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Spectral Properties of Human Lysozyme and Its Inhibitor Complexes. Fluorescence and Difference Spectra†

Roderick S. Mulvey, Richard J. Gaultieri, and Sherman Beychok*

ABSTRACT: The fluorescence spectra and fluorimetric and spectrophotometric titrations of human lysozyme and its complexes with oligomers of *N*-acetyl-D-glucosamine (AcGlcN) have been investigated. The results are compared with a number of parallel studies on hen egg-white lysozyme. The spectrophotometric titration and alkaline difference spectra of the human lysozyme $(AcGlcN)_4$ complex show that the pK_{int} of Tyr-63 (-62) is lowered from approximately 10.5 to 10.0 when the inhibitor is bound. The fluorescence of human lysozyme has a lower quantum efficiency (0.04) than that of hen egg-white lysozyme and its emission maximum is at a shorter wavelength (330 nm). The spectrofluorimetric titrations of human and hen egg-white lysozymes are very similar. In contrast their complexes do not exhibit the same dependence of fluorescence on pH in the acid region. Below pH 5.5 the fluorescence of the hen egg-white lysozyme–$(AcGlcN)_3$ complex is strongly quenched whereas the fluorescence of the human lysozyme complex is as intense as the fluorescence of the free enzyme. The marked enhancement of enzyme fluorescence at pH 7.5 by inhibitor binding was used to determine the association constant of a series of saccharides from di- to penta-*N*-acetyl-D-glucosamine. At room temperature the K_a of $(AcGlcN)_3$ with human lysozyme is 1.9×10^4. Compared to hen egg-white lysozyme, this represents a difference of about 800 cal/mol in the free energy of binding. These different properties of the two lysozymes may largely be related to the substitution of Trp-62 of hen egg-white lysozyme by Tyr-63 (-62) in human lysozyme.

Human and hen egg-white lysozyme possess a high degree of homology, 78 positions out of a total of 130 having identical residues (Canfield, *et al.*, 1971; Jolles and Jolles, 1972). Alignment of the two sequences to maximize homology requires only a single deletion in the hen egg-white lysozyme between residues 47 and 48. X-Ray crystallographic studies of hen egg-white lysozyme at 2-Å resolution (Blake *et al.*, 1965) and of human lysozyme at 6-Å resolution (Blake and Swan, 1971) suggest that the two enzymes have very similar secondary structures, as do studies of the far-ultraviolet circular dichroism (Halper *et al.*, 1971; Ikeda *et al.*, 1972). Other investigations (Osserman and Lawlor, 1966; Jolles *et al.*, 1968; Cohen, 1969) point to similarities in the structure and function of the active sites of these enzymes.

Despite the overall similarity of these two lysozymes, a number of differences in enzymatic and physical properties have been noted. Human lysozyme is more active than hen egg white (Osserman Lawlor, 1966; Mouton and Jolles, 1969; Isaka *et al.*, 1971), the measured difference in activity depending on the type of assay employed. Using a turbidimetric assay with *Micrococcus lysodeikticus* (Gorin *et al.*, 1971), we found the human enzyme to be four to five times more active than hen egg-white lysozyme. The near-ultraviolet circular dichroic spectrum of human lysozyme (Halper *et al.*, 1971; Ikeda *et al.*, 1972) is quite distinct from that of hen egg-white lysozyme reflecting the altered arrangements of aromatic residues in the two enzymes.

Of the 51 different residues in hen egg-white and human lysozymes 27 occur at external[1] positions. Most of the remaining 24 changes, at surface and internal positions, are

† From the Departments of Biological Sciences and Chemistry, Columbia University, New York, New York 10027. *Received January 15, 1973.* This work was supported in part by Grant GB 29481x1 from the National Science Foundation and Grant 5 R01 CA 13014 from the National Institutes of Health.

[1] The terms "internal," "surface," and "external" are used as defined by Browne *et al.* (1969).

conservative and should have little effect on the three-dimensional structures of these two enzymes. One substitution is, however, of particular interest since it occurs in the substrate binding cleft. In subsite C (Rupley and Gates, 1967) Trp-62 of hen egg-white lysozyme becomes Tyr-63 (-62)[2] in human lysozyme. In hen egg-white lysozyme this residue contributes to the binding of saccharides at subsite C by moving toward the cleft and forming a hydrogen bond from its imidazole nitrogen to the C-6 oxygen of the sugar ring. It has also been identified as a major source of fluorescence (Imoto *et al.*, 1971) and optical activity (Ikeda and Hamaguchi, 1970). In an attempt to evaluate the effects of this substitution on the structure and activity of lysozyme, a detailed investigation of the spectroscopic properties of the human enzyme and its inhibitor complexes has been carried out. In this paper we report the effect of binding small oligomers of *N*-acetylglucosamine on the spectrophotometric titration, fluorescence emission and fluorimetric titration of human lysozyme.[3] The binding constants of these inhibitors were also determined and compared with the corresponding values obtained with hen egg-white lysozyme.

Materials and Methods

Materials. Human lysozyme isolated from the urine of patients with monocytic or monomyelocytic leukemia was a generous gift of Dr. Elliott F. Osserman. This was prepared as described by Halper *et al.* (1971), except that as a final step the protein solution was deionized on Bio-Rad AG 501-X8 mixed-bed resin, and then adjusted to neutral pH with HCl and lyophilized. Hen egg-white lysozyme was obtained from Pentex, Inc., and was used without further purification.

Oligosaccharides of *N*-acetylglucosamine were prepared by partial hydrolysis of chitin and separation by charcoal–Celite chromatography (Rupley, 1964). They were characterized by $[\alpha]_D$ values and nuclear magnetic resonance (nmr) spectroscopy, and their purity was checked by paper chromatography (Sharon and Seifter, 1964) and column chromatography on Bio-Gel P2 (Raftery, 1969).

Spectrophotometric Titration. Ultraviolet absorption spectra were measured on a Cary 14 spectrophotometer. Data was gathered both by measurement of direct spectra and by difference spectroscopy. In the latter case two matched cuvettes were used, the sample cell containing a solution of lysozyme at high pH and the reference cell a lysozyme solution at pH 6.

