Self-Association of Hen Egg-White Lysozyme as Studied by Nuclear Magnetic Resonance†

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ABSTRACT: The ionization constant of His-15 of hen egg-white lysozyme, determined by proton nuclear magnetic resonance (NMR) titration at 40 °C in 0.1 N NaCl, exhibits a dependence on concentration over the range 20 to 150 mg of protein/mL. The pK_a values of His-15 of lysozyme with bound saccharide and of ester-35,108 lysozyme were observed to be almost independent of protein concentration. The tryptophan residues of lysozyme were examined by carbon-13 NMR as functions of pH, protein concentration, and temperature. The γ-carbon resonances of residues Trp-63 and Trp-108 were perturbed in native lysozyme by a change in pH from 5 to 7 but were either not perturbed or perturbed differently in lysozyme with bound saccharide or in ester-35,108-lysozyme. The concentration and temperature dependences of the chemical shifts are consistent with the pH dependence of the shifts and reflect the self-association reaction. On the basis of these results, it is proposed that the portion of the lysozyme surface involved in the head-to-tail self-association includes His-15 and possibly one or more adjacent positively charged residues. The other spatially distinct region involved in the self-association comprises Glu-35, Trp-108, and Trp-63. There is an interaction between His-15 and Glu-35 within the intermolecular interface.

Since the finding of the weak self-association of lysozyme by Sophianopoulos and Van Holde (1961), the nature of this reaction has been clarified using various techniques, including sedimentation equilibrium (Sophianopoulos and Van Holde, 1964; Adams and Filmer, 1966; Sophianopoulos, 1969; Deonier and Williams, 1970), proton magnetic resonance (Studebaker et al., 1971), light scattering (Bruzzesi et al., 1965), and calorimetry (Banerjee et al., 1975). Sophianopoulos (1969) concluded that a head-to-tail model for the process best fit data obtained in the presence and absence of saccharides, which block the self-association. Sophianopoulos also suggested that Glu-35 is perturbed through the self-association.

Banerjee et al. (1975) examined lysozyme derivatives and native lysozyme, using absorbance and calorimetric methods. Their results indicated that Glu-35 and Trp-62 are involved in the self-association. The head-to-tail model of the association process was supported by the observation of hybrid polymers of native lysozyme and oxindolealanine-62-lysozyme.

Proton NMR studies at 100 MHz of the titration properties of the His-15 C-2 ring proton resonance of lysozyme and lysozyme with substrate bound have been reported by Cohen and Jardetzky (1968). They found no significant effect of the substrate on the pK_a value of His-15. A more detailed analysis is presented here and shows that the pK_a of His-15 exhibits a protein concentration dependence and that the pK_a is almost independent of concentration for lysozyme with bound saccharide or ester-35,108-lysozyme.

In order to clarify the nature of the self-association, carbon-13 chemical shifts of tryptophan residues were also examined as functions of pH, temperature, and concentration of lysozyme. The observed changes in chemical shifts were consistent with a self-association reaction of lysozyme.

Experimental Procedures

Materials. Hen egg-white lysozyme was purchased from Worthington Biochemical Corp. (LYSF) and dialyzed exhaustively against deionized water at pH 3. Ester-35,108-lysozyme was prepared by iodine oxidation as described previously (Imoto et al., 1973a), followed by purification using chromatography on Bio-Rex 70. The derivative was subjected to ultrafiltration, using an Amicon Model MMC to remove a trace of acetate, and then lyophilized. The (1→4)-linked dimer and trimer of *N*-acetylglucosamine (GlcNAc)[1] were prepared from chitin (Rupley, 1964). Protein concentrations were determined using values of $E_{280}^{1\%}$ of 25.5 for lysozyme and 26.5 for ester-35,108-lysozyme at a neutral pH (Imoto et al., 1973a,b).

NMR Measurements. All solutes used for proton NMR were lyophilized several times from D_2O (99.8%, Wilmad Glass Corp.). Proton samples for measurements were freshly dissolved in D_2O (0.1 N NaCl) and adjusted to the desired pH using 0.2 or 1 N NaOD and DCl. Measurements of pH were made at 40 °C for proton NMR titrations and at room temperature for carbon-13 NMR, using a Radiometer Model 26 instrument equipped with a long, thin Ingold combination electrode fitting inside the NMR tube. Direct meter readings are reported. Differences in concentration before and after a NMR measurement were usually less than 3% at low pH and 6% at high concentration and high pH.

