	25		2.41	0.20	4.81(0.10)
	35		2.46	0.16	4.42(0.12)
	40		2.42	0.19	4.78(0.10)
Cys 56 α	35	$C_\alpha H$	4.38	0.18	2.29(0.12)
	40		4.41	0.21	2.74(0.11)
	15	$C_\beta H$	3.14	0.19	2.22(0.11)
	25		3.14	0.19	2.23(0.10)
	35		3.14	0.16	2.45(0.19)
	40		3.14	0.18	2.34(0.14)

[a] The parameters δ_{A^-}, δ_{AH}, and pH_{mid} were obtained from fits to eq 2. The fitting errors for δ_{A^-} and δ_{AH} are all less than or equal to the 0.02 ppm uncertainty in the experimental measure of chemical shift. [b] $\Delta\delta = \delta_{AH} - \delta_{A^-}$; a positive value of $\Delta\delta$ means a downfield shift upon protonation. [c] Numbers in parentheses are fitting errors at 1 SD.

Table 2: pH Dependence of OMTKY3 Amide Proton Chemical Shifts in 10 mM KCl[a]

amino acid	T (°C)	δ_{A^-} (ppm)	$\Delta\delta^b$ (ppm)	$\Delta\delta(T)^c$	pH_{mid}[d]
Ser 9	15	8.78	−0.31		3.20(0.22)
	25	8.74	−0.39		2.99(0.24)
	35	8.69	−0.42		3.13(0.12)
	40	8.67	−0.41	−4.5, −8.6	3.09(0.12)
Glu 10	15	8.92	−0.20		4.07(0.08)
	25	8.85	−0.20		4.07(0.07)
	35	8.76	−0.19		4.11(0.13)
	40	8.72	−0.19	−8.1, −7.6	4.12(0.07)
Thr 17	15	8.20	0.25		3.27(0.06)
	25	8.14	0.24		3.25(0.06)
	35	8.07	0.25		3.21(0.06)
	40	8.04	0.36	−6.5, −6.5	2.94(0.12)
Glu 19	15	8.30	−0.66		3.21(0.03)
	25	8.26	−0.63		3.21(0.02)
	35	8.22	−0.62		3.21(0.02)
	40	8.20	−0.61	−4.0, −2.1	3.27(0.03)
Asp 27	15	8.40	−0.85		2.19(0.03)
	25	8.41	−0.85		2.25(0.03)
	35	8.44	−0.92		2.27(0.02)
	40	8.45	−0.93	2.1, −1.5	2.25(0.09)
Lys 29	15	7.84	−0.92		2.20(0.03)
	25	7.83	−0.90		2.27(0.02)
	35	7.81	−0.94		2.29(0.02)
	40	7.81	−0.99	−1.3, −4.0	2.29(0.04)
Leu 48	40	7.69	−0.10		4.60(0.17)
Lys 55	40	7.85	0.14		2.54(0.29)

[a] δ_{A^-}, δ_{AH}, and pH_{mid} were obtained from fits to eq 2. Fitting errors for δ_{A^-} and δ_{AH} are all ≤0.02 ppm. [b] $\Delta\delta = \delta_{AH} - \delta_{A^-}$; a positive value of $\Delta\delta$ means a downfield shift upon protonation. [c] Thermal coefficients (ppb/°C) for the fitted NH chemical shifts, δ_{A^-} and δ_{AH}, respectively. [d] Numbers in parentheses are fitting errors at 1 SD.

resolved in all DQF-COSY spectra, but the Glu 19 cross peak overlaps with many other cross peaks.

All of the resolved resonances for the aspartate and glutamate residues shift upfield, by about 0.2−0.4 ppm, with

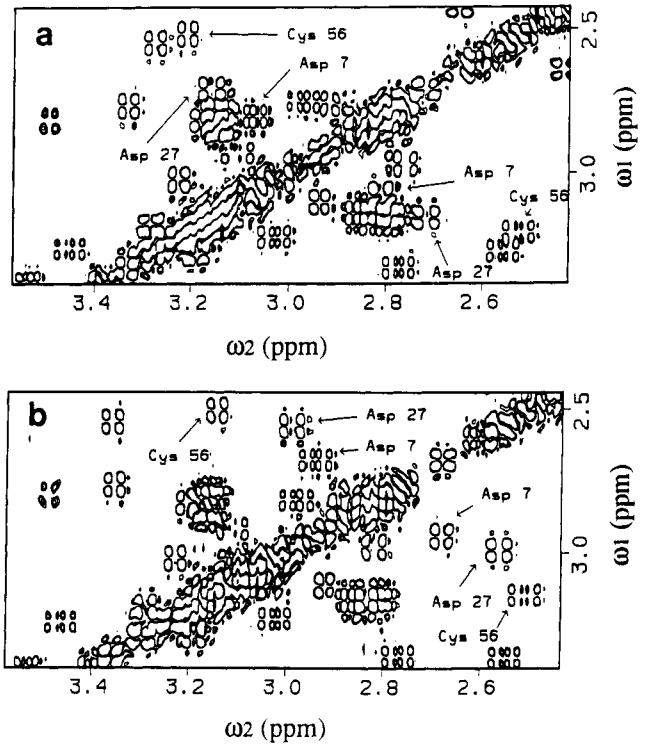

FIGURE 2: Expansion of the aliphatic region of the DQF-COSY spectrum of OMTKY3 at 35 °C. Solutions contained 5 mM OMTKY3 and 10 mM KCl and were adjusted to (a) pH 2.51 and (b) pH 5.28. The labeled crosspeaks are some of the $C_\beta H/C_\beta H'$ correlations showing significant shifts with varying pH.