Difference Spectroscopy. The difference spectra of (lysozyme–inhibitor complex) − (lysozyme) were measured over the pH range 6–11. Following the procedure of Herskovits and Laskowski (1962) tandem cells were employed, and the Cary 15 spectrophotometer was used with a 0.1 slide-wire and dynode voltage 3. Initially the two compartments of each cell contained 2 ml of lysozyme solution and 2 ml of buffer, respectively. This arrangement was used to record a base line. An aqueous solution (1 ml) of $(AcGlcN)_3$[4] or $(AcGlcN)_4$ was then added to the protein compartment on the sample side and to the buffer compartment of the reference side. The remaining compartments were diluted appropriately with water so that the final ionic strength of the buffer in all compartments was 0.1. The same pipette was used for the addition of inhibitor or water to each compartment, and the pH was checked before and after recording the spectra. The protein concentration ranged from 0.03 to 0.05%. With human lysozyme the large increase in ΔOD_{250} at pH's near 10, necessitated a reduction of protein concentration for measurements in this pH region.

Fluorescence Spectra. A Perkin-Elmer MPF3 spectrofluorimeter was used for the measurement of fluorescence spectra. The excitation wavelength was, unless otherwise specified, 280 nm and the protein concentration was about 0.03 mg/ml, so that the OD_{280} was below 0.1. All spectra were corrected for photomultiplier and emission monochromator sensitivity.

Quantum Efficiencies. Relative quantum efficiencies were determined by comparing the protein emission spectrum with that of a tryptophan solution in water. Relative areas beneath the fluorescence spectra were estimated by cutting out and weighing the graph paper. The protein quantum efficiency was calculated by the following relationship (Edelhoch *et al.*, 1969)

$$QE = \frac{Area_{protein}}{Area_{Trp}} \frac{OD_{Trp}}{OD_{protein}} QE_{Trp}$$

Following the convention adopted by other workers the quantum efficiency (QE) of tryptophan was assumed to be 0.2 (Lehrer and Fasman, 1966; Kronman *et al.*, 1971).

Fluorimetric Titrations. The fluorescence emission of a series of lysozyme solutions made from the same stock solution was determined over the pH range 2–12. In between the measurement of each fluorescence spectra the emission of a standard solution of tryptophan was recorded to allow correction for fluctuations in lamp intensity.

Binding Constants. Binding constants were determined in phosphate buffer at pH 7.5, where the enhancement of lysozyme fluorescence due to inhibitor binding is greatest. The fluorescence of a series of enzyme–inhibitor complex solutions was measured with inhibitor concentrations of 4×10^{-6} to 1×10^{-3} M. The fluorescence of the enzyme alone was measured before and after recording each enzyme–complex spectrum, and the data were expressed as the percentage increase in fluorescence intensity at 323 nm over the intensity of the free enzyme.

Buffers. In all experiments the ionic strength of buffers was 0.1. The following buffer systems were used: pH 2.0–3.6, glycine–HCl; pH 3.6–5.6, acetate; pH 5.7–8.0, potassium phosphate buffer; pH 8.5–10.5, glycine–KOH; pH 11.0–12.0, K_2HPO_4–KOH.

Protein Concentrations. The concentrations were determined in solution using $E_{280}^{1\%} = 24.6$ for human lysozyme (Latovitzki *et al.*, 1971), and $E_{280}^{1\%} = 26.4$ hen egg-white lysozyme (Imoto *et al.*, 1972).

Results

Spectrophotometric Titration. The spectrophotometric titration of human lysozyme is shown in Figure 1. The number

[2] Where the sequence number of a residue in human lysozyme differs from the number of the corresponding residue in hen egg-white lysozyme, the human sequence number is given first followed by the hen egg-white number in parentheses.

[3] When the final draft of this paper was completed, Dr. N. Sharon kindly provided the authors with a preprint of a paper describing some of the fluorescence properties of human lysozyme (Teichberg *et al.*, 1972). Those studies parallel some of the experiments reported here and there is generally good agreement, particularly with regard to the binding constant of $(AcGlcN)_3$ and human lysozyme.

[4] Abbreviation used is: AcGlcN, *N*-acetyl-D-glucosamine.

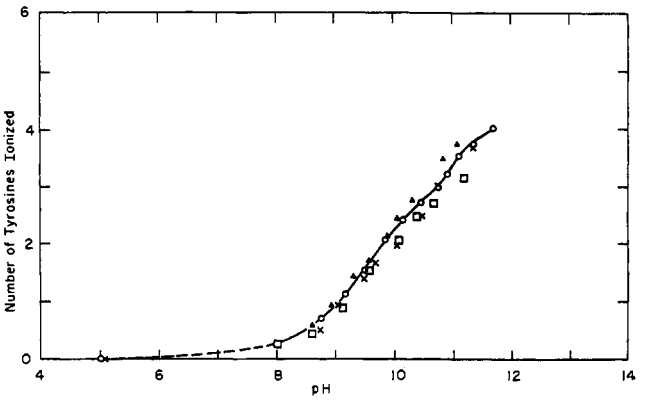

FIGURE 1: Spectrophotometric titration of human lysozyme measured by the increase in molar extinction coefficient at 245 nm (×), 290.5 nm (□), and 245 nm by ionization difference spectroscopy (▲). The circles are taken from previously published data (Latovitzki *et al.*, 1971). Protein concentration was 0.013 % for measurements at 245 nm, and 0.06% for measurements at 290.5 nm. For buffers used, see Methods.

of tyrosines ionized at a given pH is calculated on the basis of $\Delta\epsilon_{290.5} = 2000$ and $\Delta\epsilon_{245} = 10{,}500$ for the ionization of a single tyrosine (Latovitzki *et al.*, 1971; Donovan, 1969). The data collected by direct spectroscopy at 290.5 and 245 nm and by difference spectroscopy at 245 nm agree closely with previously published results (Latovitzki *et al.*, 1971), the experimental error in the pH region 10–11 being ±0.3 ionized tyrosine.

