Insensitivity of Perturbed Carboxyl pK_a Values in the Ovomucoid Third Domain to Charge Replacement at a Neighboring Residue†

William R. Forsyth‡ and Andrew D. Robertson*

Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242

Received December 28, 1999; Revised Manuscript Received April 12, 2000

ABSTRACT: A number of carboxyl groups in turkey ovomucoid third domain (OMTKY3) have low pK_a values. A previous study suggested that neighboring amino groups were primarily responsible for the low carboxyl pK_a values. However, the expected elevation in pK_a values for these amino groups was not observed. In the present study, site-directed mutagenesis is used to investigate the origins of perturbed carboxyl pK_a values in OMTKY3. Electrostatic calculations suggest that Lys 34 has large effects, 0.4−0.6 unit, on Asp 7, Glu 10, and Glu 19 which are 5−11 Å away from Lys 34. Two-dimensional ^{1}H NMR techniques were used to determine pK_a values of the acidic residues in OMTKY3 mutants in which Lys 34 has been replaced with threonine and glutamine. Surprisingly, the pK_a values in the mutants are very close to those of the wild-type protein. The insensitivity of the acidic residues to replacement of Lys 34 suggests that long-range electrostatic interactions play less of a role in perturbing carboxyl pK_a values than originally thought. We hypothesize that hydrogen bonds play a key role in perturbing some of the carboxyl ionization equilibria in OMTKY3.

Protein function and stability are intimately linked to the protonation equilibria of ionizable groups. Many such groups exhibit altered pK_a values whose shifts result from a variety of possible interactions (*1*−*3*). pK_a values thus reflect the intricate relationship between structure and energetics within a protein. In favorable cases, ionization equilibria are one of the few types of reversible reactions that can be monitored simultaneously at many residues in a protein. They thus provide a sensitive and precise measure of the energetic consequences of perturbations, such as a mutation, throughout the structure of a protein.

The origins of pK_a perturbations have been previously investigated by mutagenesis (*4*−*16*). In most cases, these studies focused on a small subset of ionizable residues. We have embarked on a systematic investigation into the mechanisms of pK_a perturbation for numerous residues throughout turkey ovomucoid third domain (OMTKY3).[1] OMTKY3 is an excellent system for this study by virtue of its stability over a wide range of solution conditions (*17*−*20*) and the availability of X-ray and NMR-derived structures (*21*−*24*).

The current study takes advantage of insight from electrostatic calculations and the ability to determine multiple pK_a values by NMR to investigate the mechanisms of pK_a perturbations for multiple residues simultaneously. Electrostatic calculations suggest that Lys 34 lowers the pK_a values of Asp 7, Glu 10, and Glu 19 by 0.6, 0.4, and 0.6 unit, respectively (*25*). Mutagenesis was used to test this hypothesis. Interestingly, the results of this study suggest that Lys 34 has little influence on the acidic pK_a values. We hypothesize that hydrogen bonding makes a significant contribution to perturbing carboxyl pK_a values.

MATERIALS AND METHODS

Materials. Deuterium oxide (99.9 atom %) and sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (98 atom %) were from Cambridge Isotope Laboratories (Cambridge, MA). Standardized buffers at pH 2, 4, 7, and 10 were from VWR Scientific (West Chester, PA) and Fisher Scientific (Pittsburgh, PA). A certified 1 N HCl solution (Fisher Scientific) served as a pH 0.10 standard (*26*). Reagent grade potassium chloride was from EM Science (Gibbstown, NJ). Lysozyme and CNBr were from Sigma (St. Louis, MO).

Protein Expression and Purification. Recombinant OMTKY3 (rOM3) was expressed and purified using a protocol modified from Lu et al. (*27*). Briefly, a single colony of *E. coli* strain RV308 containing the pEZZ318.tky plasmid grown from a LB-ampicillin plate incubated overnight at 30 °C was used to inoculate 10 mL of 2 × TY media [16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 100 mg/L ampicillin, and 0.1% (w/v) glucose]. This starter culture was incubated overnight with constant shaking at 30 °C. The culture was then used to inoculate 500 mL of 2 × TY medium which was then incubated overnight at 30 °C with constant shaking. Seventy-five milliliters of this culture was

† This work was supported by the National Institutes of Health (GM 46869).

* To whom correspondence should be addressed. Telephone: 319-335-6515. Fax: 319-335-9570. E-mail: andy-robertson@uiowa.edu.

‡ Present address: Department of Chemistry and The Center for Biomolecular Structure and Function, The Pennsylvania State University, University Park, PA 16802.

[1] Abbreviations: δ_A, chemical shift of the fully unprotonated species; δ_{HA}, chemical shift of the fully protonated species; δ_{obs}, observed chemical shift; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy; *n*, Hill coefficient; OMTKY3, turkey ovomucoid third domain (purified from egg whites); rOM3, recombinant OMTKY3; K34Q, rOM3 mutant with lysine 34 replaced with glutamine; K34T, rOM3 mutant with lysine 34 replaced with threonine; TOCSY, total correlation spectroscopy; DQCOSY, double quantum correlation spectroscopy; TSP, sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$.