Proton NMR spectra were obtained at 220 MHz on a Varian Associates HR-220 spectrometer equipped with an FT

† From the Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20014. *Received November 22, 1976; revised manuscript received May 11, 1977.* During the processing of this communication, a paper describing the self-association of hen egg-white lysozyme appeared (R. S. Norton and A. Allerhand (1977), *J. Biol. Chem. 252,* 1795). The authors implicated Trp-62 in the self-association process above pH 6. We will present evidence implicating His-15, Trp-108, and Trp-63 in the self-association of lysozyme. In our study a concentration dependence of the chemical shift of the γ-carbon resonance of Trp-62 was not observed at pH 4.75 but was observed for Trp-63. This suggests that the self-association modes may somewhat differ below and above pH 6, possibly due to a change in the extent of the intermolecular surface and the protonation and deprotonation of the Glu-35 and His-15 residues in the pH range 5–7.

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[1] Abbreviations used: GlcNAc, *N*-acetylglucosamine; NMR, nuclear magnetic resonance; DSS, sodium 3-(trimethylsilyl)-1-propanesulfonate.

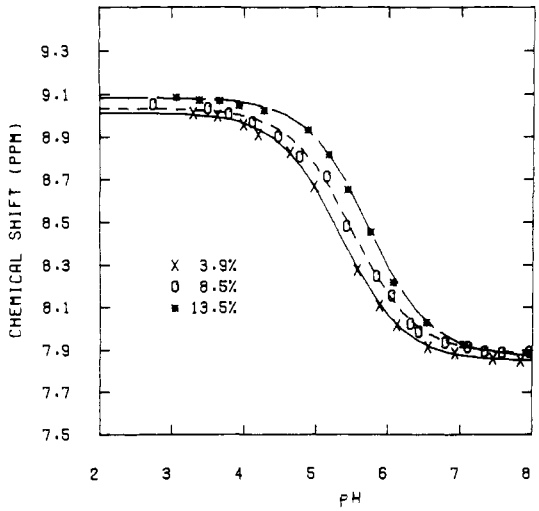

FIGURE 1: Proton NMR titration curves of the C-2 proton of histidine residue 15 at different concentrations of lysozyme, 40 ± 1 °C, 0.1 N NaCl (D_2O).

TABLE I: Parameters of the NMR Titration Curve of the His-15 C-2 Proton of Lysozyme Obtained from Computer Curve Fitting.

Lysozyme	Concn (%)	δ_{min} (ppm)[a]	$\Delta\delta$ (ppm)[a]	pK_a
Native	13.5	7.87	1.21	5.73 ± 0.02
	8.5	7.88	1.15	5.50 ± 0.03
	3.9	7.85	1.16	5.35 ± 0.02
Native-trisaccharide (1:1.5)	14.4	7.87	1.16	5.67 ± 0.02
	3.8	7.81	1.17	5.58 ± 0.03
Ester-35,108-lysozyme	9.8	7.87	1.14	5.47 ± 0.02
	2.0	7.82	1.14	5.38 ± 0.01

[a] δ_{min} is the chemical shift at high pH and $\Delta\delta$ the change of chemical shift on titration.

accessory with a 620L computer. Sample temperature was 40 ± 1 °C. Chemical-shift values are reported in parts per million downfield from DSS as an internal reference (ca. 1:20 molar ratio of DSS to protein). Sample concentration for C-13 NMR studies was about 15% by weight unless otherwise indicated. Carbon-13 spectra were obtained at 40 ± 2 °C and at 67.9 MHz on a homebuilt spectrometer with a Bruker magnet and probe, equipped with a Nicolet Model 1080 computer with maximum 32K data memory. The Bruker probe was replaced in the course of these experiments by a homemade 12-mm carbon-13 probe, which gave better sensitivity and temperature control. Carbon-13 signals were usually collected using 15 KHz sweep width, 16K data points unless otherwise stated, and 15 to 22K scans (corresponding to 8 to 12 h accumulation time). Chemical shifts were referred to a small amount of added dioxane and were converted to the Me_4Si scale using $\delta_{dioxane}$ = 67.37 ppm.

