Effect of Chemical Modifications at Tryptophan-108 on Binding of Lanthanide Ions to Hen Egg-White Lysozyme. Application of Natural-Abundance Carbon-13 Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: Natural-abundance ^{13}C NMR spectroscopy (at 15.18 MHz) is used to investigate the effect of chemical modifications at Trp-108 of hen egg-white lysozyme on the lanthanide ion-binding properties of the enzyme. The strong binding of Gd^{3+} in the vicinity of Glu-35 and Asp-52 of lysozyme is weakened (by a factor $\gtrsim 20$) when Trp-108 is converted into the internal Glu-35 ester of δ_1-hydroxytryptophan-108. The modified protein has a weak binding site for lanthanide ions near Asp-101. Unlike intact lysozyme, the modified protein does not show evidence of aggregation at neutral pH. When the internal Glu-35 ester of [δ_1-hydroxytryptophan-108]lysozyme is converted into [oxindolealanine-108]lysozyme (and the pH is maintained at a value $\lesssim 5$), the modified Trp-108 residue exists as an equimolar mixture of the two diastereoisomers of oxindolealanine-108. However, when the pH is raised to 6 or higher, one diastereoisomer becomes predominant. When the pH is lowered again (to a value in the range 3–5), the system does not revert back to the equimolar mixture of diastereoisomers (even after several weeks at pH 3 and 40 °C). The predominant diastereoisomer of [oxindolealanine-108]lysozyme binds Gd^{3+} in the vicinity of Glu-35 and Asp-52 with a binding constant similar to that of the intact protein.

Rupley and co-workers (Hartdegen and Rupley, 1964, 1967, 1973; Imoto et al., 1973; Imoto and Rupley, 1973) made detailed studies of the reaction of hen egg-white lysozyme with a half-molar amount of I_2 at pH 5.5. The reaction yielded a protein mixture which could be separated chromatographically into three major components (Imoto et al., 1973). Rupley and co-workers (Imoto et al., 1973; Hartdegen and Rupley, 1973) showed that one of these components is lysozyme with a chemical modification only at Trp-108, and that the modified residue is not oxindolealanine[1] (Figure 1C,D). There is strong evidence that the modified residue 108 is δ_1-hydroxytryptophan (the enol form of oxindolealanine) esterified to Glu-35 (Figure 1B) (Imoto and Rupley, 1973; Beddell et al., 1975; Norton and Allerhand, 1976). We shall call the modified protein [(RCOO)Trp-108]lysozyme.[1] It can be hydrolyzed irreversibly to [oxindolealanine-108]lysozyme[1] after it is unfolded with guanidinium chloride (Imoto et al., 1973; Imoto and Rupley, 1973; Norton and Allerhand, 1976). Removal of the denaturing agent yields two folded proteins which are diastereoisomeric at oxindolealanine-108 (Figure 1C,D) (Norton and Allerhand, 1976).

Hen egg-white lysozyme has a site in the vicinity of the carboxylate groups of Glu-35 and Asp-52 which strongly binds lanthanide ions (Dwek et al., 1971; Campbell et al., 1973a, 1975; Secemski and Lienhard, 1974; Kurachi et al., 1975). In this report, we use the resonances of nonprotonated aromatic carbons in natural-abundance ^{13}C NMR spectra of [(RCOO)Trp-108]lysozyme and [oxindolealanine-108]lysozyme to study the binding of Gd^{3+} to these proteins. Our results yield information about the extent of secondary binding of lanthanide ions to intact lysozyme. We also show that the ratio of the two diastereoisomers of [oxindolealanine-108]-lysozyme depends on pH in a complex manner.

Experimental Procedure

Materials. Hen egg-white lysozyme (six times crystallized, substantially salt-free) from Miles Laboratories, Inc., Elkhart, Ind., was used as received. Purity was checked by electrophoresis on cellulose acetate and by chromatography on Bio-Rex-70 (Imoto et al., 1973). Lanthanum oxide (ultrapure grade) was obtained from Alfa Products, Beverly, Mass. Gadolinium oxide (99.9% pure) was purchased from Sigma Chemical Co., St. Louis, Mo. Other chemicals were reagent grade.

Methods. [(RCOO)Trp-108]lysozyme was prepared and purified essentially as described by Imoto et al. (1973), except that an I_2/protein molar ratio of 1.0 instead of 0.5 was used (see Norton and Allerhand, 1976). Typically, potassium triiodide solution (0.04 M I_2, 0.48 M KI) was added to a stirred

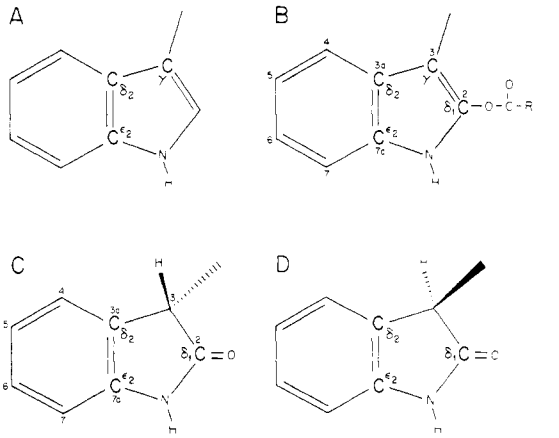

FIGURE 1: Structures. (A) Indolyl group of a tryptophan residue. (B) Modified indolyl group of residue 108 of [(RCOO)Trp(108)]lysozyme.[1] (C,D) Oxindolyl groups of the two diastereoisomers of an oxindolealanine residue.