increasing pH (Figure 3). These shifts are very similar to the changes observed in small peptides (Bundi & Wüthrich, 1979a), m-EGF (Kohda et al., 1991), and hirudin (Szyperski et al., 1994). No low pH plateau is observed for either of the aspartate residues or Cys 56 (Figure 3a), so the reported pK_a values are upper estimates (Table 1). Clearly, all three pK_a values are significantly lower than the values for model compounds (3.9−4.0; Creighton, 1993). In contrast, the entire transition for both Glu 10 and Glu 43 is observed in the NMR experiments (Figure 3b), and their pK_a values are only moderately perturbed relative to model compound values (4.3−4.5; Creighton, 1993). All of the observed transitions are completely reversible (Figure 3b and data not shown).

Amide Proton Resonances. In small peptides, peptide NHs of most acidic residues are expected to show modest (<0.1 ppm) upfield shifts upon deprotonation of the carboxyl group, with two exceptions (Bundi & Wüthrich, 1979b). First, the C-terminal NH group undergoes large (0.3−0.6 ppm) upfield shifts when the α-carboxyl group is deprotonated, and second, an NH hydrogen-bonded to an ionized carboxyl group will show a downfield shift, which may exceed 1 ppm. (Bundi & Wüthrich, 1979b; Mayer et al., 1979; Ebina & Wüthrich, 1984; Szyperski et al., 1994).

Eight NH resonances in the spectrum of OMTKY3 shift by at least 0.1 ppm over the pH range employed in this study (Figures 4 and 5; Table 2). Six of the eight are shifted downfield with increasing pH, suggesting that some of these NHs may be involved in hydrogen bonds with carboxyl groups. Neither of the other two NHs, belonging to Thr 17 and Lys 55, are on acidic residues, so their upfield shifts cannot be attributed to through-bond effects; changes at these NHs must result from a local perturbation in structure. Unfortunately, the $C_\alpha H$/NH cross peak of Cys 56 falls in a

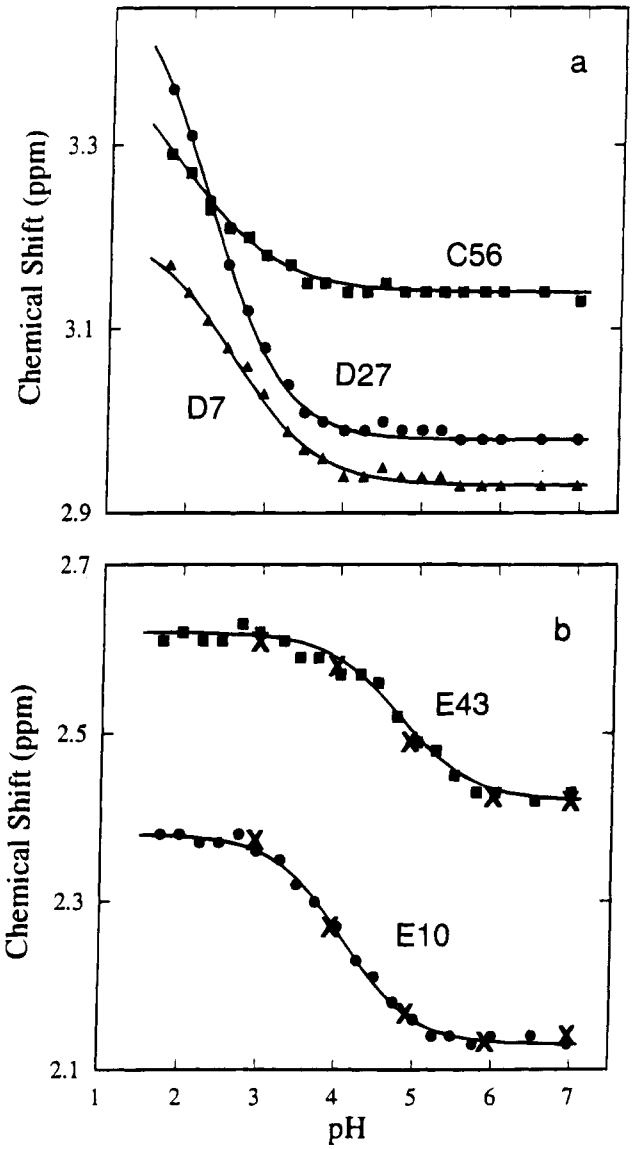

FIGURE 3: Aliphatic chemical shifts versus pH in 5 mM OMTKY3 and 10 mM KCl at 40 °C: (a) $C_\beta H'$ of Asp 7 (▲) and Asp27 (●) and $C_\beta H$ of Cys 56 (■); (b) $C\gamma H$ of Glu 10 (●) and Glu 43 (■). Data obtained by returning the acidified sample to neutral pH in 1 pH unit increments (X) are plotted for Glu 10 and Glu 43. The solid lines represent fits to eq 3.

very crowded region of the DQF-COSY spectrum, so the NH chemical shift could not be tracked accurately.