10.1021/bi992967p CCC: $19.00
Published on Web 06/10/2000

used to inoculate each of six 2.8 L flasks containing 1.5 L of 2 × TY media. The 9 L were grown at 30 °C with constant shaking for 24 h. The cells were then harvested by centrifugation at 5000*g* for 20 min at 4 °C.

An osmotic shock procedure adopted from Randall and Hardy (*28*) was then used to liberate the periplasmic proteins. The cells were thoroughly suspended in 75 mL of ice-cold sucrose buffer (0.5 M sucrose, 0.1 M Tris-HCl, 1 mM EDTA, pH 8.2) and incubated for 10 min in an ice/water bath. Lysozyme (1 mL of a 12 mg/mL solution) was added followed immediately by 150 mL of ice-cold distilled, deionized water. Following an incubation period of 5 min in an ice/water bath, 2.7 mL of 1 M $MgSO_4$ was added. The cells were then centrifuged at 100000*g* for 60 min at 4 °C.

The supernatant was collected and loaded onto an IgG Sepharose 6 Fast Flow (Pharmacia, Piscataway, NJ) affinity column (12.7 × 5 cm) equilibrated with TS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). Eighteen milliliter fractions were collected at a flow rate of approximately 0.9 mL/min. The column was washed with TS buffer after loading of the supernatant fraction until a baseline was established. The fusion protein was eluted using 0.5 M acetic acid adjusted to pH 3.3 with concentrated ammonium acetate. Usually the column was overloaded, so the flow-through (unbound fraction) was reintroduced to the column following equilibration in TS buffer, and the fusion protein was eluted. This process of rerunning the column was repeated until no protein peak was detected in the acetic acid elution. A different IgG column was used for each variant of rOM3.

Following lyophilization, the fusion protein was dissolved (2% w/v) in 5% TFA containing 60 mg/mL CNBr to liberate rOM3 from the Z fragments. The reaction was carried out in the dark with constant stirring at ambient temperature and halted after 24 h by addition of 10 volumes of water and immediate freezing and lyophilization.

Gel filtration chromatography using a Bio-gel P-10 (Bio-Rad) column (1.8 × 60 cm) equilibrated in 15 mM ammonium bicarbonate allowed for the separation of rOM3 from the Z fragments and uncleaved fusion protein. Approximately 9.6 mL fractions at a flow rate of 0.8 mL/min were collected. Typical yields of purified rOM3 were 1.5−2.5 mg/L of bacterial culture.

Purified protein was extensively dialyzed against 4 L of distilled deionized water at pH 9 followed by dialysis against 4 L of distilled deionized water at neutral pH. The protein was then lyophilized and analyzed by SDS−PAGE, MALDI-TOF mass spectrometry, amino acid analysis, and NMR (data not shown).

Mutagenesis. Site-directed mutagenesis was carried out on isolated wild-type pEZZ318.tky plasmid (*27*) using the Stratagene QuikChange kit. Both mutations involved a single nucleotide change that was incorporated into mutagenic primers of 39 nucleotides. The proper mutation was verified by DNA sequencing analysis (DNA Facility, University of Iowa). Each mutant plasmid was transformed into *E. coli* strain RV308 (*29*) for expression.

NMR Sample Preparation. A stock solution of approximately 1 mM rOM3 was prepared by dissolving lyophilized protein in 90% H_2O/10% D_2O (v/v) containing 10 mM potassium chloride and 0.5 mM TSP as an internal chemical shift standard. The pH of the stock solution was adjusted using concentrated HCl and KOH. The pH was measured at room temperature using an Orion Research pH meter (Model 611) with a 3 mm Ingold combination electrode (Wilmad Glass Co., Buena, NJ) standardized at two points. Sample pH was measured both before and after the experiment with the average value being used in the data analysis. Agreement was typically within 0.04 pH unit. Reported pH values are not corrected for the deuterium isotope effect. Data were recollected at various pH values after the titration was completed in order to assess reversibility.

NMR Spectroscopy. All DQCOSY (*30, 31*) and TNTOCSY (*32, 33*) spectra were acquired using a Varian $^1H\{^{13}C, ^{15}N\}$ 5 mm triple resonance probe on a Varian UNITY INOVA spectrometer, located in the University of Iowa College of Medicine NMR facility and operating at a ^1H frequency of 499.717 MHz. The carrier frequency was set on the water signal. Solvent suppression was achieved by continuous-wave irradiation of the solvent resonance during the 1.5 s relaxation delay. The spectral width was 6000 Hz, and the TNTOCSY spectra utilized a 50 ms mixing time. Each experiment consisted of 4 transients with 2048 time-domain points and either 256 or 330 increments in the indirectly detected dimension for the DQCOSY and TNTOCSY spectra, respectively. All experiments were performed at 25 °C, as verified by a methanol standard (*34*).

NMR data were processed using Varian software installed on a Sun Microsystems ULTRA 5 workstation. The time-domain data were weighted using an unshifted Gaussian filter prior to the Fourier transformation. The Gaussian time constants were 0.079 and 0.020 s in the directly and indirectly detected dimensions, respectively. The final digital resolution was 2.93 Hz/point in both dimensions, and estimated uncertainties in chemical shifts are 0.007 ppm in ω_2 and 0.013 ppm in ω_1.