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[1] Abbreviations used are: (RCOO)Trp-108, δ_1-hydroxytryptophan-108 esterified to the carboxylate group of Glu-35; oxindolealanine, β-(3-oxindolyl)-L-alanine; Me_4Si, tetramethylsilane.

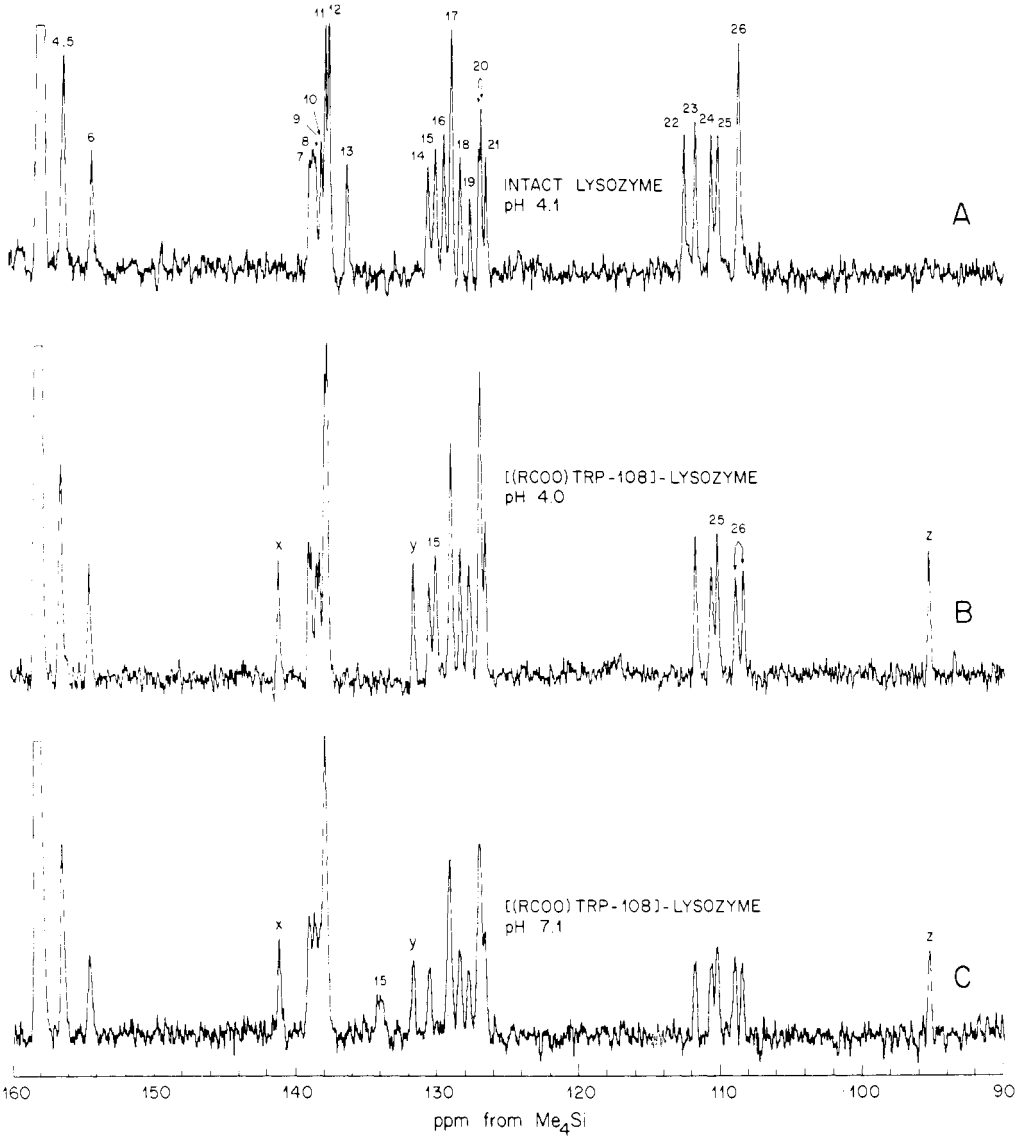

FIGURE 2: Region of aromatic carbons (and C^{ζ} of arginine residues) in convolution-difference natural-abundance ^{13}C NMR spectra of intact hen egg-white lysozyme and [(RCOO)Trp-108]lysozyme[1] in H_2O (0.1 M NaCl). Each spectrum was recorded at 15.18 MHz, with a recycle time of 1.1 s. Peak designations are discussed in the text. (A) Intact lysozyme (11 mM) at pH 4.1 and 33 °C, after 65 536 scans (20 h). The truncated peak at about 158 ppm arises from C^{ζ} of the 11 arginine residues (Oldfield et al., 1975b). Peaks 4–26 arise from the 28 nonprotonated aromatic carbons. Assignments to specific residues are given in Table I. (B) [(RCOO)Trp-108]lysozyme (8 mM) at pH 4.0 and 37 °C, after 96 566 scans (30 h). (C) [(RCOO)Trp-108]lysozyme (8 mM) at pH 7.1 and 36 °C, after 65 536 scans (20 h). The chemical shift of C^{γ} of His-15 (peak 15) exhibits the expected (Allerhand et al., 1977) pH dependence.

solution of 5 g of protein in 300 mL of water at pH 5.5, as described by Norton and Allerhand (1976). Salts and unreacted iodine were removed by chromatography on Sephadex G-25 equilibrated at pH 3 (with HCl). The resulting dilute protein solution was concentrated by ultrafiltration (UM-10 membrane, Amicon Corp., Lexington, Mass.) to about 30 mL (pH was adjusted to a value between 4 and 5), and then loaded on a Bio-Rex-70 column (120 × 3 cm) equilibrated at pH 10.0 with borate–carbonate buffer (0.05 