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Optical Activity of Human Lysozyme

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ABSTRACT **The ultraviolet circular dichroism spectra of human lysozyme are presented. Effects of pH and added inhibitor (*N*-acetyl-D-glucosamine) were examined and the results were compared with similar measurements of hen egg-white lysozyme. The near-ultraviolet CD spectral bands are substantially different in the human and hen egg-white enzymes. In addition to marked dissimilarities in the spectral interval 260–300 nm, an unusual CD band occurs at an anomalous wavelength (313 nm) in human lysozyme. The pH dependence of the latter suggests a possible interaction, absent in hen egg-white lysozyme, between a tryptophan and a tyrosine residue. Analysis of the spectra furthermore suggests lesser net rotational strengths of tryptophan bands in hen egg-white lysozyme than in human lysozyme, although the latter has one less tryptophan residue. The relationship between the CD spectra and the sequence differences of the proteins is discussed, as well as the CD spectra (published by others) of a closely related protein, bovine α-lactalbumin. Contributions of cystine residues to the spectra are examined in the light of possible differences in chirality of one of the four disulfide bridges.**

The far-ultraviolet CD spectra of human and egg-white lysozyme are quite similar, though not identical. In view of the pronounced differences in side-chain optical activity, and of the effect of pH variation on the far-ultraviolet CD spectrum of human lysozyme, it is likely that at least part of the observed difference in spectra is due to nonpeptide optical activity, and that the proteins have a secondary structure in common.

Human lysozyme can be isolated in large quantities from the urine of individuals with monocytic or monomyelocytic leukemia; the enzyme so isolated appears to be identical with that obtained from normal human tissues and secretions (1). There are several similarities between human lysozyme and hen egg-white lysozyme, but there are important differences as well. Both proteins are small, with sedimentation coefficients (1) close to 1.80 S. The number of amino acid residues in the egg-white protein is 129, that in the human protein, 125 (2). Differences in the sequence commence at residue 4 and occur at many positions in the chains. However, there is extensive sequence homology; at approximately 70 positions, identical residues occur (2). Furthermore, as Canfield observed, the deletions, insertions, and substitutions are such that the three-dimensional structures of the two enzymes could be very similar. For example, as he noted, in the human enzyme a glycine residue is inserted between positions 47 and 48. This is at the hinge of the antiparallel pleated sheet (2, 3) and, consequently, the residue could be accommodated without disruption of this element of β-structure that appears to be vital to the function of the enzyme (3, 4). Moreover, the number and pairing of cystine residues are the same; the catalytically important glutamate and aspartate residues occur at the same points, when account is taken of the insertion mentioned above; and a number of other active-site residues are identical in both (2). Substrate specificities are similar, although the human enzyme is between 2 and 12 times as active against the cell wall of *M. lysodeikticus* (1). Enzyme inhibition and nmr studies point to common structural and functional features at the active site (5, 6).

Notwithstanding these similarities, the differences should not be overlooked. Perhaps most notable is that the proteins exhibit no immunological cross-reactivity (1, 7). In addition, an important active-site residue, aspartate 101 in egg lysozyme, is replaced by proline 102 in human lysozyme. Tryptophan 62 of egg lysozyme, which helps bind substrate (3, 8), is replaced by a tyrosine residue.

Preliminary crystallographic data on human lysozyme have appeared (7). Extensive comparison of solution properties thus appears highly worthwhile. In this paper, we report the circular dichroic spectra of human lysozyme solutions. Comparisons are made with CD spectra of egg-white lysozyme reported by others (9–16) as well as by ourselves. In particular, an unusual CD band occurs at an anomalous wavelength in human lysozyme only, and the pH dependence of this band is examined. Other significant similarities and differences are described and discussed in terms of the sequences.

MATERIALS AND METHODS

Three samples of human lysozyme isolated from the urine of patients with monocytic or monomyelocytic leukemia were used in this work. Two samples from the urine of an individual with monocytic leukemia were provided by Dr. Robert E. Canfield. The protein had been purified by ion-exchange chromatography on carboxymethylcellulose. In a subsequent step, the protein was desalted on Sephadex G-25 in 1% acetic acid and then lyophilized (Robert E. Canfield, personal communication‡). The third sample was provided by Dr. Elliott F. Osserman. The lysozyme had been isolated from the urine of a patient with monomyelocytic leukemia by bentonite adsorption and elution with 5% aqueous pyridine adjusted to pH 5 (1). We crystallized it twice at pH 4.5 in 5% NaCl according to a method used by Alderton and Fevold (17) in crystallizing egg-white lysozyme, as adapted to human lysozyme by Osserman (18).