The absorption of $(AcGlcN)_3$ and $(AcGlcN)_4$ at 245 nm interfered with the spectrophotometric titration of the human lysozyme–inhibitor complex at this wavelength. Consequently only the data collected at 290.5 nm is included in Figure 2. All the points for the complex lie above the titration curve for the enzyme suggesting that the binding of inhibitor might enhance the ionization of one or more tyrosines. However, the effect is small and generally within the experimental error estimated above. Also, the increased absorption of the complex will in part be a result of the perturbation of tryptophan absorption.

Difference Spectroscopy. In order to establish the presence or absence of an effect of inhibitors on the ionization of tyrosines, difference spectroscopy with tandem cells was employed. The difference spectra (complex – enzyme) for

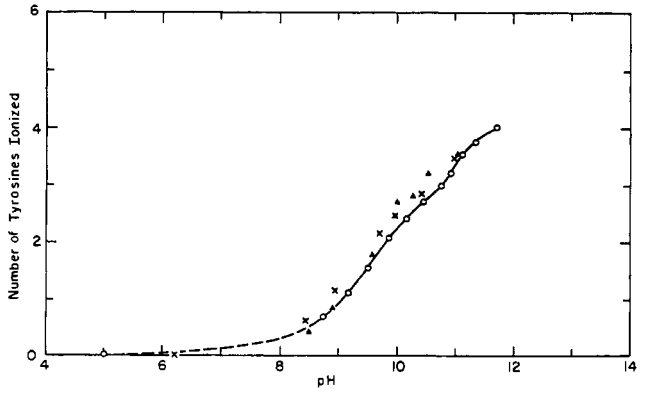

FIGURE 2: Spectrophotometric titration of human lysozyme–inhibitor complex, measured by the increase in molar extinction coefficient at 290.5 nm: (▲) $(AcGlcN)_3$ 2.1×10^{-3} M; (×) $(AcGlcN)_4$, 1.3×10^{-3} M; circles as in Figure 1. Protein concentration was 0.047 %; for buffers used, see Methods.

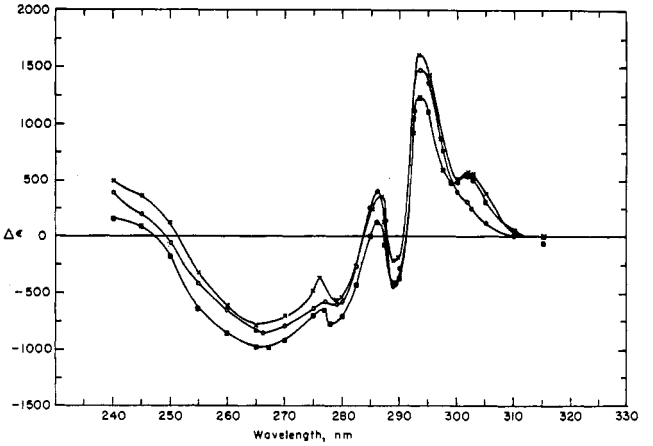

FIGURE 3: Difference spectra of hen egg-white lysozyme – $(AcGlcN)_4$ complex (complex – enzyme): (○) pH 7.0, (×) pH 8.9, (■) pH 10.0. Protein concentration, 0.04 %; $(AcGlcN)_4$ concentration, 1.0×10^{-3} M.

$(AcGlcN)_4$ and hen egg-white lysozyme are shown in Figure 3 over the pH range 7–10. Within experimental error these spectra are pH independent and have features typical of tryptophan perturbation spectra (Herskovits and Sorensen, 1968). Specifically, the spectra exhibit positive peaks at 293.5 and 286–287 nm, an inversion around 277 nm, a negative trough at 289 nm and a broad negative peak centered around 265 nm. Furthermore, the magnitude of $\Delta\epsilon_{293.5}$ and $\Delta\epsilon_{286}$ are in close agreement with those obtained by Hayashi *et al.* (1964) for hen egg-white and its glycol–chitin complex.

It is possible to make an almost identical analysis of the difference spectrum of human lysozyme and its $(AcGlcN)_4$ complex at pH 6.6 (Figure 4). Again the resemblance to a tryptophan perturbation spectrum is clear. However, in marked contrast to the hen egg-white lysozyme difference spectra, the human lysozyme alkaline spectra show positive peaks at 250 and 300 nm. These peaks grow as the pH increases to 10, beyond which they diminish (Figure 5).

Fluorescence Spectra. The fluorescence emission spectra of human lysozyme and its complexes with oligomers of *N*-acetylglucosamine are shown in Figure 6. The emission spectrum of hen egg-white lysozyme is included for comparison and the main spectral features are summarized in Table I.

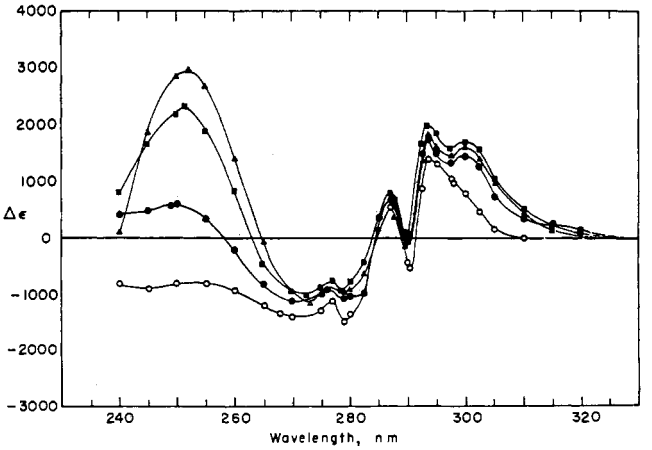

FIGURE 4: Difference spectra of human lysozyme – $(AcGlcN)_4$ complex (complex – enzyme): (○) pH 6.6, (●) pH 8.6, (■) pH 9.6, (△) pH 10.1. Protein concentration, 0.04%; $(AcGlcN)_4$ concentration, 1.14×10^{-3} M.