M). Borate–carbonate buffer (0.05 M, pH 10.0) with a linear gradient of NaCl (0.0 to 0.1 M for 9 L of buffer) was used as the eluent. The flow rate was 30 mL/h. This procedure fractionated the various modified lysozymes and the intact protein (Imoto et al., 1973). Usually, 3 g of the reacted protein mixture yielded about 1 g of [(RCOO)Trp-108]lysozyme.

[Oxindolealanine-108]lysozyme was prepared by hydrolysis of [(RCOO)Trp-108]lysozyme in the presence of guanidinium chloride (Imoto et al., 1973; Norton and Allerhand, 1976). The absorbance ratio A_{280}/A_{250} was used to monitor the progress of the hydrolysis (Imoto and Rupley, 1973). Incubation of 1 mM [(RCOO)Trp-108]lysozyme for 30 h with 4 M guanidinium chloride at 40 °C and pH 3.1 (Norton and Allerhand, 1976) yielded only 50% conversion. Incubation for 2 h in 5 M guanidinium chloride at 40 °C and pH 1.0 (Imoto et al., 1973) yielded essentially complete conversion. The unfolded [oxindolealanine[108]lysozyme was "renatured" by exhaustive dialysis against deionized water at 4 °C and pH 3.0.

Samples for NMR studies were prepared essentially as described (Norton and Allerhand, 1976). Changes of pH were made by addition of 1 M NaOH or HCl. Protein concentrations were determined on a Beckman 25 spectrophotometer, with the use of published extinction coefficients (Imoto et al., 1973; Imoto and Rupley, 1973; Banerjee et al., 1975). Solutions of proteins in the presence of La^{3+} and Gd^{3+} were prepared as described (Allerhand et al., 1977).

Natural-abundance ^{13}C Fourier transform NMR spectra were obtained at 15.18 MHz with the use of 20-mm spinning sample tubes, essentially as described (Allerhand et al., 1973a;

