Measurement of the Individual pK_a Values of Acidic Residues of Hen and Turkey Lysozymes by Two-Dimensional ^1H NMR

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ABSTRACT The pH dependence of the two-dimensional ^1H nuclear magnetic resonance spectra of hen and turkey egg-white lysozymes has been recorded over the pH range 1–7. By monitoring the chemical shifts of the resonances of the various protons of ionizable residues, individual pK_a values for the acidic residues have been determined for both proteins. The pK_a values are displaced, with the exception of those of the residues in the active site cleft, by an average of 1 unit to low pH compared to model compounds.

INTRODUCTION

Electrostatic interactions influence many aspects of protein structure and have an immediate relevance to chemical reactivity, binding, and catalysis (Perutz, 1978; Matthew, 1985; Sharp and Honig, 1990). pK_a values of ionizable groups are a direct experimental probe of electrostatic effects in proteins, and knowledge of accurate values can be important evidence in the formulation of enzymatic mechanisms and in the understanding of protein folding and stability (Fersht, 1985). They also provide a direct test for computational procedures designed to enhance our understanding of electrostatic effects in proteins from a theoretical standpoint (Sharp and Honig, 1990; Bashford and Karplus, 1990).

NMR chemical shift values are sensitive to the electronic environment around the nucleus of an atom. As a consequence of this, changes in ionization state can lead to changes in the chemical shifts of nuclei in close proximity to the ionizable group. At any pH value, the protonated and deprotonated species are usually in fast chemical exchange, and the observed chemical shift of a resonance is a weighted average of the chemical shifts of the two species. Thus, plots of chemical shift versus pH can enable, under appropriate conditions, pK_a values to be determined for individual ionizable groups (Wüthrich, 1986).

Assignments for the ^1H resonances of hen lysozyme (Redfield and Dobson, 1988) and turkey lysozyme (Bartik et al., 1993) have been reported. Both proteins contain 129 amino acid residues, but their sequences differ at seven positions. Four of these involve histidine or acidic residues (His15/Leu15; Gln41/His41; Asp101/Gly101; Gln121/His121, giving the hen lysozyme residue first). One of these residues, Asp101/Gly101, is located in the active site cleft of lysozyme. The other three amino acid changes involve histidine and could influence the electrostatic interactions around neutral pH. In this paper we present the pK_a values for all the carboxylate groups and histidine residues of hen and turkey lysozymes, and discuss the values in terms of their local environments.

METHODS

Hen and turkey egg white lysozymes were obtained from Sigma Chemical Co. (St. Louis, MO). The lysozymes were dialyzed at pH 3 to remove acetate. The proteins were dissolved in 90%H_2O:10%D_2O at a concentration of 2 mM. Separate samples were prepared for each pH value; a total of 13 samples in the pH range 1–7 were prepared for each protein. The pH of the samples was adjusted in steps of ~0.5 pH units with dilute HCl or NaOH. The total concentration of added salts in each sample was kept constant at 100 mM by the addition of appropriate quantities of 2.5 M NaCl. A single sample was additionally used for the measurement of the histidine pK_a values of turkey lysozyme. The protein was dissolved at a concentration of 5.5 mM in 500 μl of D_2O, and the pH was adjusted in ~0.5 pH steps with NaOD and DCl. The pH values given are direct pH meter readings measured at 20°C and are not corrected for isotope effects (Bundi and Wüthrich, 1979). The pH meter was calibrated with standard buffer solutions (Sigma) at pH 4 and 7. The temperature dependence of the sample pH was checked by recalibrating the pH meter at 35°C and measuring the pH of a lysozyme sample incubated at 35°C; the sample pH was found to change by less than 0.1 pH units. The uncertainty in the reported pH values arising from the temperature dependence and the pH meter is estimated, therefore, to be ±0.1 pH units. All chemical shifts are referenced to 1,4-dioxane, which was added to the samples in low concentration. The uncertainty in the measured chemical shifts is ±0.02 ppm.

Phase-sensitive DQF-COSY spectra (Piantini et al., 1982) were recorded with a standard phase cycling scheme (States et al., 1982). Experiments were performed at 35°C on the VARIAN Unity 600 MHz NMR spectrometer installed at Université Libre de Bruxelles. The solvent resonance was suppressed by low power, continuous wave irradiation during a 0.7-s preparation delay. 256 complex t_1 increments, defined by 1024 points using 8 transients, were collected. A spectral width of 8500 Hz was used in both dimensions. Spectra were resolution enhanced by double exponential multiplication and trapezoidal multiplication in the F_2 dimension and by trapezoidal multiplication in the F_1 dimension. After zero-filling the digital resolution was 4.1 Hz/point in the F_2 dimension and 8.3 Hz/point in the F_1 dimension.