TABLE I: Fluorescence and Binding Data: Comparison of Human Lysozyme (HL) and Egg-White Lysozyme (HEWL).[a]

	λ_{max} (nm)		Q.E.		K_a (M^{-1})		$-\Delta F°$ (kcal/mol)	
	HEWL	HL	HEWL	HL	HEWL[b]	HL	HEWL	HL
Enzyme	337	330	0.06	0.04				
$(AcGlcN)_2$ complex		323		0.057		9.4×10^2		4.05
$(AcGlcN)_3$ complex		323		0.06	7.0×10^4	1.9×10^4	6.06	5.08
$(AcGlcN)_4$ complex		323		0.064		5.0×10^4		6.04

[a] pH 7.5, phosphate buffer, $\mu = 0.1$, room temperature. [b] Value of hen egg-white lysozyme $(AcGlcN)_3$ K_a from Lehrer and Fasman (1966).

The emission maximum for hen egg-white lysozyme, at 337 nm, is slightly lower than values previously reported (Lehrer and Fasman, 1966; Teichberg and Sharon, 1970; Imoto *et al.*, 1971). The emission maximum for human lysozyme is 330 nm, suggesting that the average environment of the fluorescent tryptophans is less polar in the human enzyme. When inhibitor is bound to human lysozyme at pH 7.5 the fluorescence emission is enhanced and its maximum is blue shifted to 323 nm. The degree of enhancement increases with inhibitor chain length up to the tetrasaccharide. The quantum efficiency of human lysozyme (QE = 0.04) is significantly lower than that of hen egg-white lysozyme (QE = 0.06). Apart from the differences in the position of the emission maximum and the quantum efficiency values the fluorescence of human lysozyme and its inhibitor complexes closely resembles that of hen egg-white lysozyme.

Fluorimetric Titration. The pH dependence of the fluorescence intensity of hen egg-white lysozyme, human lysozyme and human lysozyme–$(AcGlcN)_3$ complex measured at their respective emission maxima are shown in Figure 7. In lowering the pH from 7.5 to 5.5 there is a small dequenching of human lysozyme fluorescence, while in the same pH region hen egg-white lysozyme shows precisely the opposite effect. As the pH is further lowered to below 3 the fluorescence of both enzymes show similar behavior, namely slight quenching presumably due to protonated carboxyls. Above pH 8 tyrosine ionization strongly quenches fluorescence.

In the human lysozyme–$(AcGlcN)_3$ complex fluorescence is quenched in the pH region 8–6. Below pH 5.5 the fluorescence of the human lysozyme complex is close to that of the free enzyme, and shows only slight quenching due to carboxyl protonation.

The absence of any marked quenching at low pH in the human lysozyme complex is confirmed by the calculated

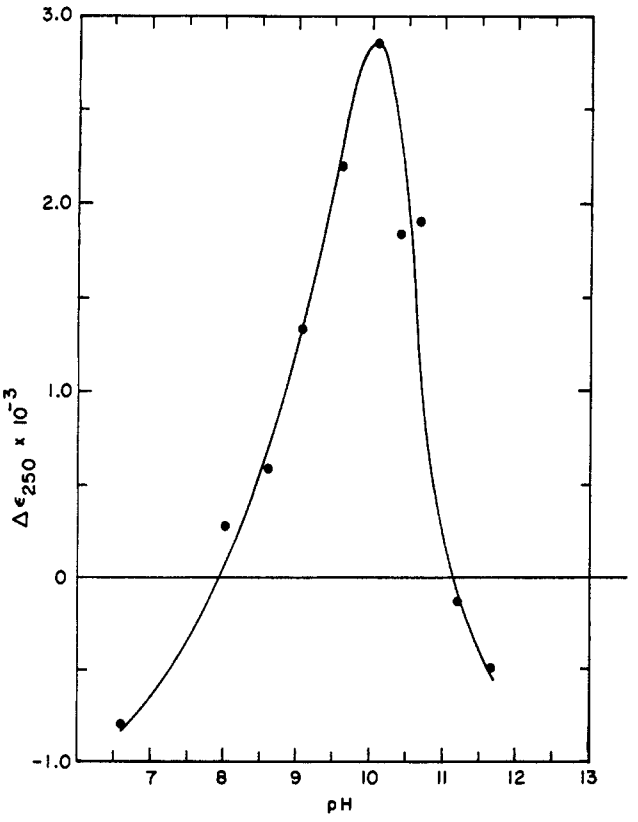

FIGURE 5: $\Delta\epsilon_{250}$ *vs.* pH, for human lysozyme–$(AcGlcN)_4$ complex. Conditions as in Figure 4.

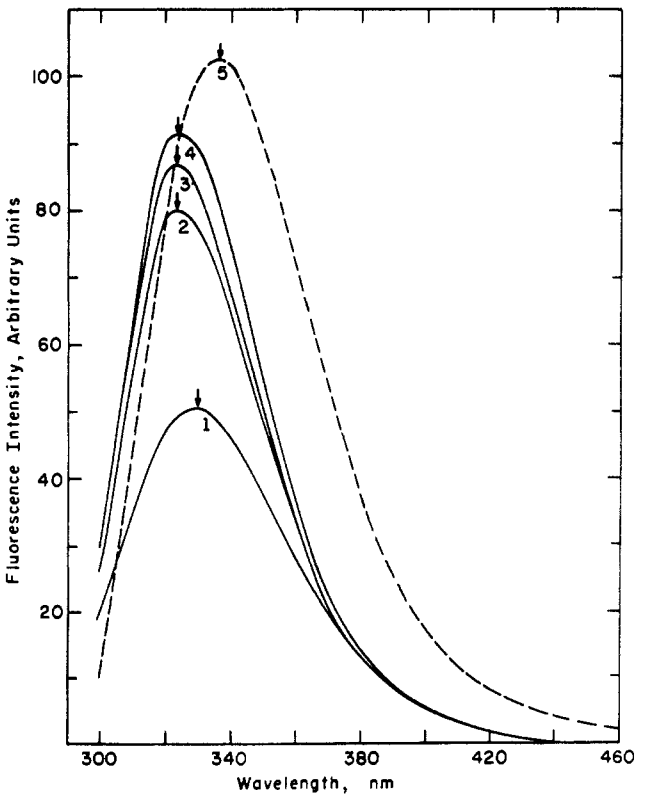

FIGURE 6: Fluorescence spectra of: (1) human lysozyme; (2) human lysozyme + $(AcGlcN)_2$, 9×10^{-3} M; (3) human lysozyme + $(AcGlcN)_3$, 1.2×10^{-3} M; (4) human lysozyme + $(AcGlcN)_4$ or $(AcGlcN)_5$, 1.2×10^{-3} M; (5) hen egg-white lysozyme. Protein concentration was 0.003% in phosphate buffer, pH 7.5, $\mu = 0.1$. Excitation at 280 nm, room temperature.