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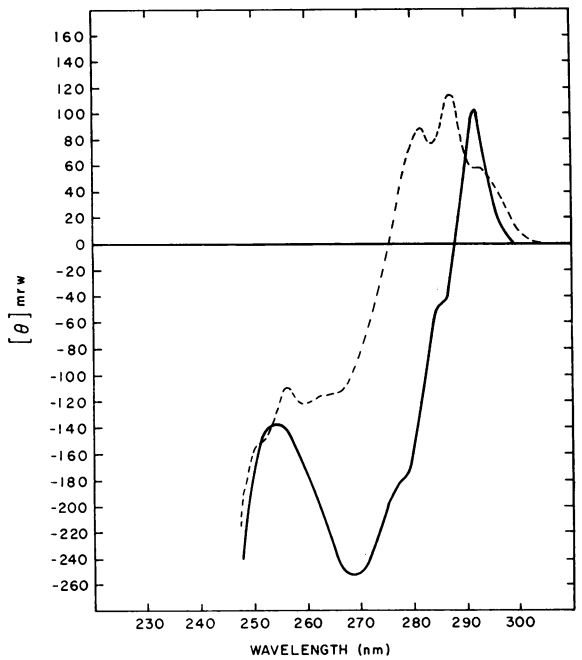

FIG. 1. Circular dichroism spectra of human and hen egg-white lysozyme at pH 5.8, 25°C. (——), human lysozyme; (— —), hen egg-white lysozyme. Each spectrum is an average of measurements on six different solutions, each measurement performed in duplicate. The concentration range included is 0.4–0.8 g/liter. Cell path, 1.0 cm. Acetate buffer, ionic strength 0.10.

The lyophilized and crystallized samples were indistinguishable from the points of view of extinction coefficient at 280 nm, $s_{20,w}$ values, enzyme assay according to the method of Parry *et al.* (19), titration behavior of tyrosine residues (20), and CD spectra as reported in this paper.

Crystalline hen egg-white lysozyme was obtained from Pentex, Inc., and used without further purification.

Circular dichroism measurements were performed on a Cary 60 spectropolarimeter with 6001 CD attachment. Most experiments were performed at room temperature. A few, as indicated in the figure legends, were done using jacketed cells with the temperature controlled at 25°C. Absorption spectra were measured on a Cary 14 spectrophotometer at 25°C.

Concentrations of human lysozyme solutions were computed from absorption values at a peak located at 281 ± 0.5 nm, using $E^{1\%}_{1cm} = 24.6$, referred to dry weight.

Curve resolutions were performed on a DuPont 301 Curve Resolver.

RESULTS

Near-ultraviolet region

Fig. 1 shows the near-UV CD spectra of human lysozyme and hen egg-white lysozyme at pH 5.8. The curve for hen egg-white lysozyme is much like those reported by others (9–13, 15, 16). The complex shape of the spectrum between 275 and 300 nm is in accord with results of Glazer and Simmons (11), Ikeda and Hamaguchi (13), and Cowburn *et al.* (15).

The spectrum of human lysozyme is very different from that of the egg-white enzyme. The most prominent features are a positive band at 292 nm, a large negative band at 268–269 nm, and shoulders at 286 and 275 nm.

Fig. 2 shows the CD spectra of human lysozyme in the same spectral region at various pH values between 5.8 and 11.60. The alkaline spectra were all reversible, at least up to 24 hr. The most significant and unexpected result shown in the figure is the pH-dependent appearance of a negative band