Determination of Apparent pK_a Values. The pH dependences of the proton chemical shifts were fit by nonlinear least-squares analysis (*35, 36*) to a modified Hill equation (*37, 38*) to determine an apparent ionization constant, pK_a:

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

where δ_{obs} is the observed chemical shift at a given pH, δ_A and δ_{HA} are the chemical shifts of the fully unprotonated and protonated species, respectively, and n is the Hill coefficient, a measure of cooperativity. Omission of the Hill coefficient does not alter the fitted pK_a values in a statistically significant manner.

All chemical shifts were corrected for the pH dependence of TSP prior to data analysis according to (*39, 40*)

$$\delta_{corr} = \delta_{obs} - 0.019\ 1 + 10^{(5-pH)^{-1}} \quad (2)$$

RESULTS

The previous work by Schaller and Robertson (*38*) served as a starting point for analysis of the DQCOSY and TNTOCSY spectra. A salt concentration of 10 mM KCl was chosen to minimize the effect of varying ionic strength by added titrant and to facilitate comparison with the previous work. The resonances for protons closest to the site of deprotonation were monitored whenever possible as these

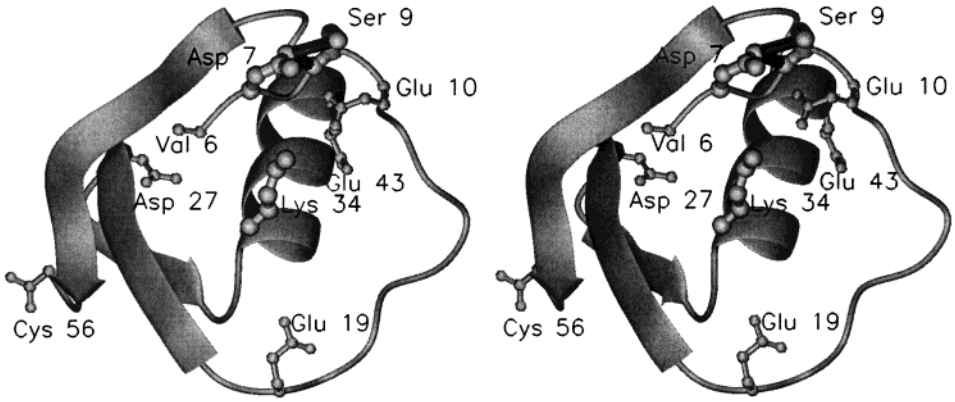

FIGURE 1: Stereo ribbon diagram of recombinant third domain (rOM3). Acidic residues as well as Ser 9, Lys 34, and the amino terminus of Val 6 are shown in ball-and-stick form. Asp 7, Lys 34, and Ser 9 are exaggerated. Hydrogen bonds between the side chain and backbone amide of Ser 9 and Asp 7 are shown in black. Figure prepared using MOLMOL (*56*).

generally show the largest changes in chemical shift. All chemical shift changes were similar to the changes in small peptides (*40*) and to those observed in the previous work (*38*).

All of the observed transitions were completely reversible. Most Hill coefficients were between 0.8 and 1.2, with a few outlying values which were heavily influenced by the completeness of the transitions. Omission of the Hill coefficient had no signficant effect on the pK_a values. rOM3 and the two variants are folded throughout the titrations, as evidenced by the fact that all chemical shifts, except those for titrating residues and amide protons to which they are hydrogen-bonded, changed <0.1 ppm in going from pH 0.93 to 7.54.

Aliphatic Proton Resonances. The cross-peaks monitored in this study are largely those described in a previous study of carboxyl groups of OMTKY3 (*38*). Cross-peaks for the $C_\beta H$ resonances of Asp 7 and Asp 27, and the carboxyl terminus of Cys 56 were well-resolved and easily monitored throughout the titration (data not shown). For Glu 10 and Glu 43, the $C_\beta H/C_\gamma H$ cross-peaks were monitored.

Incomplete transitions were observed for Asp 7, Asp 27, and Cys 56 (Figure 2). As a result, the fitted pK_a values for these residues (Table 1) represent upper limits for the true pK_a values. If we assume these titrations are simple, i.e., the Hill coefficient equals 1, then the transitions appear to be greater than 95% complete based on the breadth of the observed transitions. In this case, the fitted pK_a values are within 0.05 pH unit of the true values. Interestingly, the plateaus for the fully deprotonated species of Asp 7, Glu 10, and Glu 43 are different for the mutants and wild-type protein (Figures 2 and 3). The mutants have a slightly more downfield chemical shift in the case of Glu 10, but Asp 7 and Glu 43 are shifted upfield relative to wild-type. The magnitude of the change in chemical shifts is very small, 0.05−0.1 ppm, and the pK_a values for these residues are similar to wild-type. The $C_\beta H/C_\gamma H$ resonances for Glu 19 overlap with other cross-peaks, so for this residue the backbone amide was analyzed (*38*, *40*).

Amide Proton Resonances. Amides that exhibit a large upfield change in chemical shift with decreasing pH usually belong to an ionizable residue or are involved in a hydrogen bond (*40*−*43*). In the case of Glu 19, the amide resonance shows a large change in chemical shift of 0.6 ppm (Figure 2) which probably results from hydrogen bonding with its own carboxyl group.