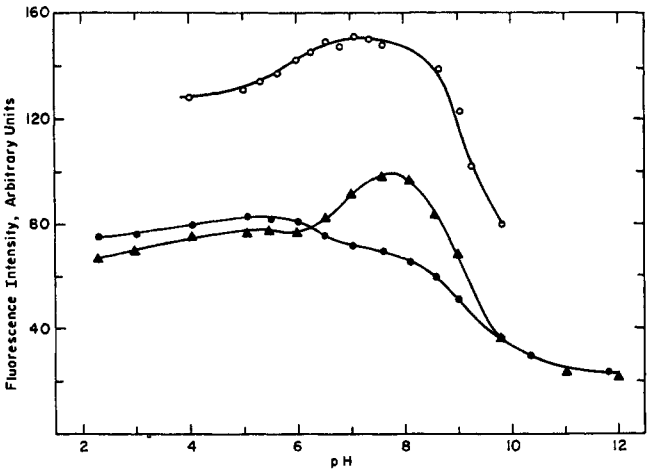

FIGURE 7: Fluorimetric titration: (○) hen egg-white lysozyme, emission at 337 nm; (●) human lysozyme, emission at 330 nm; (▲) human lysozyme + $(AcGlcN)_3$, 5.0×10^{-4} M, emission at 323 nm. Protein concentration, 0.004%; excitation wavelength: hen egg-white lysozyme 280 nm, human lysozyme 287 nm. Room temperature; for buffers used, see Methods.

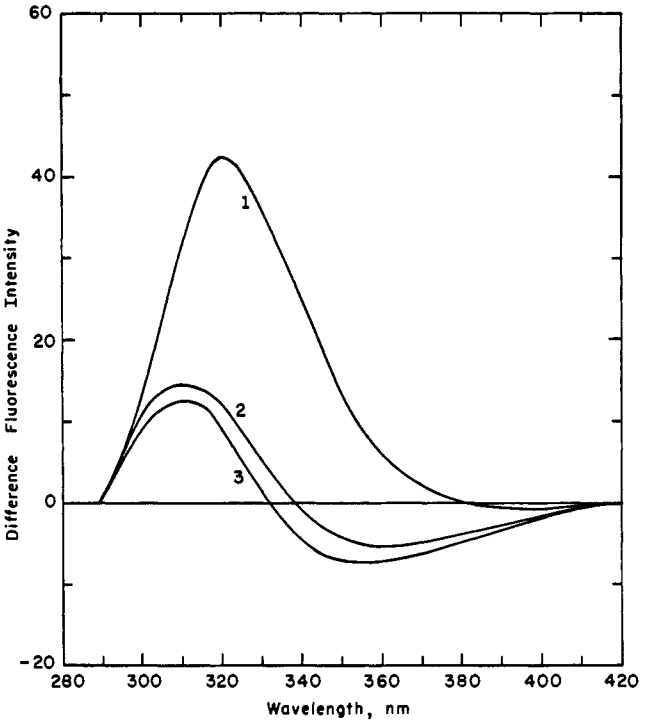

FIGURE 8: Calculated difference fluorescence spectra, (human lysozyme–$(AcGlcN)_4$ complex) − (human lysozyme) at: (1) pH 7.5, (2) pH 5.5, (3) pH 2.9. Protein concentration, 0.003%; $(AcGlcN)_4$ 1.3×10^{-3} M. Excitation at 280 nm, room temperature, for buffers see Methods.

difference fluorescence spectra shown in Figure 8. At pH 7.5 the difference spectra shows that fluorescence in the complex is considerably enhanced at low wavelengths (about 320 nm) and a blue shift is indicated by the small negative peak at long wavelengths. The difference spectra at pH 5.5 and 2.5 are almost identical and are dominated by a blue shift in the emission maximum.

Inhibitor Binding Constants. The binding of the oligosaccharides di- through penta-*N*-acetyl-D-glucosamine to human lysozyme was followed at pH 7.5 where their enhancement of the protein fluorescence is maximal. The data are presented in Figure 9 in the form of a derived Scatchard plot

$$\frac{(F - F_E)}{[I]} = K_a(F_C - F_E) - K_a(F - F_E)$$

where F_E and F_C are the fluorescence intensities of the free enzyme and the complex, respectively, and F is the fluorescence intensity in the presence of I moles of *added* inhibitor. K_a is the association constant assuming there is only one binding site per enzyme molecule. The association constants and standard free energies of binding are shown in Table I.

The association constant of $(AcGlcN)_3$ and hen egg-white lysozyme is 1.6×10^5 at pH 5.5, and at pH 7.5 this drops to 7.0×10^4 (Lehrer and Fasman, 1966). The K_a for $(AcGlcN)_3$ and human lysozyme at pH 7.5 is $1.9 (\pm 0.3) \times 10^4$ giving a standard free energy of binding of −5.8 kcal/mol, 800 cal less negative than the corresponding value for hen egg-white lysozyme. This value is in excellent agreement with estimations of the same association constant by Teichberg *et al.* (1972) and Kuramitsu *et al.* (1972). Tetra- and penta-*N*-acetyl-D-glucosamine have, within experimental error, identical binding constants, but with smaller inhibitors the binding weakens as the length of the oligosaccharide decreases.

Discussion

Hen egg-white lysozyme contains six tryptophans and three tyrosines. Of these residues only three tryptophans (62, 63, 108) appear to be in the binding cleft and they all interact with a saccharide ring placed in subsite C. Human lysozyme has five tryptophans and six tyrosines. One of the tyrosines, 63 (62), and two of the tryptophans, 64 (63) and 109 (108), are in the binding site and it might be expected that the environments of these residues will be perturbed by inhibitor binding.