Least-square fitting of NMR titration data was carried out using a Digital Equipment Corp. PDP10 computer at the Division of Computer Research and Technology, National Institutes of Health.

Results and Discussion

The pK_a value of His-15 increases as a function of protein concentration, as evidenced by the C-2 proton NMR titration curves (Figure 1). pK_a values and related parameters obtained through least-squares fitting are listed in Table I. Measurements to determine pK_a values at different protein concentrations were carried out in parallel to avoid systematic errors. This procedure gives highly precise values of pK shifts (ΔpK). As is seen from the table, the pK_a value of His-15 of native lysozyme increases with increasing concentration (ΔpK = 0.38), while the pK_a values of lysozyme with bound $(GlcNAc)_3$ and ester-35,108-lysozyme exhibit a small difference (ΔpK = 0.09) just beyond the usual error in pH measurements (0.05 pH unit). The pK_a value of the histidine residue of native lysozyme is lower than the usual value of 6.0–6.5 for free histidine, which may be due, as suggested by the results of x-ray crystallography (Blake et al., 1965), to hydrogen-bond formation with Thr-89, or an adjacent positive charge such as that of Arg-14, or both. Of course, the absolute values of pK_a in different species cannot be directly compared because very small local effects can change the pK_a value.

The concentration dependence of the pK_a value of His-15 is possibly due to an interaction of this residue with a negatively charged group on another molecule within the polymer. It has been shown that ionization of the carboxyl group of Glu-35 accounts for the pH dependence of the self-association (Sophianopoulos and Van Holde, 1964; Sophianopoulos, 1969; Banerjee et al., 1975). In the light of this information, it is possible to consider several explanations for the concentration dependence of the pK_a value of His-15: (i) There is an interaction between His-15 and Glu-35, and this is the only interaction of charged groups within the polymer interface. (ii) Glu-35 interacts with His-15 and also with other residues. For example, Glu-35 of one molecule could interact with Arg-14 and His-15 of an adjacent molecule. (iii) His-15 does not participate in electrostatic interactions within the interface, in which case the increased pK_a would reflect other interactions, such as hydrogen bonding. The same situation could obtain also for Glu-35.

Case i can be discounted as follows: if the pH dependence of the self-association is of electrostatic origin and involves only Glu-35 and His-15, then there must be a maximum association in the pH range between the pK_a values of His-15 and Glu-35; this is not observed (Banerjee et al., 1975). It is difficult to distinguish between cases ii and iii, using NMR results alone. Case ii, in which Glu-35 interacts with His-15 and one or more other residues, is supported by two lines of evidence. First, ester-35,108-lysozyme exhibits a self-association without a pH dependence (Banerjee et al., 1975), indicating that the ionization of Glu-35 is linked to the pH dependent contribution of His-15. Additionally, it has been reported that the modification of His-15 produces a weaker self-association and a different pH dependence for the process (Venkatappa et al., 1968).

The pH dependence of the chemical shifts of the γ-carbon resonances of the tryptophan residues of lysozyme and its derivatives is shown in Figure 2 (the numbering system of the peaks refers to the spectra in Figure 3). For native lysozyme, with increasing pH the chemical shift of peak 1 (due to Trp-108) is clearly observed to be shifted downfield over the pH range 5–7 by 17 Hz but, within experimental error, no dependence is observed for peaks 2 and 5 (due to Trp-23 and Trp-28 plus Trp-111, respectively); these assignments are from Oldfield et al. (1975). A small downfield shift is observed for peak 3 (Trp-63) above pH 6; peak 4 (Trp-62) was significantly broadened by the pH change and we were unable to follow this resonance above pH 6.5 (where their assignments were made by Norton and Allerhand (1976)). The resonance A is observed in this spectral range above pH 4 for native lysozyme but not observed for $(GlcNAc)_2$ bound lysozyme and for ester-