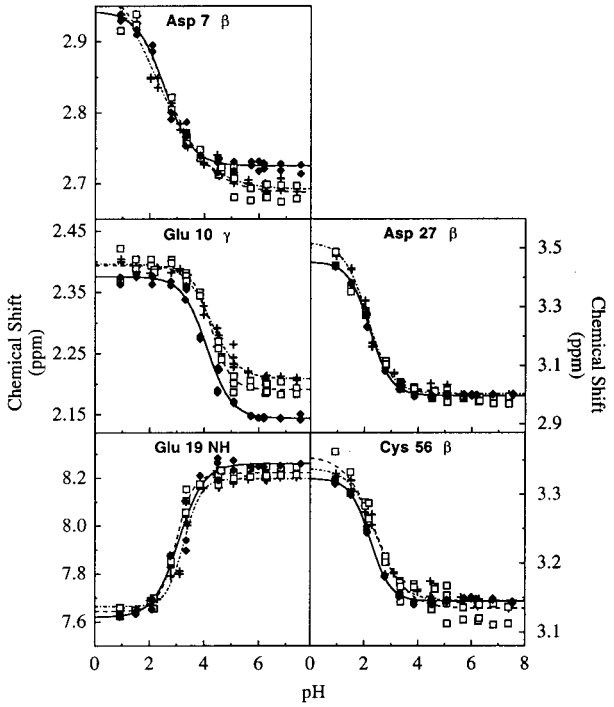

FIGURE 2: pH dependencies of wild-type rOM3 and the K34Q and K34T variants (◆, +, and □, respectively) at 25 °C in 10 mM KCl. β, γ, and NH designate the monitored resonance. Lines represent least-squares fits to eq 1. The data overlap in many instances.

The backbone amides of both Ser 9 and Lys 29 also show large changes in chemical shifts of approximately 0.6 and 1.0 ppm, respectively (Figure 3). As noted by Schaller and Robertson (*38*), these amides are hydrogen bond donors to the side chains of Asp 7 and Asp 27, respectively. The pK_a values monitored by the amides of Ser 9 and Lys 29 agree well with their respective hydrogen bonding partners, Asp 7 and Asp 27 (Table 1).

DISCUSSION

OMTKY3 and rOM3. The recombinant protein (rOM3) is truncated by five residues at the amino terminus relative to OMTKY3 prepared from egg whites. An important consequence is that the positively charged amino terminus is now

Table 1: Analysis of pK_a's of Acidic Side Chains at 25 °C, ~10 mM KCl[a]

	OMTKY3[b]	rOM3			rOM3(K34Q)			rOM3(K34T)		
amino acid	pK_a	pK_a	δ_A (ppm)	$\Delta\delta$[c] (ppm)	pK_a	δ_A (ppm)	$\Delta\delta$[c] (ppm)	pK_a	δ_A (ppm)	$\Delta\delta$[c] (ppm)
Asp 7	<2.6	2.55 (0.14)	2.73	0.21	2.44 (0.08)	3.00	0.31	2.53 (0.31)	2.69	0.30
Ser 9	3.0	2.63 (0.12)	8.73	−0.57	2.77 (0.01)	8.79	−0.56	2.75 (0.07)	8.78	−0.57
Glu 10	4.1	4.10 (0.08)	2.14	0.24	4.25 (0.10)	2.21	0.19	4.26 (0.08)	2.19	0.20
Glu 19	3.2	3.08 (0.14)	8.26	−0.64	3.26 (0.06)	8.20	−0.54	2.97 (0.06)	8.22	−0.58
Asp 27	<2.3	2.20 (0.05)	2.55	0.42	2.12 (0.11)	3.00	0.52	2.05 (0.19)	2.99	0.45
Lys 29	2.3	2.21 (0.07)	7.84	−0.98	2.22 (0.05)	7.85	−0.99	2.20 (0.08)	7.85	−0.99
Glu 43	4.8	4.66 (0.04)	2.44	0.18	4.77 (0.04)	2.34	0.29	4.77 (0.16)	2.40	0.24
Cys 56	<2.5	2.23 (0.03)	3.15	0.17	2.43 (0.13)	3.15	0.19	2.44 (0.27)	3.13	0.23

[a] pK_a values were obtained from nonlinear least-squares fits to eq 1. Fitting errors at 1 standard deviation are in parentheses. Values for Ser 9 and Lys 29 represent amide chemical shifts reporting on their respective hydrogen bond partners of Asp 7 and Asp 27. [b] Experimental values of whole OMTKY3 are from Schaller and Robertson (*38*). [c] $\Delta\delta = \delta_{HA} - \delta_A$. A positive value indicates a downfield shift upon protonation.