The spectrophotometric titration and the alkaline difference spectra of the $(AcGlcN)_4$ complex of human lysozyme suggest that the pK of one or more tyrosines is lowered by inhibitor binding. This conclusion is supported by two observations. First, as shown in Figure 5, $\Delta\epsilon_{250}$ increases with pH reaching a maximum around pH 10.1 beyond which it falls and becomes negative above pH 11. Second, subtraction of the neutral pH difference spectrum from the alkaline difference spectrum yields the calculated spectrum shown in Figure 10. This procedure eliminates the contribution of tryptophan perturbation, and the remaining spectrum resembles the ionization difference spectrum of tyrosine (Donovan, 1969). The two maxima of this spectrum, at 250 and 303 nm, are at longer wavelengths than the corresponding maxima in the ionization difference spectrum of acetyltyrosine in aqueous solution (Donovan, 1964). This might indicate that the tyrosine(s) generating the ionization difference spectrum when inhibitors are bound is in a perturbed environment relative to aqueous solution. However, it should be noted that the experimental errors involved in the measurement of difference spectra will accumulate in such calculated "difference–difference" spectra.

The proximity of Tyr-63 (-62) to the substrate binding cleft suggests that this is the tyrosine which is perturbed by inhibitor binding. Although the perturbation of other tyrosines cannot be ruled out, their distance from the inhibitor binding site makes this unlikely. The results do rule out the possibility of a contribution of Tyr-63 (-62) to the binding of inhibitors by formation of a hydrogen bond. A hydrogen bond from the phenolic hydroxyl to the sugar would tend to raise the tyrosine pK. There is a number of possible explanations for the lowered pK of this tyrosine in the complex. For instance, a sugar

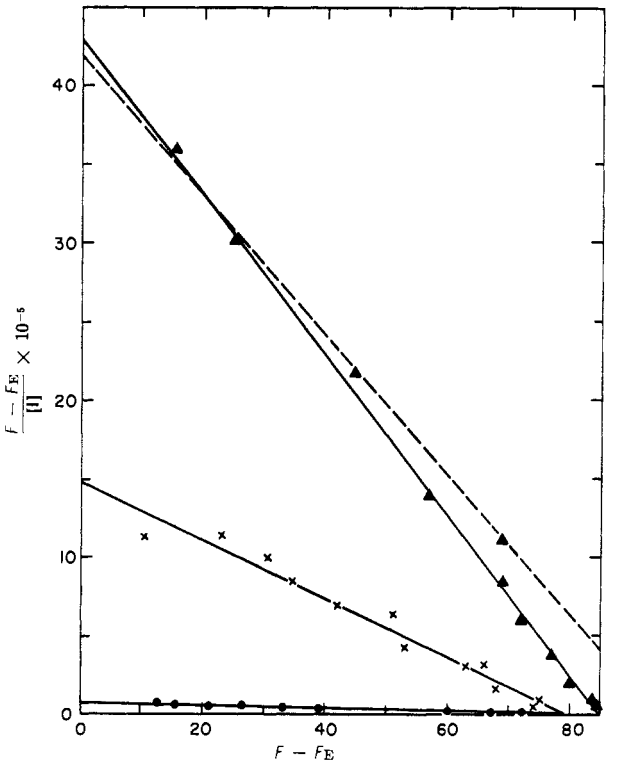

FIGURE 9: Scatchard plot of inhibitor binding to human lysozyme: (●) $(AcGlcN)_2$, (×) $(AcGlcN)_3$, (▲) $(AcGlcN)_4$; dashed line $(AcGlcN)_5$. Fluorescence emission measured at 323 nm in phosphate buffer, (pH 7.5).

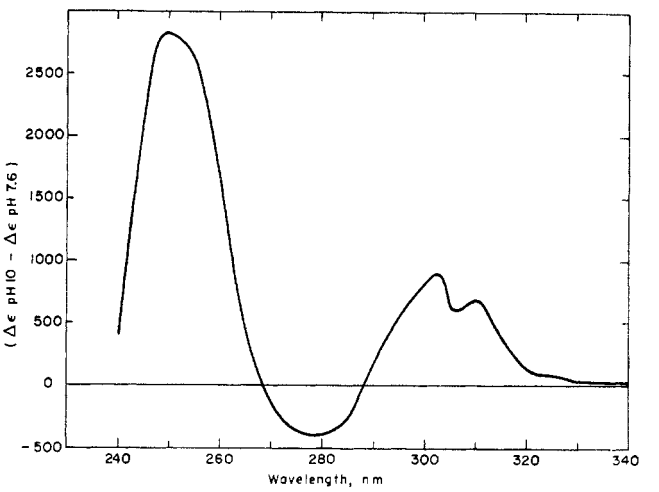

FIGURE 10: Calculated difference – difference spectrum of human lysozyme–$(AcGlcN)_4$ complex: [$\Delta\epsilon\lambda$ (pH 10) – $\Delta\epsilon\lambda$ (pH 7.6)]. Conditions as in Figure 4.

hydroxyl might donate a proton, in a hydrogen bond, to the ionized Tyr-63 (-62). Alternatively, if the tyrosine is internally H bonded to a protein carboxyl or carbonyl group a conformational change following inhibitor binding might weaken the H bond, and lower the apparent pK.

Using the data obtained from difference spectroscopy of the enzyme and its inhibitor complex it is possible to estimate the pK's of Tyr-63 (-62). The pH_{max} at which $\Delta\epsilon$ is maximal (*i.e.*, $\Delta\epsilon_{max}$) is 10.1, as measured from Figure 5, and this is the average of the apparent pK's of Tyr-63 (-62) in the complex and the free enzyme (see Appendix eq 4). The magnitude of $\Delta\epsilon_{max}$ can be estimated from Figure 10, in which the contribution to the difference spectrum from tryptophan perturbation has been removed. Using $\Delta\epsilon_{max,250\ nm} = 2820$, the intrinsic p$K$ of tyrosine-62 (-63) may be calculated in eq 5 and 6 (see Appendix). Thus in the free enzyme this tyrosine has the slightly abnormal pK_{int} of 10.55, and in the complex this is lowered to 10.07, the overall pK change on binding inhibitor being 0.5.