Histidine titrations for turkey lysozyme were monitored by one-dimensional NMR spectroscopy. Data sets were defined by 4096 complex

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Abbreviations used in this article: DQF-COSY, two-dimensional double-quantum-filtered J-correlated spectroscopy; NMR, nuclear magnetic resonance; ppm, parts per million; δ_{low}, chemical shift value at low pH plateau; δ_{high}, chemical shift value at high pH plateau.

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points and consisted of 128 transients. The data were resolution enhanced by an unshifted sinebell. After zero-filling the digital resolution was 0.96 Hz/point.

The apparent pK$_a$ values of titrated proton resonances were obtained by fitting the variation of chemical shift with pH to the Henderson-Hasselbach equation using a computer program incorporating the downhill simplex method (Press et al., 1986). Three parameters, the pK$_a$ value and the chemical shift values at the high and low pH plateaus, were optimized. The uncertainty in the individual pK$_a$ values was assessed as follows. The fitting procedure was executed 1000 times. In each run the pH and chemical shift values for each experimental data point were assigned a random value within ± 0.1 and ± 0.02 units, respectively, of the experimentally determined pH and chemical shift value. The reported pK$_a$ values and the chemical shift values at the low and high pH plateaus are the average of the 1000 calculated values. The standard deviation for the 1000 fitted pK$_a$ values gives a measure of the uncertainty. The pH dependences were also analyzed by plotting $\log[(\delta_{high} - \delta)/(\delta - \delta_{low})]$ versus pH in the pH range of pK$_a \pm 1.0$. For all residues the Hill constant was measured from these plots to be 1.0 ± 0.1, indicating no detectable cooperativity between different ionization events. There was no evidence for hysteresis in the pH dependence of any of the chemical shift values.

RESULTS AND DISCUSSION

Spectra of hen and turkey lysozymes were recorded between pH 1 and 7. There are no major changes indicative of significant cooperative conformational changes in the spectra of either protein over this pH range, although at lower pH values major spectral changes associated with unfolding to the denatured states can be readily detected (Cooper et al., 1992). A large number of small changes in chemical shifts were observed, however, in the spectra of both lysozymes and attributed to local changes in the environment associated with the ionization of individual residues in the protein. Examples of spectra showing such changes are given in Fig. 1. For hen lysozyme extensive line broadening, indicative of aggregation, was observed above pH 5.5. This aggregation is not observed in turkey lysozyme and is thought to be associated with His15 in hen lysozyme (Imoto et al., 1972).

To measure the pK$_a$ values of these individual ionizable groups, the chemical shift values of the NH, αCH, and βCH protons of the acidic residues and the ϵ_1CH and δ_2CH protons of the histidines were monitored. The resonances of all acidic residues, except those of Glu 35 which broaden out at high pH, could be followed in both proteins through the ionization step of the side chain. The amplitude of the various shifts with pH, however, varied substantially between residues; representative data are shown in Fig. 2. For example, in the case of Asp18 in hen lysozyme the two βCH protons shift by more than 0.15 ppm upfield as the pH is increased, whereas in the case of Asp52 in turkey lysozyme one of the βCH protons shifts by 0.3 ppm, and the other shifts by only 0.05 ppm. In simple model compounds, identical shifts of approximately 0.2 ppm are reported for the two protons adjacent to the titrating group (Bundi and Wüthrich, 1979). It is likely that in proteins the magnitude of the shifts depends on the conformational state of an individual residue, including which rotamer is populated, and on the relative orientation of the ionizable group to the rest of the residue. The data available for lysozyme in the present work, however, are inadequate to establish the specific effects of these various contributions. By following the protons of each residue, pK$_a$ values were defined and are listed in Table 1. The pK$_a$ values listed in Table 1 were obtained from the resonances that showed the greatest chemical shift changes with pH. When pK$_a$ values could be measured for more than one proton of a particular residue the values were found to agree within the reported errors. The pK$_a$ values of Glu35 could not be measured in this manner but were determined from the substantial shifts induced on ionization of this residue in the ring protons of the neighboring residue Trp108 (Cassels et al., 1978).