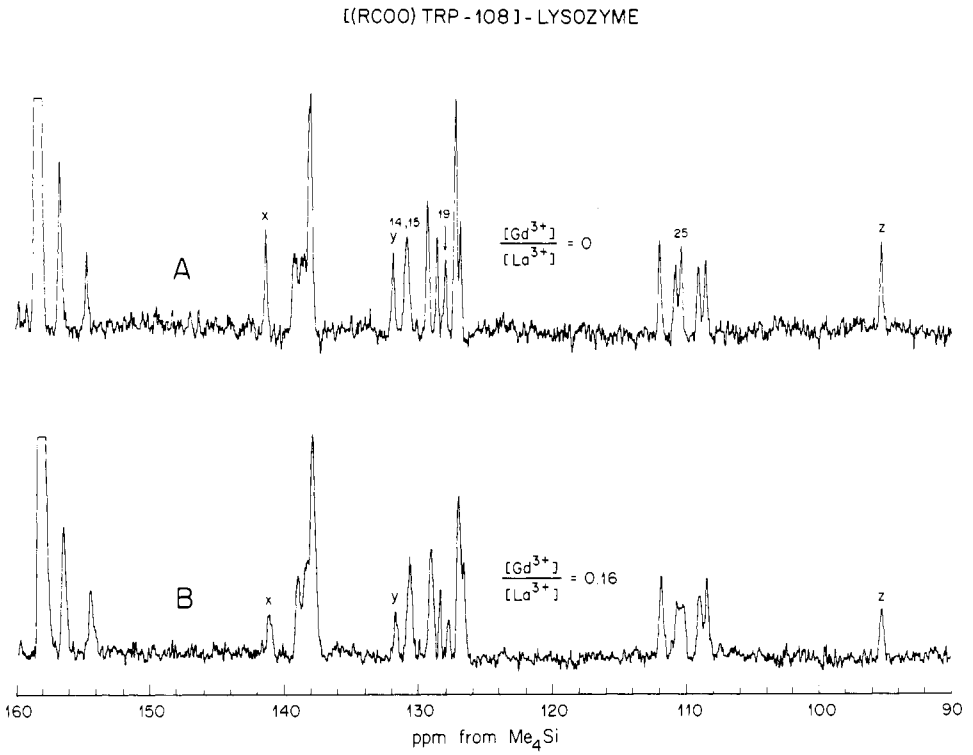

FIGURE 3: Effect of Gd^{3+} on the resonances of nonprotonated aromatic carbons of 13 mM [(RCOO)Trp-108]lysozyme[1] in H_2O at 36 °C. Each spectrum was recorded at 15.18 MHz with a recycle time of 2.2 s. The convolution-difference method was applied (see Experimental Procedure). Peak numbers are those of Figure 2. The molar ratios of $GdCl_3$ to $LaCl_3$ are indicated in each spectrum. The concentration of $LaCl_3$ was 45 mM in each case. (A) Without $GdCl_3$, at pH 5.0, after 32 768 scans (20 h). (B) With 7.2 mM $GdCl_3$, at pH 5.1, after 65 536 scans (40 h).

Oldfield et al., 1975a). All spectra were recorded under conditions of noise-modulated off-resonance proton decoupling (Allerhand et al., 1973b; Oldfield et al., 1975a,b). A spectral width of 3788 Hz was used. Time-domain data were accumulated in 8192 addresses of a Nicolet 1085 computer. Fourier transformation was carried out on 16 384 data points, by placing 8192 addresses with a zero value at the end of each block of accumulated data points. The convolution-difference method (Campbell et al., 1973b) was used to eliminate the broad methine aromatic carbon bands (Allerhand et al., 1973b; Oldfield et al., 1975a,b) as follows. Two separate Fourier transform spectra were obtained from each accumulated time-domain spectrum (stored on a disk), one with 0.9-Hz and the other with 18-Hz digital broadening. The second spectrum (multiplied by 0.9) was subtracted digitally from the first one. Chemical shifts were obtained digitally, and are reported in parts per million downfield from the ^{13}C resonance of Me_4Si. Estimated accuracy is ±0.05 ppm for fully resolved peaks, and decreases to about ±0.1 ppm with decreasing resolution. A trace of dioxane (at 67.8_6 ppm downfield from external Me_4Si) was used as an internal reference.

Spectra of [(RCOO)Trp-108]lysozyme

Figure 2 shows the region of aromatic carbons (and C^{ζ} of the 11 arginine residues) in the natural-abundance ^{13}C NMR spectra of intact hen egg-white lysozyme at pH 4.1 (Figure 2A), [(RCOO)Trp-108]lysozyme at pH 4.0 (Figure 2B), and [(RCOO)Trp-108]lysozyme at pH 7.1 (Figure 2C). The convolution-difference method (see Experimental Procedure) was applied to each spectrum in order to remove the broad bands of methine aromatic carbons (Oldfield et al., 1975b). Consider first the spectrum of intact lysozyme (Figure 2A). The 28 nonprotonated aromatic carbons give rise to peaks

TABLE I: Assignments of the Nonprotonated Aromatic Carbon Resonances of Hen Egg-White Lysozyme to Specific Residues in the Sequence.

Assignment[a]	Peak[b]	Chemical shift[c]
Tyr-20 C^{ζ} Tyr-53 C^{ζ}	4,5	156.4_4
Tyr-23 C^{ζ}	6	154.4_2
Phe-3 or 38 C^{γ}	7	139.0_7
Trp-63 $C^{\epsilon 2}$	8	138.8_2
Phe-38 or 3 C^{γ}	9	138.6_4
Trp-28 or 111 $C^{\epsilon 2}$	10	138.2_5
Trp-111 or 28 $C^{\epsilon 2}$ Trp-123 $C^{\epsilon 2}$	11	137.9_4
Phe-34 C^{γ} Trp-62 $C^{\epsilon 2}$	12	137.7_3
Trp-108 $C^{\epsilon 2}$	13	136.3_9
Tyr-23 C^{γ}	14	130.6_9
His-15 C^{γ}	15	130.1_8
Trp-108 $C^{\delta 2}$	16	129.6_0
Tyr-20 C^{γ} Tyr-53 C^{γ}	17	129.0_5
Trp-111 $C^{\delta 2}$	18	128.4_4
Trp-63 $C^{\delta 2}$	19	127.7_7
Trp-28 $C^{\delta 2}$ Trp-62 $C^{\delta 2}$	20	127.1_3 127.0_0
Trp-123 $C^{\delta 2}$	21	126.6_4
Trp-108 C^{γ}	22	112.6_3
Trp-123 C^{γ}	23	111.8_4
Trp-63 C^{γ}	24	110.7_4
Trp-62 C^{γ}	25	110.2_3
Trp-28 C^{γ} Trp-111 C^{γ}	26	108.7_3

[a] Taken from Allerhand et al. (1977). [b] Peak designations are shown in Figure 2A. [c] From the spectrum of Figure 2A (protein in H_2O, pH 4.1, 0.1 M NaCl, 33 °C).