The fluorescence properties of human and hen egg-white lysozymes display a number of differences which may be explained in terms of the different aromatic compositions of the two enzymes. First the protein quantum efficiency of human lysozyme is lower than that of hen egg-white (0.04 and 0.06, respectively). When comparing the quantum efficiencies of proteins with different numbers of tryptophans it is important to consider tryptophan quantum efficiencies (*i.e.*, the sum of the quantum efficiencies of the individual tryptophans) rather than the measured protein quantum efficiences. The tryptophan quantum efficiency is given by the following equation (Kronman *et al.*, 1971): $\Sigma_i Q_i = nQ$, where Q_i is the quantum efficiency of the ith tryptophan, Q is the protein quantum efficiency, and n the number of tryptophans per molecule. Accordingly the tryptophan quantum efficiencies of hen egg-white and human lysozymes are 0.36 and 0.20, respectively. These tryptophan quantum efficiencies do not allow for the contribution of tyrosine to either the absorption or emission spectra, that is they are based on the assumption that only tryptophan absorbs at the excitation wavelength. Since the tyrosine composition of human lysozyme is high (six residues) this assumption will lead to a low estimate of the quantum efficiency. Tyrosine will account for approximately 10 and 20% of the absorption at 280 nm of hen egg-white and human lysozymes, respectively. Adjusting the tryptophan quantum efficiencies for tyrosine absorption gives 0.4 for hen egg-white and 0.24 for human lysozyme. These are maximum values for tryptophan quantum efficiency since the tyrosine may also contribute to the fluorescence emission (Lehrer and Fasman, 1966), although the contribution is probably very small.

The tryptophan quantum efficiency of human lysozyme is, therefore, reduced by about 40% relative to hen egg-white lysozyme. In their evaluation of the contributions of various tryptophan residues to the fluorescence of hen egg-white lysozyme, Imoto *et al.* (1971) estimate that tryptophan-62 provides 35–38% of the fluorescence over the pH range 2–8. The replacement of Trp-62 by Tyr-63 (-62) in human lysozyme may therefore account for the reduced quantum efficiency of this enzyme. This hypothesis is further supported by the fact that the emission maximum of human lysozyme is blue shifted relative to hen egg-white lysozyme. Trp-62 is the most exposed tryptophan residue of hen egg-white lysozyme and may be expected to emit at the longest wavelength. Trp-108, the other major fluorophore of hen egg-white lysozyme (Imoto *et al.*, 1971), is partially buried and will emit at a shorter wavelength, and one may expect this residue to dominate the fluorescence spectrum of human lysozyme. It is of interest that α-lactalbumin which also possesses Trp-104 (108) but not Trp-62, also has an emission maximum close to 330 nm (Kronman *et al.*, 1971).

The fluorimetric titration of hen egg-white lysozyme is discussed in some detail by Lehrer and Fasman (1967). Below pH 5.5 the fluorescence intensity remains fairly constant and above pH 8.0 there is strong quenching by ionized tyrosine. Both these features are duplicated in the fluorimetric titration of human lysozyme. In raising the pH from 5.5 to 7.5 hen egg-white lysozyme fluorescence is dequenched and human lysozyme fluorescence is quenched. The pH region implicates

the ionization of Glu-35 which probably perturbs Trp-108. However, the reason for the opposing effects of this ionization on the fluorescence of the two lysozymes is not clear. The changes in fluorescence of the hen egg-white lysozyme–$(AcGlcN)_3$ complex in the acid region are attributed to the protonation of two carboxyls affecting two distinct tryptophans. The protonation of Glu-35 quenches Trp-108. The quenching of the fluorescence of human lysozyme–$(AcGlcN)_3$ complex around pH 7.5–5.5 is consistent with this mechanism. Quenching of the hen egg-white lysozyme complex between pH 5.5 and 2.0 is interpreted by Lehrer and Fasman as being due to protonation of Asp-52 or Asp-101, affecting the fluorescence of Trp-63. However, there is no corresponding effect in the fluorimetric titration of the human lysozyme–$(AcGlcN)_3$) complex, and Imoto *et al.* (1971) have concluded that Trp-63 contributes little to hen egg-white lysozyme fluorescence. These results are difficult to reconcile with the quenching of Trp-63 at low pH. It is more probable that in the hen egg-white lysozyme complex Trp-62, having moved close to the binding cleft, is available to carboxyl quenching. That there is no similar quenching in the free enzyme may reflect the different orientation of Trp-62 to the binding site. Further, since human lysozyme has no tryptophan at position 63 (62), this hypothesis is consistent with the absence of fluorescence quenching in both human lysozyme and its $(AcGlcN)_3$ complex below pH 5.0.

Perhaps the most interesting result from the binding studies of inhibitors to human lysozyme is that the enzyme binds $(AcGlcN)_3$ less strongly than does hen egg-white lysozyme. The interactions between $(AcGlcN)_3$ and the A, B, and C subsites of hen egg-white lysozyme are summarized by Imoto *et al.* (1972). In all there are 48 van der Waal's contacts and six hydrogen bonds, including the interaction between Trp-62 and the sugar C-6 hydroxyl at subsite C. Some caution should be observed in interpreting the different association constants of the two enzymes. Subtle differences in the topographies of their binding sites might alter a number of these polar and nonpolar interactions. However, the difference spectroscopy studies on human lysozyme suggest that there is no H bond between Tyr-63 (-62) and the inhibitor below pH 10. Consequently, it is tempting to conclude that the absence of this H bond accounts, to a large extent, for the loss of 800 cal in the free energy of binding.

The different association constants of $(AcGlcN)_3$ and $(AcGlcN)_4$ with human lysozyme demand attention. The tri-, tetra-, penta-, and hexasaccharides of AcGlcN all have the same association constants with hen egg-white lysozyme at 25° (Dahlquist *et al.*, 1966). It is believed that they bind in the same nonproductive mode involving an interaction between the three sugar rings at the reducing end of the molecule and subsites A–C on the enzyme. At slightly elevated temperatures (*e. g.*, 30°) $(AcGlcN)_4$ appears to bind to hen egg-white lysozyme with a higher association constant than $(AcGlcN)_3$ (Imoto *et al.*, 1972). Therefore, the binding of $(AcGlcN)_4$ to human lysozyme may resemble that to hen egg-white lysozyme at the higher temperature.