The pK_a values obtained with many of the NHs agree well with values obtained from aliphatic resonances (Table 2). Low pH plateaus are not observed for Ser 9 NH (Figure 5), which overlaps with other crosspeaks at low pH, and Asp 27 and Lys 29, whose NH chemical shifts apparently continue to shift at pH values less than 1.4 (Figure 5). Ser 9 NH nevertheless shows a low pK_a similar to that of Asp 7, while Asp 27 and Lys 29 NHs appear to report on titration of the Asp 27 side chain. The pK_a for Glu 10 NH matches that observed for the $C_\gamma H$ resonance, and the Leu 48 NH shows a pK_a that agrees best with that for Glu 43. Lys 55 NH is probably responding to changes at Cys 56. Thr 17 and Glu 19 NHs yield pK_a values that do not agree with any values obtained thus far and are probably sensitive to titration of the Glu 19 side chain.

pH dependences of chemical shifts were also fit to eq 3, a modified Hill equation. All fits for glutamate residues yield Hill coefficients between 0.9 and 1.1. Hill coefficients for

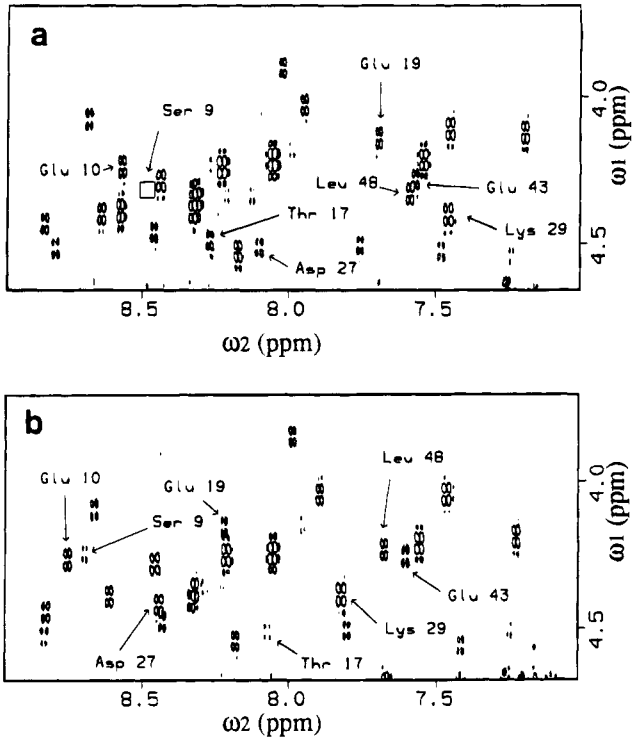

FIGURE 4: Expansion of the $C_\alpha H$/NH region of the DQF-COSY spectrum of 5 mM OMTKY3 and 10 mM KCl at 35 °C. Samples were adjusted to (a) pH 2.51 and (b) pH 5.28. The labeled cross peaks are some of the correlations showing significant shifts with varying pH.

Asp 7, Asp 27, and Cys 56 were generally between 0.7 and 0.9, but this may be a consequence of the incomplete transitions described by the data; neither the variance of the fit nor the standard errors of the pK_a values obtained with eq 2 were reduced by fitting to eq 3. Moreover, there are no significant differences in the pK_a values obtained from fits to eqs 2 and 3; the two pK_a values always overlapped at one confidence interval (Schaller, 1993).

No significant changes in pK_a were observed over the 25 °C temperature range employed in this study (Tables 1 and 2). This observation, in conjunction with the estimated uncertainties in pK_a values, leads us to conclude that none of the apparent ionization enthalpies (ΔH°_{ion}) exceeds 2 kcal/mol in magnitude.

Experiments were also performed at 35°C with samples that contained 1.0 M KCl, and the results are summarized in Table 3. Most of the very low pK_a values are increased in the presence of 1.0 M KCl, but of the previously identified low pK_a values, only that for Glu 19 now approaches model compound values (Figure 6).

DISCUSSION

Cooperativity. The net charge on OMTKY3 varies from about +1 to +6 as the pH is decreased from 7.0 to 1.4. Interactions between ionizing carboxyl groups are thus expected to perturb the pK_a values of side chains titrating over this pH range, leading to transitions that are broader (i.e., with Hill coefficients <1) than those seen in monobasic acids (Linderstrøm-Lang, 1924; Edsall & Wyman, 1958; Tanford, 1962; March et al., 1982). The observed Hill coefficients in OMTKY3 range between 0.7 and 1.2, but confidence in the outlying values is low because most of these values are derived from data describing incomplete transitions. In general, most of the Hill coefficients are close