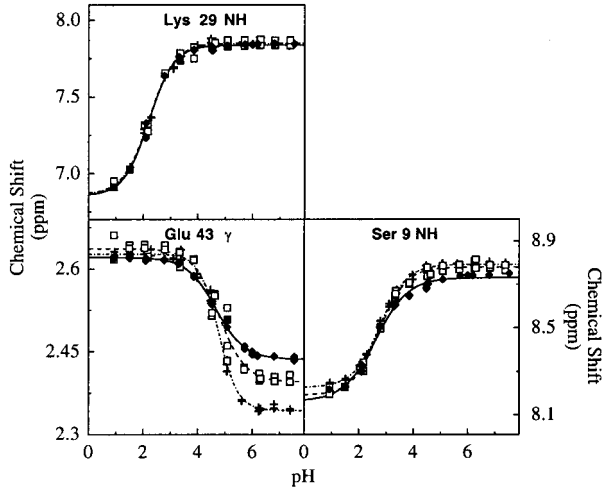

FIGURE 3: pH dependencies of wild-type rOM3 and the K34Q and K34T variants (◆, +, and □, respectively) at 25 °C in 10 mM KCl. NH and γ designate the monitored resonance. Lines represent least-squares fits to eq 1. The data overlap in many instances.

7 Å away from Asp 7 instead of 19 Å (*21*, *22*, *24*, *44*). This might be expected to decrease the pK_a of Asp 7. However, the fitted pK_a values of Asp 7 in wild-type OMTKY3 and rOM3 are not significantly different (Table 1). In fact, the overall agreement among all of the pK_a values is quite good. This suggests that the amino termini in OMTKY3 and rOM3 do not interact with the carboxyl groups in these proteins.

Choice of Mutations. Lysine 34 was predicted to significantly perturb the pK_a values of many nearby acidic residues (*25*). Lysine 34 is highly conserved in ovomucoid third domains (*45*−*47*) but is not so well conserved in the Kazal family as a whole. Gln and Thr are commonly substituted within the family and involve only a single nucleotide alteration. Both variants were constructed with the idea that if the major effect of the substitutions is removal of the positive charge, then both variants should give the same results. If, however, new interactions have been introduced upon substitution, then the two chemically different variant side chains may give different results. The $C_\alpha H$ and NH chemical shifts of the mutants relative to wild-type rOM3 differ by less than 0.1 ppm, indicating very similar overall structures.

The titration profiles and fitted pK_a values for both mutants agree quite well (Figures 2 and 3, Table 1) with a possible modest exception of Glu 19 in the rOM3(K34Q) variant. The overall similarity in profiles and pK_a values for the two variants indicates that the effects of substitution are general.

Role of Lys 34. Lysine 34 was predicted to lower the pK_a values of Asp 7, Glu 10, and Glu 19 by 0.6, 0.4, and 0.6 pK_a unit, respectively. Thus, substitution of Lys 34 with neutral residues was expected to increase the pK_a values for these groups. However, carboxyl groups show the same pK_a values in wild-type and mutant proteins, with the possible exception of a 0.1−0.2 unit elevation in the pK_a value of Glu 10 in the mutants (Table 1). Hence, it appears that Lys 34 contributes little to the perturbation of the acidic pK_a values in OMTKY3.

Structural Considerations. The proposed role of Lys 34 in perturbing carboxyl pK_a values is based on calculations using both the crystal and NMR structures for OMTKY3 (*25*). The sensitivity of the calculations for OMTKY3 on the NMR and crystal structures has been discussed previously (*25*, *48*, *49*). These studies found better overall agreement between predicted and experimental values using the NMR structures. This improvement is attributed to averaging over an ensemble of structures; this has been computationally explored in other proteins using molecular dynamic simulations (e.g., *50*, *51*). A recent study on the acidic groups of OMTKY3 found some improvement by incorporating conformational sampling of side chains into the calculations (*49*). Even in these cases, Asp 7 and Glu 19 are still predicted to have significantly perturbed pK_a values of 2.1 and 3.1, respectively.

One of the acknowledged limitations of pK_a predictions based on electrostatic calculations is the assumption that the crystal or NMR structure accurately represents the protein throughout an experimental pH titration (*48*, *51*, *52*). Some improvement in the agreement between predicted and experimental pK_a values has been observed in studies that take into account possible conformational changes (*53*−*55*). The possibility of such pH-dependent conformational changes in OMTKY3 has not yet been explored in detail.

Origin of Carboxyl pK_a Perturbations. The rOM3 mutants indicate that Lys 34 is contributing little to the pK_a perturbations of nearby acidic groups. The question remains as to the origin of the pK_a perturbations. Lys 34 has a small effect on Glu 10, but Lys 34 is clearly not responsible for perturbing the pK_a of Glu 19. Arg 21 is the most likely candidate for perturbing Glu 19 (*38*).

The origin of the low pK_a of Asp 7 is of particular interest. Even though Asp 7 is exposed to solvent, it is hydrogen bonded to the backbone amide and side chain hydroxyl of Ser 9 (Figure 1). The current work is consistent with the original hypothesis of Schaller and Robertson (*38*) that these

hydrogen bonds, rather than interactions with nearby basic residues, contribute significantly to the pK_a shift.