Comparison of the pK$_a$ values of the lysozyme residues with those of amino acid side chains in unstructured peptides and proteins (Bundi and Wuthrich, 1979; Nozaki and Tanford, 1967) shows that these values are substantially different; they are shifted on average by approximately 1 pH unit, and in two cases by more than 1.5 pH units, to low pH. The exceptions are the three residues in the active site cleft of hen lysozyme (Glu35, Asp52 and Asp101) and the two residues in the active site of turkey lysozyme (Glu35 and Asp52); their pK$_a$ values are little changed from those of model compounds or, in the case of Glu 35, shifted to higher pH. One important feature of the present data is that this pattern was not visible from earlier estimates of pK values; both Asp48 and Asp87 were presumed previously to have pK$_a$ values close to normal, rather than the displacements of 1.5 units or more to low pH evident from the values in Table 1 (Imoto et al., 1972).

One general explanation of the displacement of the pK$_a$ values may be simply that the net positive charge on the lysozymes is high at the pH values over which the acid groups titrate; the isoelectric points of the proteins are approximately 11 (Imoto et al., 1972). This should result in an overall decrease in stability of the positively charged ionization state of histidine residues and an increase in stability of the negatively charged state of carboxylate groups, in accord with the observed decreases in pK$_a$ values. Furthermore, analysis of the crystal structures of both proteins (Handoll, 1985; coordinates deposited in the Brookhaven data base 1LZ3, 1992 (Harata) indicates that the carboxylate groups of all the acidic residues, except Glu35 and Asp101, form hydrogen bonds likely to stabilize the charged forms of the ionizable groups. That the three residues of hen lysozyme and the two residues of turkey lysozyme in the active site have pK$_a$ values substantially higher than the remainder of acidic residues in the protein may arise from the hydrophobic nature of the cleft and the interaction between the carboxylate groups of Glu35 and Asp52 (Blake et al., 1967; Kurimatsu and Hamaguchi, 1980). The fact that the pK$_a$ values of Glu35 and Asp52, and indeed those of other conserved residues, are essentially identical in hen and turkey lysozymes is consistent with the identical activity profile for the enzymes as a function of pH (Fukamizo et al., 1983) and indicates that interaction with Asp101 (substituted by glycine in the turkey protein) is not a contributing factor to the relative stability of different ionization states.

The pK$_a$ values for ionizable groups in a protein are a direct experimental probe of the local environments of the