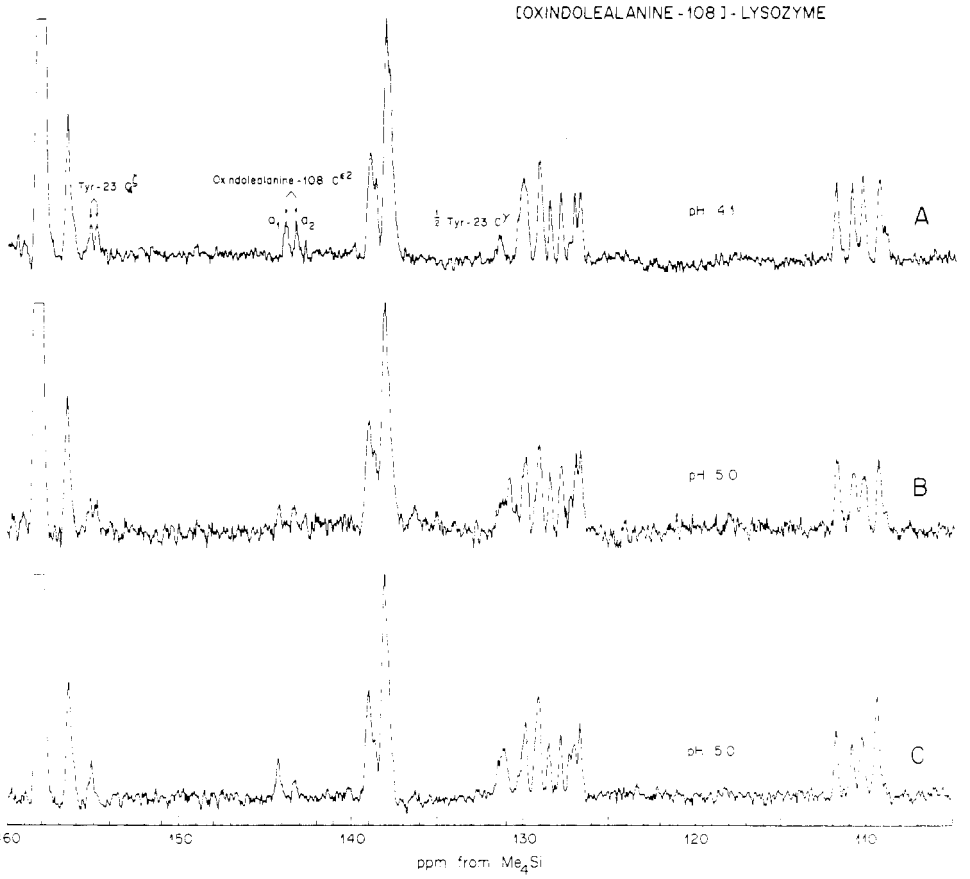

FIGURE 4: Effect of pH and sample history on the nonprotonated aromatic carbon resonances of 14 mM [oxindolealanine-108]lysozyme[1] in H_2O at 39 °C. Each spectrum was recorded at 15.18 MHz with a recycle time of 1.1 s. The convolution-difference method was applied. Assignments for Tyr-23 are based on those in Table I (intact lysozyme). The assignment for $C^{\epsilon 2}$ of oxindolealanine-108 is that of Norton and Allerhand (1976). (A) At pH 4.1, with 65 536 scans (20 h), recorded immediately after removal of guanidinium chloride (by dialysis at pH 3, see Experimental Procedure). (B) At pH 5.0, with 32 768 scans (10 h), recorded after the spectrum at pH 4.1 was obtained. (C) At pH 5.0, with 65 536 scans (20 h), recorded after the pH had been taken to 5.5 (for 10 h) and 6.3 (for 30 h).