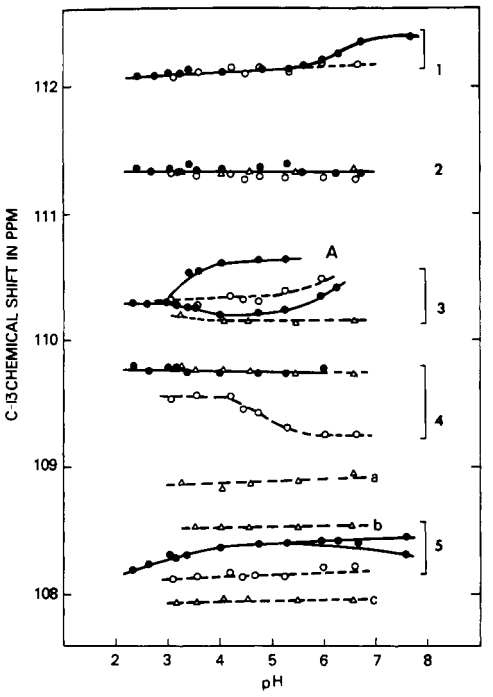

FIGURE 2: γ-Carbon chemical shifts of tryptophan residues of lysozyme at 40 ± 2 °C, 0.1 N NaCl (H_2O:D_2O = 4:1) as a function of pH. (●) native lysozyme; (○) $(GlcNAc)_2$ bound lysozyme; and (△) ester-35,108-lysozyme. Assignments of peaks a, b, and c of ester-35,108-lysozyme are not clear, but there are essentially no pH dependencies of these chemical shifts.

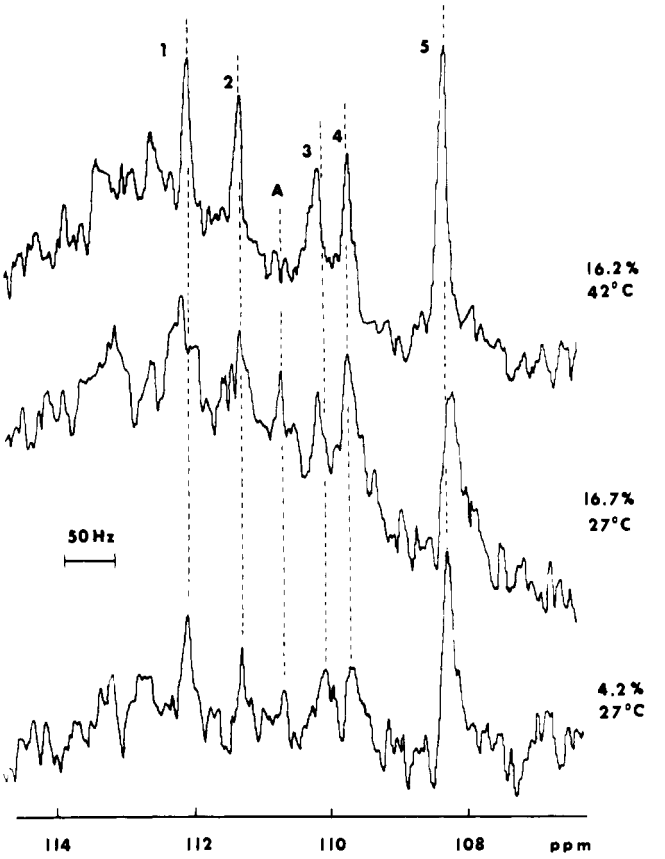

FIGURE 3: Carbon-13 spectra in the range of γ-carbon resonances of tryptophan residues of lysozyme at pH 4.75, 0.1 N NaCl (H_2O:D_2O = 1:4) at differing temperatures and concentrations. Upper trace: 16.2% lysozyme at 42 °C, 15 KHz sweep width, 16K data points plus 16K zero filling in the time domain (digital resolution 0.9 Hz in the frequency domain), 3 Hz additional line broadening, 34K scans with 1.45-s delay time (15.4 h acc.). Middle trace: 16.7% lysozyme at 27 °C, conditions the same as the above but 22.5K scans (12.5 h acc.). Lower trace: 4.2% lysozyme at 27 °C, conditions the same as the above but 58K scans (32 h acc.).

35,108-lysozyme, and may be due to a ζ_2-carbon resonance of the tryptophan residue.

Both for $(GlcNAc)_2$ bound lysozyme and ester-35,108-lysozyme, no dependence of the chemical shift for peaks 1, 2, 3, and 5 is observed; however, peak 4 of $(GlcNAc)_2$ bound lysozyme does exhibit an inflection point at about pH 4.7 which probably reflects the ionization of a carboxyl group in the vicinity of Trp-62 (probably Asp-101). Three peaks a, b, and c of ester-35,108-lysozyme are not clear for their assignments but it should be noted that these exhibit no particular change in chemical shift by pH.