Perturbation of pK_a values by hydrogen bonds is not unprecedented. A simple example is salicylic acid which has a low carboxyl pK_a of 3.0 relative to pK_a values of 4.2, 4.1, and 4.5 for the related compounds benzoic acid, *m*-hydroxybenzoic acid, and *p*-hydroxybenzoic acid, respectively, in which intramolecular hydrogen bonding is not possible (*57*). The suppressed pK_a of salicylic acid is thus much lower than can be accounted for through induction effects and is attributed to a hydrogen bond between the hydroxyl and the deprotonated carboxyl group (*58*).

Relevance to Electrostatic Calculations. The current work suggests that the electrostatic calculations overestimate the role of Lys 34 in perturbing the pK_a values of nearby carboxyl groups. A tendency to overestimate long-range electrostatic interactions has been previously noted (*25*). In calculating pK_a values, current electrostatic models focus on interactions among full, partial, and induced charges (*3*, *59*−*63*). Hydrogen bonds are handled as charge−dipole interactions. This may be problematic if hydrogen bonds have partial covalent character (*64*−*67*). This partial covalent nature may increase the contributions of hydrogen bonds in altering pK_a values. However, while we can rationalize the low pK_a of Asp 7 by hydrogen bonding, we still do not understand the overall lack of an effect from Lys 34. This puzzle highlights the complexity and difficulty in accurately predicting pK_a values.

Relation to Other Studies. Other studies have employed mutagenesis to alter pK_a values and found that the response to charge replacement is complex (*4*−*16*). Many of these studies sought to modulate catalytic activity through single-site mutations either in or around the active site and used the pH dependence of k_{cat}/K_M, stability, or fluorescence to estimate pK_a values. Such approaches require care in interpretation since the apparent pK_a may not be attributable to a single residue and, in the case of enzymatic activity, the mutations may alter the rate-limiting step, thereby appearing as an altered pK_a (*68*−*70*). Using NMR to determine the pK_a values helps to minimize this problem.

Asp 121 in RNase A plays a significant role in catalysis, as evidenced by a large reduction in activity in the D121N variant (*9*). To test the hypothesis that Asp 121 affects catalysis via modulations in histidine pK_a values, Cederholm et al. (*9*) conducted a combined computational and NMR study of histidine pK_a values in a semisynthetic RNase A containing either aspartate or an asparagine at position 121, which is 8−14 Å distant from the histidine residues. Poisson−Boltzmann calculations indicated that substitution of asparagine for aspartate would lead to decreases of 0.58, 0.55, and 0.10 pH unit in the pK_a values of His 12, His 119, and His 105, respectively. However, the experimental pK_a values for His 12 and His 119 in D121N were only reduced by 0.18 and 0.16 unit, respectively; better agreement between calculation and experiment agreement was observed at His 105. The trend in calculated versus experimental pK_a values is similar to that observed in the present study of OMTKY3.

The active site of RNase A is lined with cationic residues. To investigate the possible role of these residues in catalysis, Fisher et al. (*16*) studied a triple mutant, K7A/R10A/K66A, in which three of these residues were replaced by alanine. Distances between these residues and the active-site hisitidines, His 12 and His 119, vary from 8 to 16 Å; His 105 is 18−27 Å away. The more distant His 105 shows little or no change in pK_a in the mutant relative to wild-type protein, but pK_a values for His 12 and His 119 are increased by 0.3−0.8 unit (*16*). No electrostatic calculations were done in this study, but the observed pK_a perturbations suggest that significant electrostatic interactions are occurring over distances ≥8 Å. This contrasts significantly with the present study, where no significant interaction is detected between residues separated by only 5 Å.

Electrostatic interactions involving His 64 of subtilisin and His 18 of barnase were investigated by comprehensive mutational analysis and pK_a determinations (*8*, *13*). In subtilisin, significant long-range interactions among surface charges were observed. For example, the pK_a of His 64 in subtilisin, an active site residue, was shifted by 0.4 unit upon the replacement of Asp 99, which is 13 Å away. A similar shift was seen upon mutation of Glu 156 which is 15 Å away. In barnase, mutations generally had smaller effects over comparable distances. The authors suggested that this might be related to differences in the extent to which charged residues are exposed to solvent in the two proteins. Agreement between calculated and observed pK_a values in both subtilisin and barnase was good (*13*). The small but significant long-range electrostatic interactions observed in subtilisin and barnase are in contrast to what is observed in the present study of OMTKY3.

The current study analyzed the influence of a basic residue on all acidic residues in a protein using NMR to directly establish the pK_a values. The data indicate that Lys 34 has little influence on the carboxyl pK_a values. This result contrasts with the results of some other studies in which even very long-range interactions can influence pK_a values (*8*, *11*, *13*). In general, however, the response to charge replacements within a protein is mixed and difficult to anticipate. These differences in results are not easily explained and perhaps illustrate that electrostatic interactions have a deeper level of complexity than is currently appreciated. One way to address this complexity is a broad systematic investigation combining mutagenesis with measurement of multiple ionization equilibria simultaneously.

ACKNOWLEDGMENT

We thank Drs. Mike Laskowski, Jr. (Purdue University), Stephen Anderson (Rutgers University), and M. Qasim (Purdue University) for providing the pEZZ318.tky plasmid, RV 308 *E. coli* cells, and technical assistance. We thank Drs. Michael K. Gilson (Center for Advanced Research in Biotechnology, National Institute of Standards and Technology) and Jan Antosiewicz (University of Warsaw, Poland) for helpful discussions.