Appendix (with Duane W. Sears)

The spectrophotometric titration curve for a single tyrosine in a protein is given by

$$\log \frac{\Delta E}{\Delta E_m - \Delta E} = \mathrm{pH} - \mathrm{p}K + 0.868wz \qquad (1)$$

$$\Delta E = \frac{\Delta E_m 10^{(\mathrm{pH}-\mathrm{p}K+0.868wz)}}{1 + 10^{(\mathrm{pH}-\mathrm{p}K+0.868wz)}} \qquad (2)$$

ΔE is the increase in the molar extinction coefficient for partial ionization of the tyrosine, ΔE_m is the increase for complete ionization, and the pK is intrinsic. Values for the electrostatic factor, w, and the charge on the protein z are taken from Latovitzki *et al.* (1971). In this derivation it is assumed that the values of w and z are the same for the enzyme and its inhibitor complex. Strictly this is not true. However, the differences are negligible. If ΔE_1 and ΔE_2 are the increments in the extinction coefficient with tyrosine ionization for the complex and enzyme respectively, the change, $\Delta\epsilon$, brought about by binding inhibitor with resultant alteration of a tyrosine pK, is given by

$$\Delta\epsilon = \Delta E_1 - \Delta E_2 = \Delta E_m \frac{10^{(\mathrm{pH}+0.868wz-\mathrm{p}K_1)}}{1 + 10^{(\mathrm{pH}+0.868wz-\mathrm{p}K_1)}} - \frac{10^{(\mathrm{pH}+0.868wz-\mathrm{p}K_2)}}{1 + 10^{(\mathrm{pH}+0.868wz-\mathrm{p}K_2)}} \qquad (3)$$

By differentiating $\Delta\epsilon$ with respect to pH, and setting $\partial\Delta\epsilon/\partial\mathrm{pH} = 0$, the pH_{max} at which $\Delta\epsilon$ is maximal ($\Delta\epsilon_{max}$) is obtained

$$\mathrm{pH}_{max} = \frac{\mathrm{p}K_1 + \mathrm{p}K_2}{2} - 0.868wz \qquad (4)$$

Using this relationship eq 3 can be solved for pK_1 (the intrinsic pK of tyrosine in the complex) and pK_2 (the intrinsic pK of tyrosine in the enzyme)

$$\mathrm{p}K_1 = \mathrm{pH}_{max} + \log \frac{1 - (\Delta\epsilon_{max}/\Delta E_m)}{1 + (\Delta\epsilon_{max}/\Delta E_m)} + 0.868wz \qquad (5)$$

$$\mathrm{p}K_2 = \mathrm{pH}_{max} - \log \frac{1 - (\Delta\epsilon_{max}/\Delta E_m)}{1 + (\Delta\epsilon_{max}/\Delta E_m)} + 0.868wz \qquad (6)$$

The values of $\Delta\mathrm{pH}_{max}$ and $\Delta\epsilon_{max,250\ nm}$ are obtained, as explained in the text, from Figures 5 and 10, respectively. The value for $\Delta\epsilon_{m,250\ nm}$ is taken as 10,500.

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Conformation and Immunochemistry of Methylated and Carboxymethylated Derivatives of Lysozyme†

Ching-Li Lee and M. Z. Atassi*

ABSTRACT: Reduction of the disulfide bonds of lysozyme with 2-mercaptoethanol in 8 M urea followed by reaction with iodoacetic acid or with methyl *p*-nitrobenzenesulfonate gave two derivatives. One derivative was carboxymethylated (SCM-lysozyme[1]) and the second was methylated (SM-lysozyme) at the eight cysteine residues resulting from reduction of the four disulfide bonds. Optical rotatory dispersion (ORD) and circular dichroism (CD) measurements in water showed that the two derivatives were greatly unfolded relative to native lysozyme. There was some indication from CD measurements that SM-lysozyme was somewhat more folded than SCM-lysozyme in water. Conformational studies in increasing concentrations of methanol suggested that SM-lysozyme assumed some structural stability around 35% methanol while SCM-lysozyme showed no discreetly stabilized structure in the range 0–60% methanol. The stabilized structure of SCM-lysozyme had ORD parameters that approximated those of native lysozyme. Further confirmation of this was obtained from immunochemical studies. SCM-lysozyme showed no reaction (0%) with antisera to lysozyme. On the other hand, SM-lysozyme showed appreciable (38%) cross-reaction with these antisera. However, the enzymic activity in each derivative was completely eliminated, suggesting that more rigid structural requirements are needed for this property than for immunochemical cross-reaction. The present findings indicated that it was indeed feasible, at least to a limited extent, to effect a stabilized structure in SM-lysozyme due to the ability of the *S*-methyl groups to participate in nonpolar interactions. In SCM-lysozyme, the directive effect of long-range interactions is ineffective because a refolded, stabilized structure is prevented by steric effects and by the like-charge repulsion between the carboxymethyl anions as they approach one another.

It is now well established that protein conformation is a direct consequence of primary structure (Lumry and Eyring, 1954; Sela *et al.*, 1957; White and Anfinsen, 1959; Anfinsen, 1961, 1964; White, 1960). The major role in stabilizing protein folding is contributed by long-range interactions (Singhal and Atassi, 1970; Atassi and Singhal, 1970), since an intact whole

† From the Department of Chemistry, Wayne State University, Detroit, Michigan 48202. *Received February 23, 1973*. This paper is the sixth in the series: Conformational Studies on Modified Proteins and Peptides. This work was supported by the American Heart Association (Grant No. 71-910) and in part by a grant (AM-13389) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service.

[1] The abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: SCM-lysozyme, a derivative of lysozyme in which the disulfide bonds were reduced and then the resulting cysteine residues were carboxymethylated by reaction with iodoacetic acid; SM-lysozyme, a lysozyme derivative obtained by reduction of the disulfide bonds followed by methylation with the use of methyl *p*-nitrobenzenesulfonate.