There is a close correspondence between the chemical-shift behavior due to a pH change and the temperature dependence observed by Cozzone et al. (1975), who attributed the changes in chemical shift to a conformational process. However, other evidence suggests that the changes in chemical shift may be due to the increased self-association of lysozyme at lower temperatures and higher pH values. Thus, lysozyme with bound $(GlcNAc)_2$ and ester-35,108-lysozyme, which are known to remain monomeric or associate only very weakly (Banerjee et al., 1975; Sophianopoulos, 1964), exhibit no pH-dependence comparable to native lysozyme.

Additionally, we have carried out measurements of the concentration and temperature dependences of the carbon-13 chemical shifts of γ-carbon resonances of tryptophan residues over the concentration range $3\text{-}12 \times 10^{-3}$ M lysozyme (i.e., 4-17% w/v) at pH 4.75, 0.1 N NaCl (H_2O:D_2O = 1:4). Figure 3 shows spectra of the γ-carbon resonances of the tryptophan residues at different concentrations and temperature. It is clear from a comparison of chemical shifts at a given concentration but at different temperatures (27 and 42 °C) that peaks 1 and 3 (due to the residues Trp-108 and Trp-63) are shifted downfield by ca. 8.1 and 3.3 Hz, respectively, and that peak 5 (due to either Trp-28 or -111) is shifted upfield by 3.9 Hz with decreasing temperature. From a comparison of the middle and lower spectra, peaks 1 and 3 are shifted downfield by 7.5 and 6.0 Hz, respectively, and peaks 5 upfield by 3.0 Hz with an increase in concentration by a factor of 4. Similar chemical-shift behavior was observed at pH 5.5 and 47 °C; namely, peaks 1 and 3 exhibited downfield shifts by 4.8 and 3.4, respectively, and peak 5 no shift with change in concentration from 5.3 to 16.0% lysozyme. The effect on chemical shifts of decreasing temperature at constant concentration and pH is similar to the effect of increasing concentration or pH at constant temperature. This observation is in accord with the association constant increasing with decreasing temperature (Sophianopoulos, 1969; Banerjee et al., 1975). It should be noted that our observation of concentration and temperature-dependent chemical shifts did not indicate a perturbation of Trp-62 upon self-association. This does not mean that the residue Trp-62 is not located on the intermolecular interface as chemical-shift changes are not always observed to occur in the presence of perturbations. Norton and Allerhand (1976) showed that chemical modification of Trp-62 perturbs the chemical shift of the γ-carbon resonance of Trp-63. A study of the chiroptical properties of lysozyme indicated an effect on Trp-62 and Trp-63 following self-association (Holladay and Sophianopoulos, 1972).

Thus the selective effects of change in pH, protein concentration, and temperature upon the chemical shifts of the γ-carbon of Trp-108 and Trp-63 appear to have a common origin in the specific self-association of lysozyme. This association process has been shown previously to be head-to-tail and to

involve elements of the active site (Sophianopoulos, 1969; Banerjee et al., 1975; see also the discussions of the introductory section and the following paragraph).

The principal conclusions to be drawn from this work about the structure of the associated lysozyme species are the following: (i) His-15 is part of the intermolecular surface. In view of His-15 being located on the lysozyme surface opposite to the active site, which is known to be part of the interface, these studies define a second region of the lysozyme molecule distant from the active site that also participates in the self-association reaction. The head-to-tail model for the self-association proposed previously (Sophianopoulos, 1969; Banerjee et al., 1975) is therefore demonstrated by experiment, and the "tail" region that interacts with the active site "head" region must comprise His-15 and adjacent residues. (ii) The substantial dependence of the γ-carbon chemical shifts of residues Trp-108 and Trp-63 on the extent of association indicate that these residues, which are on the surface of the molecule (Shrake et al., 1973), are part of the "head" region together with Glu-35. The NMR results confirm the previously proposed (Banerjee et al., 1975; Holladay and Sophianopoulos, 1972) presence of tryptophan residues in the intermolecular interface. (iii) The charged forms of His-15 and Glu-35 interact within the interface. It is possible that His-15, which at pH above 6 is deprotonated, is also involved in nonpolar and hydrogen-bonding interactions that stabilize the polymer.

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