REFERENCES

1. Matthew, J. B., Gurd, F. R. N., Garcia-Moreno, E. G., Flanagan, M. A., March, K. L., and Shire, S. J. (1985) *CRC Crit. Rev. Biochem. 2*, 91−197.
2. Warshel, A., and Åqvist, J. (1991) *Annu. Rev. Biophys. Chem. 20*, 267−298.
3. Juffer, A. H. (1998) *Biochem. Cell Biol. 76*, 198−209.
4. Spomer, W. E., and Wootton, J. F. (1971) *Biochim. Biophys. Acta 235*, 164−171.
5. Valenzuela, P., and Bender, M. L. (1971) *Biochim. Biophys. Acta 230*, 538−548.

6. Rees, D. C. (1980) *J. Mol. Biol. 141*, 323−326.
7. Thomas, P. G., Russel, A. J., and Fersht, A. R. (1985) *Nature 318*, 375−376.
8. Russell, A. J., Thomas, P. G., and Fersht, A. R. (1987) *J. Mol. Biol. 193*, 803−813.
9. Cederholm, M. T., Stuckey, J. A., Doscher, M. S., and Lee, L. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8116−8120.
10. Dao-pin, S., Söderlind, W., Baase, W. A., Wozniak, J. A., Sauer, U., and Matthews, B. W. (1991) *J. Mol. Biol. 221*, 873−887.
11. Day, P. J., and Shaw, W. V. (1992) *Biochemistry 31*, 4198−4205.
12. Inoue, M., Yamada, H., Yasukochi, T, Kuroki, R., Miki, T., Horiuchi, T., and Imoto, T. (1992) *Biochemistry 31*, 5545−5553.
13. Loewenthal, R., Sancho, J., Reinikainen, T., and Fersht, A. R. (1993) *J. Mol. Biol. 232*, 574−583.
14. Dyson, J. H., Jeng, M.-F., Tennant, L. L., Slaby, I., Lindell, M., Cui, D.-S., Kurpin, S., and Holmgren, A. (1997) *Biochemistry 36*, 2622−2636.
15. DeSantis, G., and Jones, J. B. (1998) *J. Am. Chem. Soc. 120*, 8582−8586.
16. Fisher, B. M., Schultz, L. W., and Raines, R. T. (1998) *Biochemistry 37*, 17386-17401.
17. Ardelt, W., and Laskowski, M., Jr. (1991) *J. Mol. Biol. 220*, 1041−1053.
18. Swint, L., and Robertson, A. D. (1993) *Protein Sci. 2*, 2037−2049.
19. Swint-Kruse, L., and Robertson, A. D. (1995) *Biochemistry 34*, 4724−4732.
20. Arrington, C. B., and Robertson, A. D. (1997) *Biochemistry 36*, 8686−8691.
21. Read, R. J., Fujinaga, M., Sielecki, A. R., and James, M. N. G. (1983) *Biochemistry 22*, 4420−4433.
22. Bode, W., Wei, A.-Z., Huber, R., Meyer, E., Travis, J., and Neumann, S. (1986) *EMBO J. 5*, 2453−2458.
23. Krezel, A. M., Darba, P., Robertson, A. D., Fejzo, J., Macura, S., and Markley, J. L. (1994) *J. Mol. Biol. 242*, 203−214.
24. Huang, K., Lu, W., Anderson, S., Laskowski, M., Jr., and James, M. N. G. (1995) *Protein Sci. 4*, 1985−1997.
25. Forsyth, W. R., Gilson, M. K., Antosiewicz, J., Jaren, O. R., and Robertson, A. D. (1998) *Biochemistry 37*, 8643−8652.
26. Weast, R. C., and Astle, M. O., Eds. (1982) in *Handbook of Chemistry and Physics*, 63rd ed., p D-156, Chemical Rubber Publishing Co., Boca Raton, FL.
27. Lu, W. Y., Apostol, I., Qasim, M. A., Warne, N., Wynn, R., Zhang, W. L., Anderson, S., Chiang, Y. W., Ogin, E., Rothberg, I., Ryan, K., and Laskowski, M., Jr. (1997) *J. Mol. Biol. 266*, 441−461.
28. Randall, L. L., and Hardy, S. J. (1986) *Cell 46*, 921−928.
29. Maurer, R., Meyer, B. J., and Ptashne, M. (1980) *J. Mol. Biol. 139*, 147−161.
30. Piantini, U., Sorensen, O. W., and Ernst, R. R. (1982) *J. Am. Chem. Soc. 104*, 6800−6801.
31. Rance, M., Sorensen O. W., Bodenhau, G., Wagner, G. Ernst, R. R., and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479−485.
32. Levitt, M., Freeman, R., and Frenkiel, J. (1982) *J. Magn. Reson. 47*, 328−330.
33. Bax, A., and Davis, D. G. (1985) *J. Magn Reson. 65*, 355−360.
34. Van Geet, A. L. (1968) *Anal. Chem. 40*, 2227−2229.
35. Johnson, M. L., and Frasier, S. G. (1985) *Methods Enzymol. 117*, 301−342.
36. Johnson, M. L., and Faunt, L. M. (1992) *Methods Enzymol. 210*, 1−37.