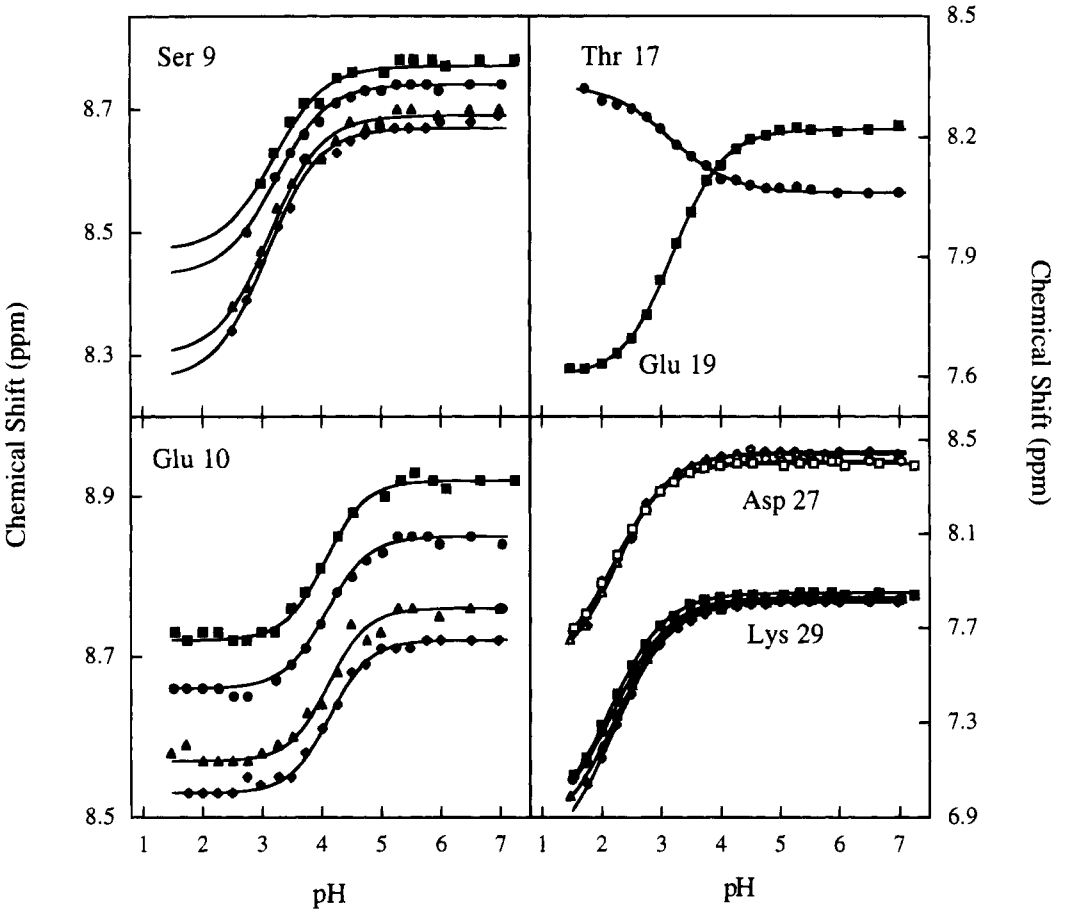

FIGURE 5: Peptide NH chemical shifts versus pH in 5 mM OMTKY3 and 10 mM KCl. Data are shown for Ser 9, Glu 10, Asp 27, and Lys 29 at 15 (■), 25 (●), 35 (▲), and 40 °C (◆). Data for Thr 17 and Glu 19 were acquired at 35 °C. Solid lines represent fits to eq 3.

Table 3: pH Dependence of OMTKY3 Proton Chemical Shifts in 1.0 M KCl at 35 °C[a]

amino acid	proton	δ_{A^-} (ppm)	$\Delta\delta$[b] (ppm)	pH_{mid}[c]	ΔpH_{mid}[d]
Asp 7	$C_\beta H$	2.68	0.22	2.78(0.26)	−0.07(0.28)
	$C_\beta H'$	2.99	0.25	3.11(0.12)	0.57(0.21)
	S9 NH	8.71	−0.52	3.08(0.04)	−0.05(0.13)
Glu 10	$C_\gamma H$	2.17	0.22	4.29(0.08)	0.25(0.14)
	NH	8.79	−0.20	4.35(0.05)	0.24(0.14)
Glu 19	NH	8.07	−0.45	3.98(0.04)	0.77(0.05)
Asp 27	$C_\beta H$	2.55	0.41	2.75(0.08)	0.43(0.09)
	$C_\beta H'$	2.98	0.47	2.60(0.12)	0.35(0.14)
	NH	8.45	−0.89	2.79(0.08)	0.72(0.08)
	K29 NH	7.78	−0.89	2.70(0.03)	0.51(0.04)
Glu 43	$C_\gamma H$	2.40	0.21	4.72(0.08)	0.30(0.14)
Cys 56	$C_\alpha H$	4.36	0.21	2.41(0.16)	0.12(0.20)
	$C_\beta H$	3.12	0.19	2.56(0.07)	0.11(0.20)

[a] The parameters δ_{A^-}, δ_{AH}, and pH_{mid} were obtained from fits to eq 2. The fitting errors for δ_{A^-} and δ_{AH} are all less than or equal to the 0.02 ppm uncertainty in the experimental measure of chemical shift. [b] $\Delta\delta = \delta_{AH} - \delta_{A^-}$; a positive value of $\Delta\delta$ means a downfield shift upon protonation. [c] Numbers in parentheses are fitting errors at 1 SD. [d] ΔpH_{mid} is the difference between pH_{mid} values obtained in the presence of 1 M KCl (above) and those obtained in the presence of 10 mM KCl (Tables 1 and 2): $\Delta pH_{mid} = pH_{mid}$(1 M KCl) − pH_{mid}(10 mM KCl).

to 1.0, indicating that there are few, if any, energetically significant interactions between the carboxyl groups. Similar results were obtained in studies of egg white lysozyme (Bartik et al., 1994) and hirudin (Szyperski et al., 1994).