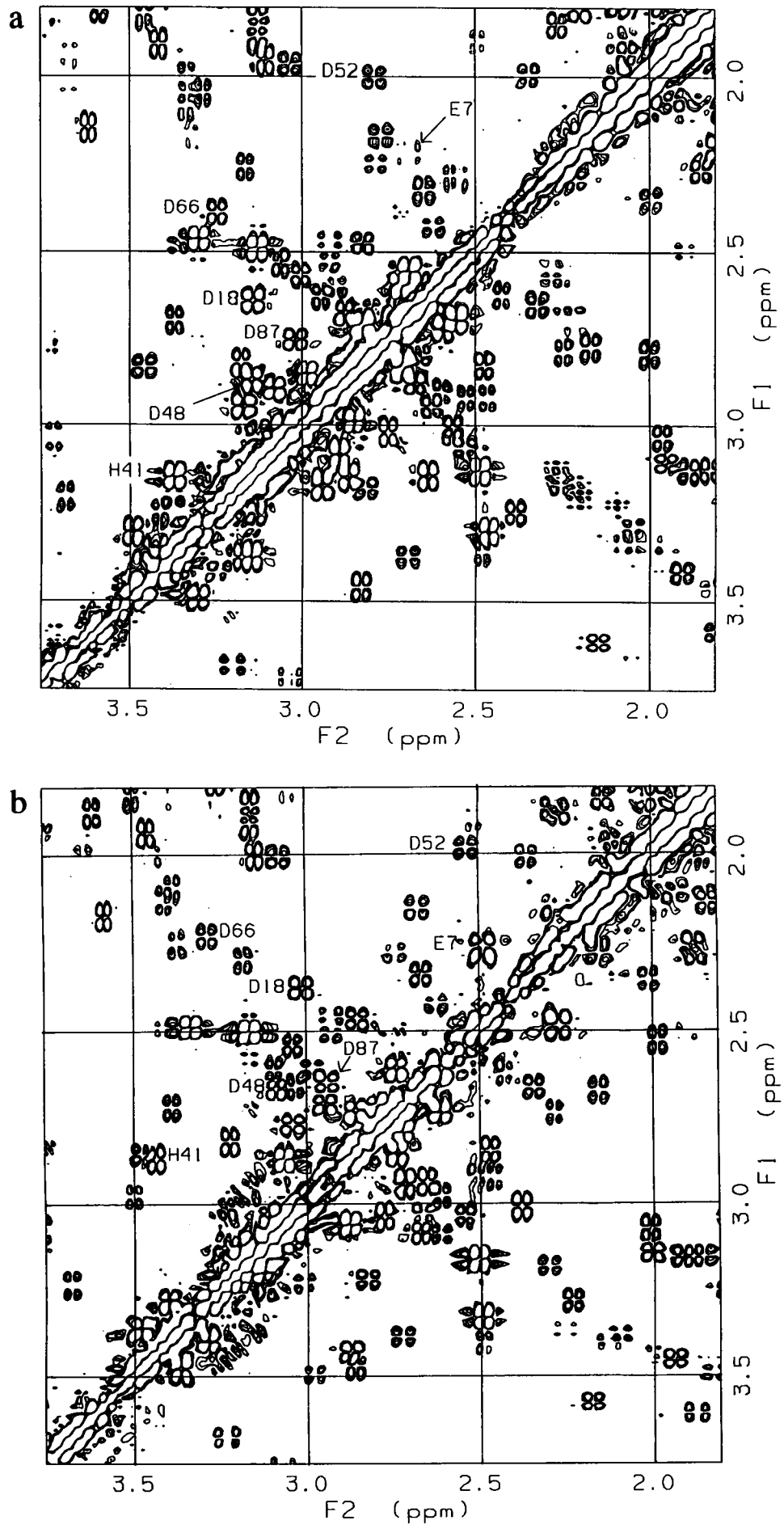

FIGURE 1 Part of the 600 MHz DQF-COSY spectra of turkey egg-white lysozyme at (*a*) pH 1.1 and (*b*) pH 5.9. Spectra were recorded at 35°C. βCH-βCH cross peaks of His41, Asp18, Asp48, Asp52, Asp66, and Asp87 are labeled along with a βCH-γCH peak of Glu7.