37. Markley, J. L. (1975) *Acc. Chem. Res. 8*, 70−80.
38. Schaller W., and Robertson, A. D. (1995) *Biochemistry 34*, 4714−4723.
39. DeMarco, A. (1977) *J. Magn. Reson. 26*, 527−528.
40. Bundi, A., and Wüthrich, K. (1979) *Biopolymers 18*, 299−311.
41. Mayer, R., Lancelot, G., and Spach, G. (1979) *Biopolymers 18*, 1293−1296.
42. Ebina, S., and Wüthrich, K. (1984) *J. Mol. Biol. 179*, 283−288.
43. Szyperski, T., Antuch, W., Schick, M., Betz, A., Stone, S. R., and Wüthrich, K. (1994) *Biochemistry 33*, 9303−9310.
44. Papamakos, E., Weber, E., Bode, W., Huber, R., Empie, M. W., Kato, I., and Laskowski, M., Jr. (1982) *J. Mol. Biol. 158*, 515−537.
45. Laskowski, M., Jr., Apostol, I., Ardelt, W., Cook, J., Giletto, A., Kelly, C. A., Lu, W., Park, S. J., Qasim, M. A., Whatley, H. E., Wieczorek, A., and Wynn, R. (1990) *J. Protein Chem. 9*, 715−725.
46. Laskowski, M., Jr., Kato, I., Ardelt, W., Cook, J., Denton, A., Empie, M. W., Kohr, W. J., Park, S. J., Parks, K., Schatzley, B. L., Schoenberger, O. L., Tashiro, M., Vichot, G., Whatley, H. E., Weiczorek, A., and Wieczorek, M. (1987) *Biochemistry 26*, 202−221.
47. Apostol, I., Giletto, A., Komiyama, R., Zhang, W., and Laskowski, M., Jr. (1993) *J. Protein Chem. 12*, 419−433.
48. Antosiewicz, J., McCammon, J. A., and Gilson, M. K. (1996) *Biochemistry 35*, 7819−7833.
49. Havranek, J. J., and Harbury, P. H. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 11145−11150.
50. Bashford, D., Case, D. A., Dalvit, C., Tennant L., and Wright, P. E. (1993) *Biochemistry 32*, 8045−8056.
51. van Vlijmen, H. W. T., Schaefer, M., and Karplus, M. (1998) *Proteins: Struct., Funct., Genet. 33*, 145−158.
52. Warshel, A., and Papazyan, A. (1998) *Curr. Opin. Stuct. Biol. 8*, 211−217.
53. Wlodek, S. T., Antosiewicz, J., and McCammon, J. A. (1997) *Protein Sci. 6*, 373−382.
54. Zhou, H.-X., and Vijayakumar, M. (1997) *J. Mol. Biol. 267*, 1002−1011.
55. Luo, R., Head, M. S., Moult, J., and Gilson, M. K. (1998) *J. Am. Chem. Soc. 120*, 6138−6146.
56. Koradi, R., Billeter, M., and Wüthrich, K. (1996) *J. Mol. Graphics 14*, 51−55.
57. Lide, D. R., and Frederikse, H. P. R., Eds. (1996) in *Handbook of Chemistry and Physics*, 77th ed., pp 8−51, Chemical Rubber Publishing Co., Boca Raton, FL.
58. Eigen, M. (1964) *Agnew. Chem., Int. Ed. Engl. 3*, 1−72.
59. Honig, B., and Nicholls, A. (1995) *Science 268*, 1144−1149.
60. Gilson, M. K. (1995) *Curr. Opin. Struct. Biol. 5*, 216−223.
61. Yang, A.-S., Gunner, M. R., Sampogna, R., Sharp, L., and Honig, B. (1993) *Proteins: Struct., Funct., Genet. 15*, 252−265.
62. Burley, S. K., and Petsko, G. A. (1988) *Adv. Protein Chem. 39*, 125−189.
63. Ullmann, G. M., and Knapp, E.-W. (1999) *Eur. Biophys. J. 28*, 533−551.
64. Blake, P. R. Park, J. B., Adams, M. W. W., and Summers, M. F. (1992) *J. Am. Chem. Soc. 114*, 4931−4933.
65. Cordier, F., and Grzesiek, S. (1999) *J. Am. Chem. Soc. 121*, 1601−1602.
66. Cornilescu, G., Hu, J. S., and Bax, A. (1999) *J. Am. Chem. Soc. 121*, 2949−2950.
67. Isaacs, E. D., Shukla, A., Platzman, P. M., Hamann, D. R., Barbiellini, B., and Tulk, C. A. (1999) *Phys. Rev. Lett. 82*, 600−603.
68. Plapp, B. V. (1995) *Methods Enzymol. 249*, 91−119.
69. Czerwinski, R. M., Harris, T. K., Johnson, W. H., Jr., Legler, P. M., Stivers, J. T., Mildvan, A. S., and Whitman, C. P. (1999) *Biochemistry 38*, 12358−12366.
70. Fersht, A. (1999) *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*, pp 173−181, W. H. Freeman and Company, New York.

BI992967P