The absence of significant cooperativity between the carboxyl groups is consistent with the high-resolution structure of OMTKY3 where most of the carboxyl groups are at least 10 Å from one another; the lone exception is the 4.9 Å separating carboxyl oxygens of Asp 7 and Glu 10 (Read et al., 1983). Data for Asp 27 in OMTKY3 suggest the possibility of a broadened transition (Figure 6), but more extensive data at low pH are needed for unambiguous identification of the complete transition. Most of the data therefore suggest that the perturbed pK_a values in OMTKY3 result from interactions with cationic and polar groups.

NH Chemical Shifts and Hydrogen Bond Interactions. The magnitude of the pH-dependent change in an NH chemical shift, $\Delta\delta$(pH), has been interpreted as a measure of the population of that NH involved in hydrogen bonds (Bundi & Wüthrich, 1979b; Mayer et al., 1979; Szyperski et al., 1994). Larger values of $\Delta\delta$(pH) are thus believed to reflect well-populated hydrogen bonds with the charged side chain serving as the hydrogen bond acceptor. $\Delta\delta$(pH) values for OMTKY3 span a wide range of values which, interestingly, correlate very well with the extent to which the pK_a values of the acceptor side chains are perturbed (ΔpK) relative to values for model compounds (Figure 7a). This correlation persists in the presence of 1 M salt (see Tables 2 and 3). If indeed the NHs showing large $\Delta\delta$(pH) are participating in strong hydrogen bonds with ionized carboxyl groups (Pimental & McClellan, 1960; Bundi & Wüthrich, 1979b; Taylor & Kennard, 1984; Fersht & Serrano, 1993), then one might also expect low thermal coefficients ($\Delta\delta/\Delta T$) for the same NHs at pH values favoring the ionized side chains [Pimental & McClellan, 1960; Eisenberg & Kauzmann, 1969; Kopple, 1971; Waltho et al., 1993; for critical discussion see also Gellman et al. (1991) and references therein]. This expectation is borne out in the results reported in Table 2 and summarized in Figure 7b.

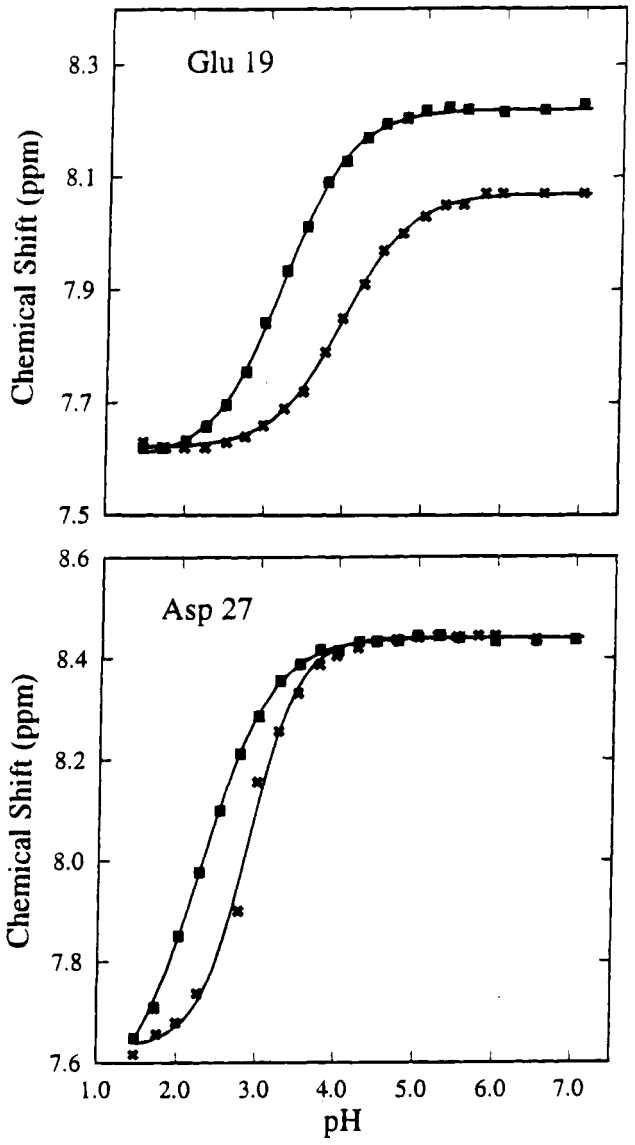

FIGURE 6: Peptide NH chemical shifts for Glu 19 and Asp 27 versus pH in 5 mM OMTKY3 in 10 mM KCl (■) or 1.0 M KCl (×) at 35 °C. The solid lines represent fits to eq 3.

Most of the reduced pK_a values in OMTKY3 appear to be linked to hydrogen bonding with backbone NH groups. The evidence for such interactions is the magnitude and direction of the pH-dependent chemical shift changes (Pimental & McClellan, 1960; Bundi & Wüthrich, 1979b) as well as the pH midpoints of these changes, which are coincident with pK_a values for carboxyl groups determined using aliphatic proton resonances. Only a very few of the hundreds of resonances in the proton spectrum of OMTKY3 show large (>0.2 ppm) pH-dependent changes in chemical shift (Schaller, 1993), so major perturbations in the tertiary fold of OMTKY3 are probably not responsible for these changes. Moreover, the hydrogen bonds identified in this study are entirely consistent with observations from X-ray studies (Papamakos et al., 1982; Read et al., 1983; Bode et al., 1985; Fujinaga et al., 1987). Similar correlations between pH-dependent NH chemical shifts and hydrogen bonds to carboxyl groups have been observed in BPTI (Wüthrich & Wagner, 1979; Tüchsen & Woodward, 1985; Berndt et al., 1992) and hirudin (Szyperski et al., 1994). Further evidence for a link between ionization equilibria and the extent of hydrogen bonding in OMTKY3 may be found in the intriguing correlation between $\Delta\delta$(pH) and ΔpK (Figure 7a), although the physical origin of this correlation is unknown at present.