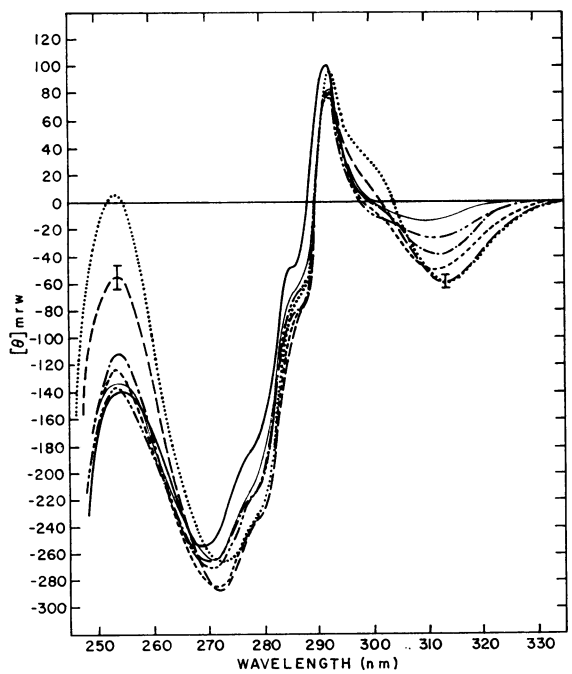

FIG. 2. Circular dichroism spectra of human lysozyme at several pH values between 5.8 and 11.4; ionic strength 0.10, 25°C. (——), pH 5.8, acetate buffer. (——), pH 8.89, glycine-KOH. (— - - —), pH 9.15, glycine-KOH. (— - —), pH 9.60, glycine-KOH. (- - -). pH 9.96, glycine-KOH. (— —), pH 10.83, glycine-KOH. (· · ·), pH 11.40, KOH-KCl.

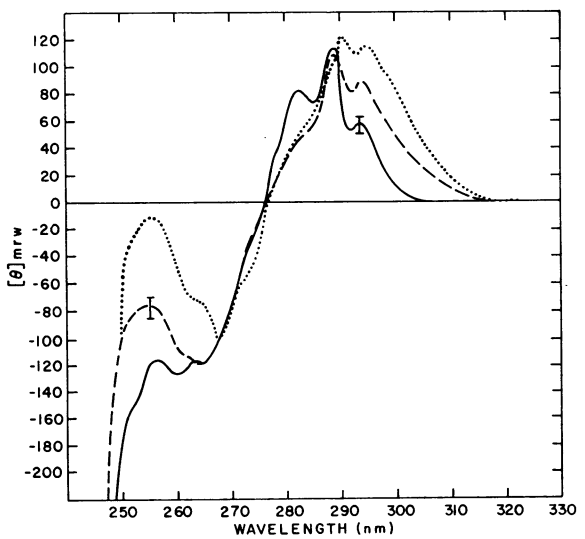

FIG. 3. Circular dichroism spectra of hen egg-white lysozyme at pH 5.83, 9.5, and 10.3, ionic strength 0.10, room temperature. (——), pH 5.8; (— —), pH 9.5; (· · ·), pH 10.3. In each case, the spectrum is an average of measurements in two different buffer systems: pH 5.8, average using phosphate and acetate buffers; pH 9.5, average using borate and glycine; pH 10.3 average using glycine and ammonium hydroxide. Vertical bars indicate noise at wavelengths indicated.

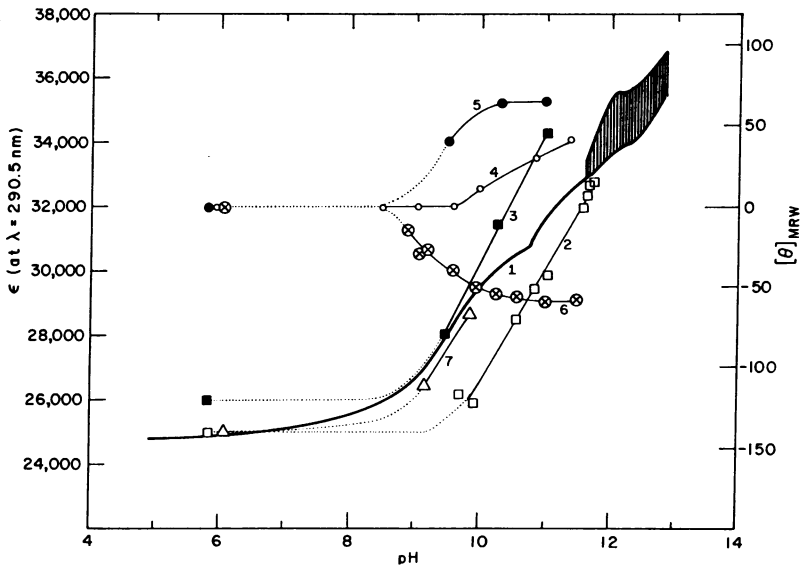

FIG. 4. Variation with pH of mean residue ellipticity of human and hen egg-white lysozyme and extinction coefficients of human lysozyme at selected wavelengths.