4-26. The peak numbering system is that of Oldfield et al. (1975a,b). Most of peaks 4-26 have been assigned to specific residues in the sequence (Allerhand et al., 1977). The assignments are given in Table I. Peaks 13, 16, and 22 are the resonances of $C^{\epsilon 2}$,$C^{\delta 2}$, and C^{γ}, respectively, of Trp-108. Peaks x, y, and z in the spectra of [(RCOO)Trp-108]lysozyme (Figure 2B,C) arise from $C^{\epsilon 2}$, $C^{\delta 2}$, and C^{γ}, respectively, of the modified Trp-108 residue (Norton and Allerhand, 1976). We have not detected the resonance of $C^{\delta 1}$ of (RCOO)Trp-108. This resonance probably overlaps with the large peak of C^{ζ} of the 11 arginine residues (truncated peak at about 158 ppm in Figure 2) (Norton and Allerhand, 1976). The conversion of Trp-108 into (RCOO)Trp-108 causes minor changes in the chemical shifts of some unmodified aromatic residues. Most affected are the γ-carbons of Trp-28 and Trp-111, which yield a two-carbon resonance in the spectrum of intact lysozyme (peak 26 in Figure 2A): One component of peak 26 moves about 0.3-ppm upfield and the other moves about 0.3-ppm downfield upon conversion of Trp-108 into (RCOO)Trp-108 (Figure 2B). Thus, it appears that the chemical modification of Trp-108 causes a slight conformational reorganization near Trp-28 and Trp-111.

It is well known that hen egg-white lysozyme readily self-associates at pH $\gtrsim$6 (Imoto et al., 1972). Under the sample conditions of Figure 2A (11 mM protein, pH 4.1, 33 °C), intact lysozyme is essentially monomeric, but becomes strongly self-associated if the pH is raised to 7 (Sophianopoulos and Van Holde, 1964; Bruzzesi et al., 1965). The resonance of C^{γ} of Trp-62 (peak 25) is a convenient monitor of the extent of self-association, because this resonance undergoes a significant downfield shift when self-association takes place (Norton and Allerhand, 1977). Under the sample conditions of Figure 2A, the observed downfield shift is about 0.5 ppm when the pH of intact lysozyme is raised from 4 to 7 (Norton and Allerhand, 1977). In contrast, there is no change in the chemical shift of C^{γ} of Trp-62 of [(RCOO)Trp-108]lysozyme when going from pH 4 (Figure 2B) to 7 (Figure 2C). This result suggests that formation of the ester bond between Glu-35 and the modified Trp-108 residue greatly reduces the extent of (or entirely suppresses) self-association. The thermodynamic data of Banerjee et al. (1975) support this conclusion.

Hen egg-white lysozyme has a strong binding site for lanthanide ions which involves the carboxylate groups of Glu-35 and Asp-52 (Dwek et al., 1971; Campbell et al., 1973a, 1975; Secemski and Lienhard, 1974; Kurachi et al., 1975). The binding of Gd^{3+} has been of particular interest, because the bound Gd^{3+} acts as a relaxation probe (Dwek, 1973); i.e., it causes a broadening of ^1H and ^{13}C resonances that is inversely proportional to r^6, where r is the distance from the relaxation probe to the pertinent nucleus (Campbell et al., 1975, Allerhand et al., 1977). The use of the observed broadening effects to assign ^1H and ^{13}C resonances and to determine interatomic distances for lysozyme in solution is simplest if the effects of secondary binding sites can be ignored. Campbell et al. (1975) have used a "detailed analysis of titration curves" of intact lysozyme to conclude that binding of lanthanides to the main site is stronger by a factor $\gtrsim$100 than binding to secondary sites such as Asp-101 and Asp-87. Here we investigate secondary