The backbone NHs of Leu 48 and Lys 55 are not involved in hydrogen bonds to ionizing side chains, and yet the chemical shifts for both show significant pH dependences (Table 2). Titration of Glu 43 clearly affects the chemical shift of Leu 48 NH, probably by way of its hydrogen bond to the hydroxyl oxygen of Ser 44 (Papamakos et al., 1982; Read et al., 1983; Bode et al., 1985; Fujinaga et al., 1987). Lys 55 NH is probably reporting the titration of C-terminal Cys 56, although the physical origin of this effect is unclear. The pH dependence of Lys 55 NH is probably not due to through-bond effects (Bundi & Wüthrich, 1977) or hydrogen-bonding interactions: X-ray and NMR studies place Lys 55 NH on the solvent-exposed surface of the third domain and far from any potential hydrogen bond acceptors. Alternatively, coupling of perturbations in His 52 to Lys 55 NH could be through an interaction between the carboxyl oxygen of Lys 55 and the C_ϵ of His 52, which is 3.13 Å away (Read et al., 1983). Much of the stereochemistry describing this close contact is consistent with the C−H···O hydrogen bond recently proposed for the C_ϵH of histidine residues (Derewenda et al., 1994).

A remarkable feature of the hydrogen bonds involving the side chains of Asp 7 and Glu 19 is their exposure to solvent. Considerable debate centers on the stability of hydrogen bonds in proteins dissolved in water (Dill, 1990; Rose & Wolfendon, 1993). Most researchers agree that hydrogen bonds buried in the protein interior are stable, but there is less agreement concerning the stability of solvent-exposed hydrogen bonds (Rose & Wolfenden, 1993). Unfortunately, the chemical shift changes seen for OMTKY3 and the present state of theory concerning these changes do not yet permit a quantitative evaluation of the strength of these hydrogen bonds; we can only conclude that either hydrogen bonds are populated as the pH is increased or the length of the hydrogen bonds is reduced (Wagner et al., 1983), or both.

Ionization Constants. The pK_a values for both aspartate and two of the three glutamate residues in OMTKY3 are significantly reduced relative to values obtained with model compounds. A large number of the acidic residues in bovine ribonuclease A (Rico et al., 1991), egg white lysozyme (Bartik et al., 1994), and ribonuclease HI (Oda et al., 1994) show similar deviations. All of these proteins, including OMTKY3, have pI values greater than 7 and bear net positive charges over the entire range of titration, which may play a role in depressing the pK_as of a large fraction of the carboxyl groups (Bartik et al., 1994). This is not, however, a universal phenomenon: BPTI is a very basic protein in which only one of the five carboxyl groups in the native protein has a pK_a value reduced by more than 1 pH unit (March et al., 1982).

The low pK_a values for acidic residues in OMTKY3 are consistent with specific features of its three-dimensional structure. Moreover, residues with ΔpK_a values exceeding 0.5 (Figure 7) are conserved in over 90% of the over 150 third domains sequenced to date (Apostol et al., 1993), which argues for important structural or functional roles for these residues. Two possible contributions to the low pK_a for Asp 7 are (1) a bifurcated hydrogen bond between the β-carboxyl group of Asp 7 and the NH and β-OH groups of Ser 9 and (2) the proximity of the ϵ-amino group of Lys 34, which is about 5 Å from the carboxyl group of Asp 7 (Papamakos et al., 1982; Read et al., 1983; Bode et al., 1985; Fujinaga et

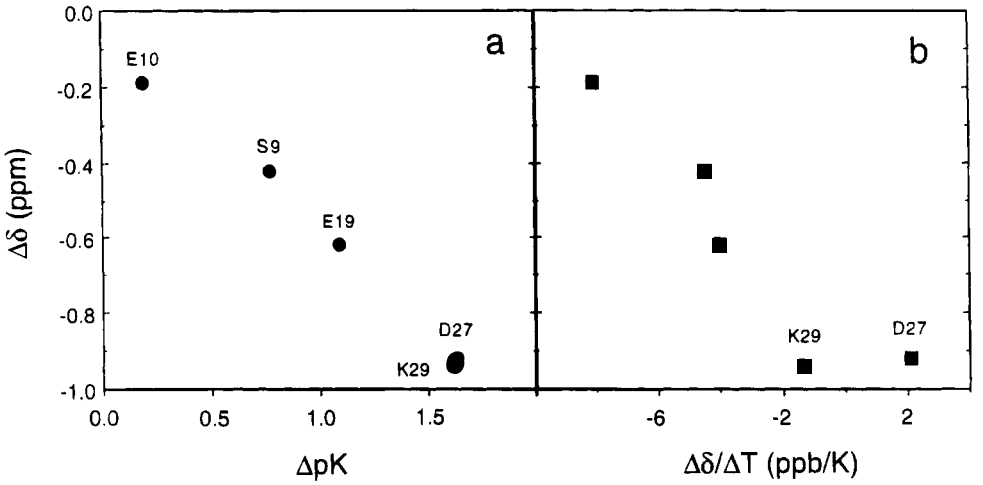

FIGURE 7: pH-dependent changes in NH chemical shifts ($\Delta\delta$) are correlated with (a) pK_a perturbations (ΔpK) in the hydrogen bond acceptor groups and (b) the temperature dependence of the NH chemical shifts ($\Delta\delta/\Delta T$). $\Delta\delta$ values are taken from Table 2, and ΔpK values are the differences between pK_a values for model compounds (3.9 for Asp and 4.4 for Glu) and pK_a values observed in native OMTKY3 (Table 2). Values of $\Delta\delta/\Delta T$ are taken from Table 2 and correspond to values observed at pH values well above the pK_a for the putative hydrogen bond acceptor group.