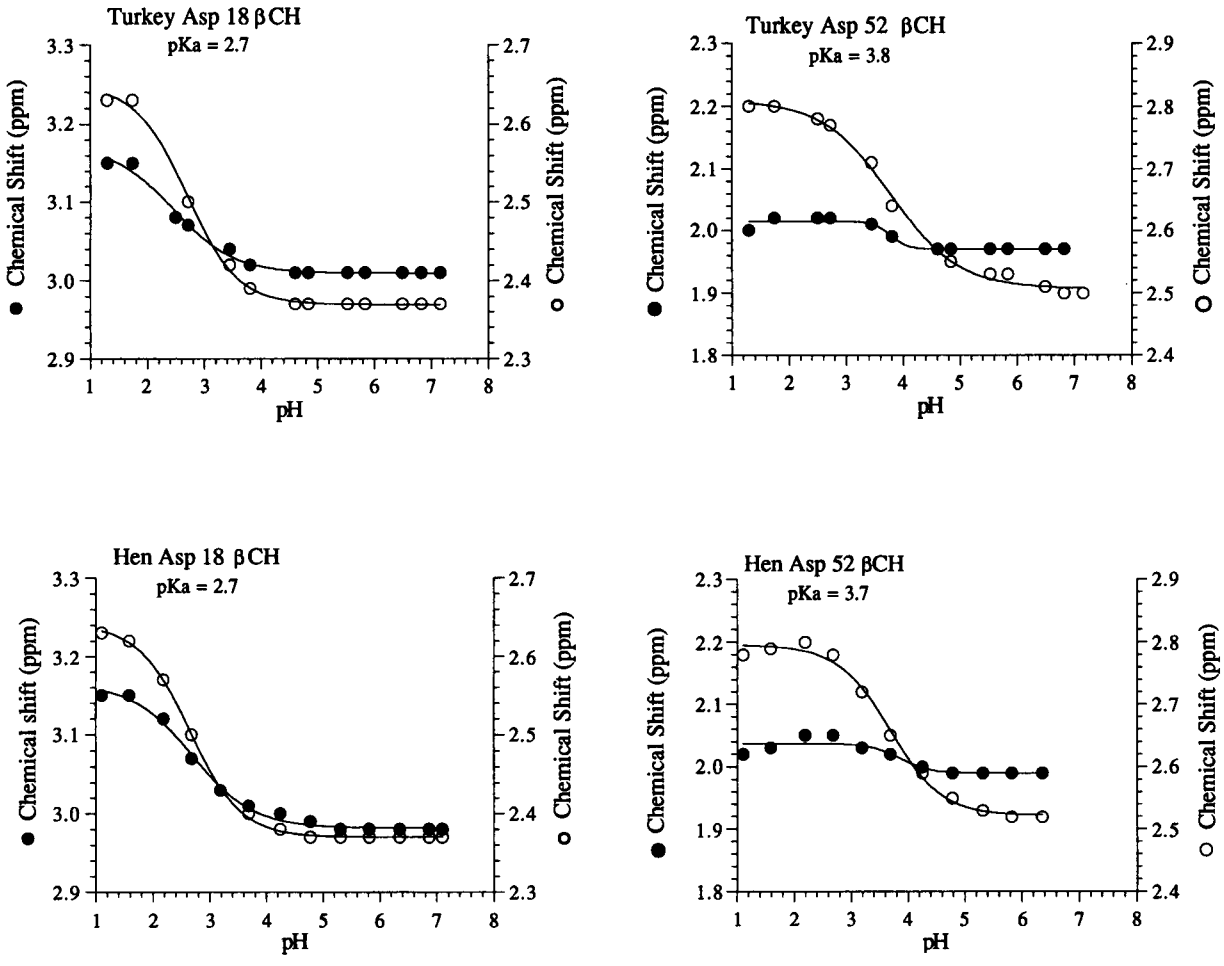

FIGURE 2 pH dependence of the chemical shifts of the βCH protons of Asp18 and Asp52 in hen and turkey lysozymes. The chemical shifts were obtained from DQF-COSY spectra. The fitted titration curves and calculated pK$_a$ values are shown.

TABLE 1 pK$_a$ values of individual groups in hen and turkey lysozymes at 35°C in H_2O

Residue	pK$_{mod}$*	Turkey pK$_a$	Turkey $\delta_{low}/\delta_{high}$	Hen pK$_a$	Hen $\delta_{low}/\delta_{high}$
			ppm		*ppm*
Glu7	4.4	2.68 ± 0.09‡	2.72/2.48 (β)§	2.85 ± 0.25	4.18/4.11 (α)
Asp18	4.0	2.68 ± 0.10	2.66/2.37 (β)	2.66 ± 0.08	2.67/2.37 (β)
Glu35	4.4	6.06 ± 0.12	10.02/10.22 (Trp108ε1)	6.20 ± 0.10	10.01/10.22 (Trp108ε1)
Asp48	4.0	<2.5 (1.5 ± 0.5)¶	(2.80)/2.66 (β)	<2.5 (1.6 ± 0.4)¶	(2.82)/2.66 (β)
Asp52	4.0	3.78 ± 0.08	2.80/2.52 (β)	3.68 ± 0.08	2.80/2.52 (β)
Asp66	4.0	<2.0 (1.4 ± 0.4)¶	(2.39)/2.23 (β)	<2.0 (0.9 ± 0.5)¶	(2.42)/2.23 (β)
Asp87	4.0	2.13 ± 0.16	4.95/4.79 (α)	2.07 ± 0.15	5.07/4.93 (α)
Asp101	4.0			4.09 ± 0.07	3.21/2.45 (β)
Asp119	4.0	3.35 ± 0.11	3.17/2.93 (β)	3.20 ± 0.09	3.13/2.92 (β)
Leu129‖	3.8	2.92 ± 0.13	4.43/4.26 (α)	2.75 ± 0.12	4.43/4.26 (α)
His15	6.3			5.36 ± 0.07	3.72/3.37 (β)
His41	6.3	5.56 ± 0.05	8.70 / 7.80 (ε1)		
His121	6.3	5.25 ± 0.04	8.68/7.51 (ε1)		

* pK$_{mod}$ values are taken from the titration behavior of denatured proteins.

‡ pK$_a$ ± standard deviation. See Methods for a description of the fitting procedure.

§ The Greek letters indicate the proton whose chemical shift was monitored.

¶ Upper limits are given for the pK$_a$ values of Asp48 and Asp66 because an insufficient number of chemical shift values were obtained at low pH. The pK$_a$ values given in parentheses are the values obtained from the fitting program; the large standard deviations arise because of the lack of low pH data.

‖ C-terminal residue.

different residues. NMR has the unique ability to monitor individual pK_a values and, hence, to provide data to test electrostatic models and to evaluate the influence of electrostatic effects on the structure and stability of the proteins. Hen lysozyme is one of the proteins receiving most attention in theoretical studies of these phenomena (e.g., Delepierre et al., 1987; Bashford and Karplus, 1990; Oberol and Allewell, 1993). The results reported here should contribute to such studies by providing accurate experimental data for this protein, and for the turkey homologue, for comparison with theoretical pK_a values.

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