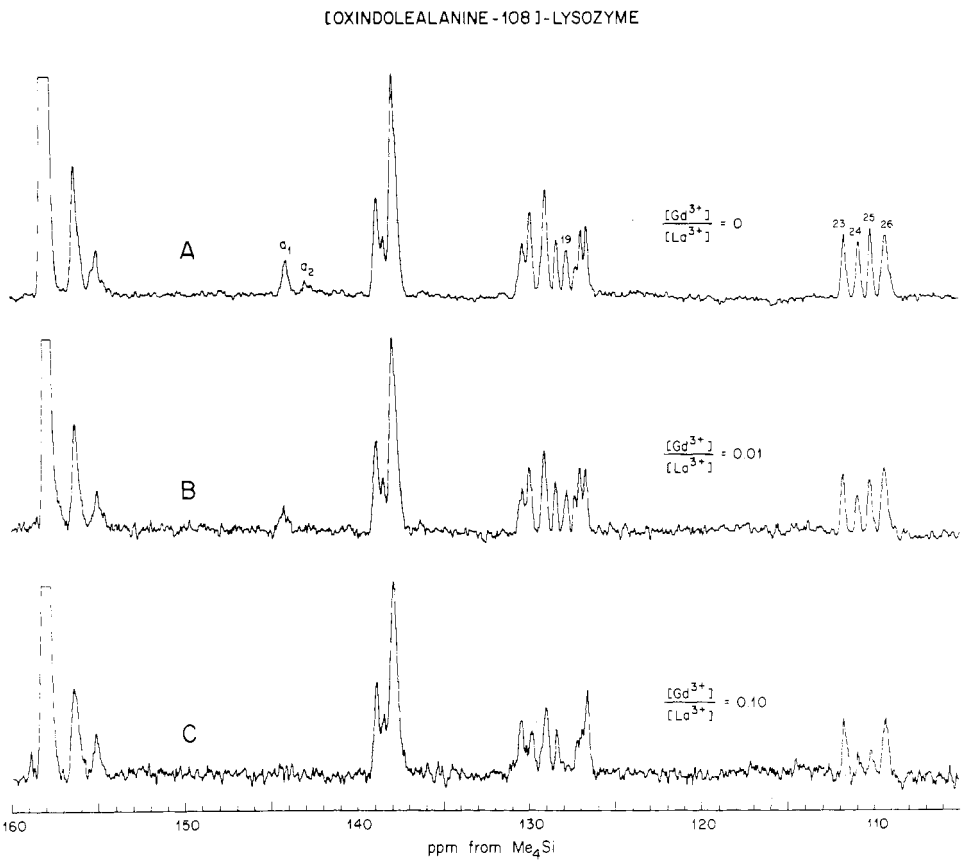

FIGURE 5: Effect of Gd^{3+} on the resonances of nonprotonated aromatic carbons of 14 mM [oxindolealanine-108]lysozyme[1] in H_2O (pH 5.0, 39 °C). Each spectrum was recorded at 15.18 MHz with a recycle time of 1.1 s. The convolution-difference method was applied. Peak designations are those of Figures 2A and 4A. The molar ratios of $GdCl_3$ to $LaCl_3$ are indicated in each spectrum. The concentration of $LaCl_3$ was 44.6 mM in each case. (A) Without $GdCl_3$, after 229 376 scans (70 h). The sample was prepared by adding $LaCl_3$ to the sample of Figure 4C. (B) With 0.45 mM $GdCl_3$, after 98 304 scans (30 h). (C) With 4.6 mM $GdCl_3$, after 98 304 scans (30 h).

binding by examining the effect of Gd^{3+} on the ^{13}C NMR spectrum of [(RCOO)Trp-108]lysozyme, a species which does not have an available carboxylate group at residue 35.

In Figure 3A, we show the aromatic region of the convolution-difference ^{13}C NMR spectrum of 13 mM [(RCOO)-Trp-108]lysozyme at pH 5 in the presence of a 45 mM concentration of the diamagnetic La^{3+} ion. As in the case of intact lysozyme (Allerhand et al., 1977), addition of La^{3+} does not significantly perturb the spectrum (compare Figure 3A with 2B). Figure 3B is the spectrum of the same sample as in 3A, but after addition of a 7.2 mM concentration of Gd^{3+}. A spectrum of intact lysozyme in the presence of similar concentrations of La^{3+} and Gd^{3+} as used for Figure 3B is given in Figure 3F of Allerhand et al. (1977). In the case of intact lysozyme, the resonances of Trp-108 are broadened beyond detection. In contrast, the resonances of (RCOO)Trp-108 (peaks x, y, and z of Figure 3B) are only slightly broadened by the Gd^{3+} ions. The broadening of peaks x, y, and z (Figure 3B) is similar to that observed for the resonances of Trp-108 of intact lysozyme when the Gd^{3+} concentration is about $1/20$ of that used for Figure 3B (see Figure 3 of Allerhand et al., 1977).

With the use of the crystallographic coordinates of intact lysozyme (Imoto et al., 1972), we estimated values of the distances (r) from the nonprotonated aromatic carbons to various possible Gd^{3+} binding sites (Campbell et al., 1975). For binding to Asp-52, we used the values of r^6 given in Table IV of Allerhand et al. (1977). The paramagnetic contributions to the line widths in Figure 3B are too small to allow a quantitative comparison of the effect of Gd^{3+} on the resonances of (RCOO)Trp-108 and those of other affected residues. Nevertheless, the observed effects cannot be explained on the basis of Gd^{3+} binding to Asp-52 exclusively. It is necessary to invoke binding of Gd^{3+} to at least one additional site in order to explain the fact that peaks x, y, and z are not significantly more affected than peaks 19 and 25, which arise from $C^{\delta 2}$ of Trp-63 and C^{γ} of Trp-62, respectively (Allerhand et al., 1977). On the basis of the estimated values of r^6, we conclude that the observed broadening effects in Figure 3B are most consistent with two weak binding sites for Gd^{3+}, one in the vicinity of Asp-101 and the second near Asp-52. However, we cannot rule out the possibility that Gd^{3+} binding near Asp-101 is entirely responsible for the broadening effects in Figure 3B. In any case, a comparison of our results (Figure 3B) with the reported effects of Gd^{3+} on the ^{13}C NMr spectrum of intact lysozyme (Figure 3 of Allerhand et al., 1977) indicates that the binding constant for Gd^{3+} in the vicinity of Asp-52 and Glu-35 decreases by a factor $\gtrsim 20$ when going from intact lysozyme to the modified protein. Our results also suggest that secondary binding makes only a minor contribution to the observed line-broadening effects in spectra of *intact* lysozyme solutions which contain Gd^{3+} ions.