al., 1987). Ser 9 and Lys 34 are found in 99% of third domain sequences (Apostol et al., 1993). The small magnitude of $\Delta\delta$(pH) for the NH of Ser 9 suggests that the hydrogen bond may not be 100% occupied in solution, which may explain why this hydrogen bond is not observed in some of the X-ray structures (Bode et al., 1985). Similar arguments may explain both the small $\Delta\delta$(pH) for the NH of Glu 10 and the low frequency with which a hydrogen bond between the NH and side chain of Glu 10 has been identified in X-ray studies (Fujinaga et al., 1987). The moderately perturbed pK_a for Glu 10, which is sensitive to ionic strength, may be a consequence of its proximity to Lys 13.

The carboxyl group of Glu 19 accepts a hydrogen bond from the hydroxyl group of Thr 17 and is only a few angstroms distant from the guanidino group of Arg 21 (Papamakos et al., 1982; Read et al., 1983; Bode et al., 1985; Fujinaga et al., 1987). A hydrogen bond between the side chain of Glu 19 (or Asp 19 in the third domain of Japanese quail ovomucoid) and its peptide NH has been observed in only one of the X-ray structures of free inhibitor (Weber et al., 1981), but is often detected in structures of complexes between inhibitor and serine proteinase [Fujinaga et al., 1982, 1987, Read et al., 1983; but see Bode et al. (1986)]. Results of the present work are most consistent with an intraresidue hydrogen bond for Glu 19 in both low- and high-salt solutions. Additional evidence for a restricted side chain conformation is the observation of two distinct $C_\gamma H$ resonances for Glu 19, in contrast to the degenerate resonances for Glu 10 and Glu 43 (Robertson et al., 1990). Crystals for many, if not all, of the X-ray studies are grown in high salt, which may reduce the population of hydrogen bonds and preclude facile observation of this interaction. The present results also confirm the suggestion of Ardelt and Laskowski (1991) that participation by the Glu 19 side chain in hydrogen bonds with the hydroxyl group of Thr 17 and the NH of Glu 19 reduces the pK_a of Glu 19 in intact OMTKY3.

The pH dependence for the NH of Thr 17 has a midpoint that is coincident with the pK_a for Glu 19 (Figure 5 and Table 2), but the sign of $\Delta\delta$(pH) is not consistent with hydrogen bonding to the carboxylate of Glu 19; no such hydrogen bond is observed in any of the X-ray crystal structures for third domains (Papamakos et al., 1982; Read et al., 1983; Bode et al., 1985; Fujinaga et al., 1987). One possible explanation for the chemical shift change at the NH of Thr 17 is an inductive effect: an increase in hydrogen bond strength between the side chains of Thr 17 and Glu 19 upon deprotonation of the latter may lead to a more electronegatively hydroxyl oxygen and, consequently, increased shielding [i.e., $\Delta\delta$(pH) > 0] at the threonine NH.

The low pK_a for Asp 27 confirms its role in the large pH-dependent changes in the spectral properties of tyrosine residues in intact ovomucoid and its third domain (Donovan, 1967; Markley et al., 1984; Laskowski et al., 1987; Hildebrandt et al., 1988). The side chain of Asp 27 is almost completely buried in the interior of OMTKY3, yet the very low pK_a for this residue suggests that the native structure favors the *ionized* side chain over the neutral protonated species. The carboxylate of Asp 27 is stabilized by hydrogen bonds with three buried polar groups: the phenolic hydroxyl group of Tyr 31 and the NHs of Asp 27 and Lys 29 (Papamakos et al., 1982; Read et al., 1983; Bode et al., 1985; Hildebrandt et al., 1988; Robertson et al., 1988). The hydrogen bond to the NH of Asp 27 has been identified in only one of the many X-ray structures for third domains (Musil et al., 1991), but results from the present study in conjunction with the observation of slow hydrogen exchange for this NH (Robertson et al., 1988; L. Swint and A. D. Robertson, unpublished results) argue for a significant interaction.