Spectra of [Oxindolealanine-108]lysozyme

Figure 4A shows the aromatic region of the convolution-difference ^{13}C NMR spectrum of [oxindolealanine-108]lysozyme at pH 4.1. Peaks a_1 and a_2 arise from $C^{\epsilon 2}$ of the oxindolealanine-108 residue (Norton and Allerhand, 1976). The splitting into two "half-carbon" resonances is caused by the diastereoisomerism of oxindolealane-108 (see Figure 1C,D)

(Norton and Allerhand, 1976). The proportions of the two diastereoisomers are about equal under the sample conditions of Figure 4A, but can be varied by changing the pH (see below). Note that the chemical shifts of some unmodified residues are affected by the diastereoisomeric state of oxindolealanine-108. For example, C^{ζ} of Tyr-23 yields two resolved "half-carbon" resonances (and C^{γ} of Tyr-23 yields one *resolved* "half-carbon" resonance) in Figure 4A. Other "half-carbon" resonances are also discernible, but are more difficult to assign because of signal overlap.

The δ_2-carbons of the two forms of oxindolealanine-108 should contribute in the range 126–132 ppm (Norton and Allerhand, 1976). The δ_1-carbons of the two diastereoisomers of residue 108 yield identifiable "half-carbon" resonances at the downfield edge of the carbonyl region (Norton and Allerhand, 1976). Note that C^{γ} of an oxindolealanine residue is a methine aliphatic carbon (Figure 1C,D) and therefore does not contribute in Figure 4.

It is pertinent to our discussion that the sample of "native" [oxindolealanine-108]lysozyme whose spectrum is shown in Figure 4A was obtained by dialysis at pH 3 of unfolded [oxindolealanine-108]lysozyme (see Experimental Procedure). After removal of guanidinium chloride, the sample was concentrated by ultrafiltration, the pH was raised to 4.1, and the spectrum of Figure 4A was recorded. The pH was then raised to 5.0 and the spectrum of Figure 4B was obtained. The main effect of the higher pH is the expected downfield shift of C^{γ} of His-15, from about 130 to about 131 ppm (Allerhand et al., 1977). However, one component of the $C^{\epsilon 2}$ doublet of oxindolealanine-108 (peak a_1 of Figure 4) moves about 0.3 ppm downfield when the pH is raised from 4 to 5, while the other component (peak a_2) does not move appreciably. This effect may be caused by the ionization of Asp-52, which has a pK_a of about 4.5 in intact lysozyme (Imoto et al., 1972). It is possible that the oxindole group of one diastereoisomer of oxindolealanine-108 is closer to the carboxylate group of Asp-52 than the oxindole group of the other diastereoisomer.

After the spectrum of Figure 4B was obtained, additional spectra were recorded at pH 5.5 and 6.3, with 10 and 30 h of spectral accumulation time, respectively. These spectra revealed an increase in the proportion of the diastereoisomer which gives rise to peak a_1. The pH was then lowered to 5.0 again, and the spectrum shown in Figure 4C was obtained. Clearly, the ratio of the two diastereoisomers in Figure 4C is very different from the initial value (Figure 4A,B). We were not able to reverse the equilibrium back to the equimolar ratio of Figures 4A and 4B even after keeping the sample at 40 °C and pH 3 for several weeks. However, the equimolar mixture was obtained again when the protein (diluted to 1 mM concentration) was unfolded with 4 M guanidinium chloride at 40 °C (for 24 h at pH 3 and 30 min at pH 1) and refolded by dialysis against H_2O at pH 3. It appears that the equimolar mixture of diastereoisomers is the equilibrium state of unfolded [oxindolealanine-108]lysozyme but a metastable condition for the folded protein. This metastable condition (Figure 4A) is the consequence of the method of preparation of [oxindolealanine-108]lysozyme, i.e., by hydrolysis of *unfolded* [(RCOO)Trp-108]lysozyme and subsequent refolding of the protein at low pH (see Experimental Procedure).

We examined the effect of Gd^{3+} on the spectrum of [oxindolealanine-108]lysozyme (Figure 5). Under our sample conditions (see caption of Figure 5), the amount of only one stereoisomer was sufficient to yield clearly observable resonances. The broadening effects in Figure 5B,C are quite similar to those observed for the corresponding resonances of intact lysozyme in the presence of comparable amounts of Gd^{3+} (see Figure 3 of Allerhand et al., 1977). We conclude that Gd^{3+} binds in the vicinity of the carboxylate groups of Asp-52 and Glu-35 of at least one diastereoisomer of [oxindolealanine-108]lysozyme, with a binding constant similar to that observed for intact lysozyme. The difference in the binding behavior of [(RCOO)Trp-108]lysozyme and that of [oxindolealanine-108]lysozyme underscores the importance of the carboxylate group of Glu-35 in the strong binding of lanthanide ions to intact lysozyme.

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