Buried ionizable groups present special challenges to workers engaged in theoretical calculations of electrostatic effects in proteins. Macroscopic or continuum models treat the protein interior as a low dielectric environment [$\epsilon = 2-4$; see Antosiewicz et al. (1994) and references therein], which disfavors ionized groups, especially those not involved in salt bridges. Warshel and co-workers prefer a microscopic approach that explicitly includes dipoles of protein groups, obviating the need for a single dielectric constant for the protein interior (Warshel, 1981; Warshel et al., 1984; Warshel & Åqvist, 1991). The carboxyl group of Asp 27, with 11 Å^2 of solvent-accessible surface for one oxygen and no solvent exposure for the other, is largely buried, and the experimental data are thus not consistent with a low dielectric interior: the polar protein groups surrounding the Asp 27 side chain are acting as a "supersolvent", wherein the free energy

of ionization is lower than that observed in aqueous solvent (Warshel, 1981; Honig & Hubbell, 1984). A very similar set of interactions is responsible for the low pK_a of Asp 66 in hen egg white lysozyme, which is also shielded from solvent (Yang & Honig, 1993). Destabilization of OMTKY3 may accompany protonation of Asp 27 because of the concomitant reduction in its hydrogen bond accepting capability. This will leave one or more buried polar groups, Tyr 31 OH or either of the NHs of Asp 27 or Lys 29, without a suitable acceptor.

The apparent low pK_a for the α-carboxyl group of Cys 56 may result from the inductive acidifying effects of the nearly disulfide bond (Edsall & Wyman, 1958; Greenstein & Winitz, 1961) and proximity to the side chain of His 52. Indirect evidence for an interaction with His 52 is its pK_a of 7.5, which is about 0.5−1.0 pH unit higher than values for model compounds (Tanford, 1962; Markley, 1973; Ogino et al., 1982). In addition, a low pH transition with a pH_{mid} of about 2.5 was noted in the pH dependence of the $C_\epsilon H$ resonance of His 52 (Markley, 1973; Ogino et al., 1982). This transition was assigned to the titration of Cys 56, and results of the present work are entirely consistent with this assignment.

Ebina and Wüthrich examined the pH dependence of the NH chemical shifts in bull seminal inhibitor IIA (BUSI IIA), a Kazal-type proteinase inhibitor which shares approximately 55% sequence identity with OMTKY3, by collecting COSY data at about 0.5 pH unit intervals from pH 3.2 to pH 5.4 (Ebina & Wüthrich, 1984). Conserved residues, using the OMTKY3 numbering, include Asp 7, Glu 10, Glu 19, and Cys 56. In contrast to OMTKY3, most of the carboxyl groups of BUSI IIA appear to have pK_a values that closely resemble those of simple model compounds. One exception is Cys 56, whose estimated pK_a of about 2.5 is very similar to that observed in OMTKY3. Possible explanations for the different pK_a values for Asp 7 and Glu 19 in the two proteins lie in key sequence differences: BUSI IIA contains an alanine at position 9 instead of serine, and an asparagine at position 21 in lieu of arginine. As discussed earlier, Ser 9 and Arg 21 are believed to contribute to the low pK_a values of Asp 7 and Glu 19, respectively, in OMTKY3.

Effect of High Ionic Strength. Asp 7 and Asp 27 continue to exhibit low pK_a values (Table 3, Figure 7, and data not shown) under conditions where Coulombic interactions are expected to be screened (Russell & Fersht, 1987; Horovitz et al., 1990; Serrano et al., 1990; Scholtz et al., 1993). Persistent interactions involving ionized side chains in high salt have been attributed to residual hydrogen bond interactions (Dill, 1990; Horovitz et al., 1990; Scholtz et al., 1993). The possibility of specific ion binding has been largely ruled out for OMTKY3 [see accompanying paper by Swint and Robertson (1995) for discussion]. The results are thus consistent with the known environment for the carboxyl group of Asp 27, which is involved in three hydrogen bonds to uncharged polar groups in the protein's interior (Papamakos et al., 1982; Read et al., 1983; Hildebrandt et al., 1988; Robertson et al., 1988; Musil et al., 1991). Apparently, Asp 27 has few long-range interactions with other charged groups in OMTKY3. One interpretation of the different salt dependences for Asp 7 and Glu 19, both of which are involved in solvent-exposed hydrogen bonds to backbone NHs, is that the depressed pK_a of the Asp 7 carboxyl group is due entirely to hydrogen bonding with NH and OH groups on Ser 9, while long-range electrostatic interactions contribute to the low pK_a for Glu 19.

Ionization Enthalpies. The present work represents one of the first attempts to measure individual ionization enthalpies for all carboxyl groups in a native protein. The standard free energies of ionization can be calculated from the pK_a values [$\Delta G^\circ_{ion} = 2.3RT(pK_a)$], so in principle, the entropic and enthalpic contributions to the perturbations of pK_a values (ΔpK) in native OMTKY3 can be determined. In practice, the uncertainites in ΔH°_{ion} are of the order of perturbations in ΔG°_{ion} for all but Asp 27. ΔG°_{ion} for Asp 27 in native OMTKY3 favors ionization by about 2.5 kcal/mol relative to model compound values for aspartate residues. As ΔH°_{ion} is approximately zero, ΔS°_{ion} for Asp 27 must favor ionization by about 8 cal/(mol K) relative to model compounds. Most of the unfavorable entropy of ionization for carboxyl groups arises from orientation and close packing of water around the charged species (Edsall & Wyman, 1958). In the case of native OMTKY3, protein groups are largely responsible for intramolecular "solvation" of the Asp 27 carboxyl group, which is likely to reduce the entropic cost of ionization. In addition to providing valuable information on the energetics of ionization in OMTKY3, the observed enthalpies also validate the assumption implicit in many scanning calorimetric studies that ΔH°_{ion} for carboxyl groups in native proteins is small and similar to model compound values (Privalov & Khechinashvili, 1974; Privalov & Potekhin, 1986).

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