1, spectrophotometric titration of tyrosine residues in human lysozyme [taken from Latovitzki *et al.* (20)]. The molar extinction values at 290.5 nm are given on the left ordinate. The shaded region indicates a pH interval in which the titration exhibits time dependence. The lower line represents 10-min values; the upper line 24-hr values. One buried residue becomes exposed during this time interval. *2*, observed mean residue ellipticity at 254 nm of human lysozyme. *3*, observed mean residue ellipticity at 254 nm of hen egg-white lysozyme. *4*, mean residue ellipticity at 298 nm of a resolved CD band of human lysozyme. *5*, mean residue ellipticity at 298 nm of a resolved CD band of hen egg-white lysozyme. *6*, observed mean residue ellipticity at 313 nm of human lysozyme. *7*, observed mean residue ellipticity at 254 nm of human lysozyme in solutions containing 0.25 M D-GlcNAc.

Ellipticity values on right ordinate scale; extinction coefficients on left ordinate scale. The solid lines are included only to connect the points and for ease in examination of the figure. Except where points are shown, the lines are arbitrarily drawn. The dots represent continuations of the solid lines; in dotted intervals, no experimental points were obtained.

centered at 313 nm. So far as we know, this is the longest-wavelength ellipticity band that has been observed until now in a nonconjugated protein. This band is distinct from a smaller positive band, or shoulder, at 298 nm (see below), which may be responsible for the displacement of the peak at 292 nm.

Increasing pH also brings about increasingly positive ellipticity values at 253–254 nm and at 298 nm, as well as alteration in the positions of the shoulders on the large negative band near 270 nm.

Fig. 3 shows the CD spectra of egg-white lysozyme at several pH values. By and large, the pH dependence illustrated in Fig. 3 for egg-white lysozyme is similar to that found by Ikeda and Hamaguchi (13).

In Fig. 4, some of the changes in ellipticity of human lysozyme at specific wavelengths are plotted as a function of pH, together with changes in extinction coefficient (alkaline titration curve) at 290.5 nm and changes in ellipticity at two wavelengths observed in egg-white lysozyme. The extinction coefficients of human lysozyme drawn in Fig. 4 are from the paper by Latovitzki *et al.* (20).

From the data in Fig. 4, it is seen that the change in ellipticity at 254 nm does not parallel the tyrosine titration curve. No noticeable increase occurs at that wavelength until pH 10, at which pH an average of two tyrosine residues has already become ionized. Between pH 10 and 11.6, two additional tyrosine residues per molecule become ionized and the ellipticity at 254 increases by 140. With the egg-white lysozyme, the ellipticity at 254 nm increases uniformly between pH 9 and 11; this range corresponds closely to the range in which the two titratable residues have been shown (21) to become ionized. The change in ellipticity is somewhat greater per residue than is seen for human lysozyme. The main difference is that only half of the four reversibly-ionized tyrosine residues of human lysozyme appear to contribute to the ellipticity at 254 nm, whereas both of two reversibly-ionized residues in egg-white lysozyme contribute.

Curve resolution of the spectra of hen egg-white lysozyme in Fig. 3 revealed that addition of a single band at 298 nm is sufficient to accommodate the CD changes at wavelengths longer than 288 nm, and the intensities at three pH values are shown in Fig. 4. A comparable positive band is required to fit the human lysozyme spectra at high pH, and resolved intensity values over a similar pH range are shown in Fig. 4. The difference in pH dependence of these bands is much like that at 254 nm, in that two tyrosine residues are ionized in human lysozyme before the band at 298 nm appears, while with the egg-white lysozyme the band appears together with the ionization of the first of the two reversibly-ionized residues. In contrast, however, to the 254-nm band in egg-white lysozyme, the intensity levels off and is unchanged with the ionization of the second. These results are very similar to those of Ikeda and Hamaguchi (13).

The pH dependence of the anomalous negative band at 313 nm, exhibited only by human lysozyme, is also shown in Fig. 4. Within the limits of experimental error, the band follows the titration of the first three reversibly-ionized tyrosine residues (20), which is complete by pH 10.7.

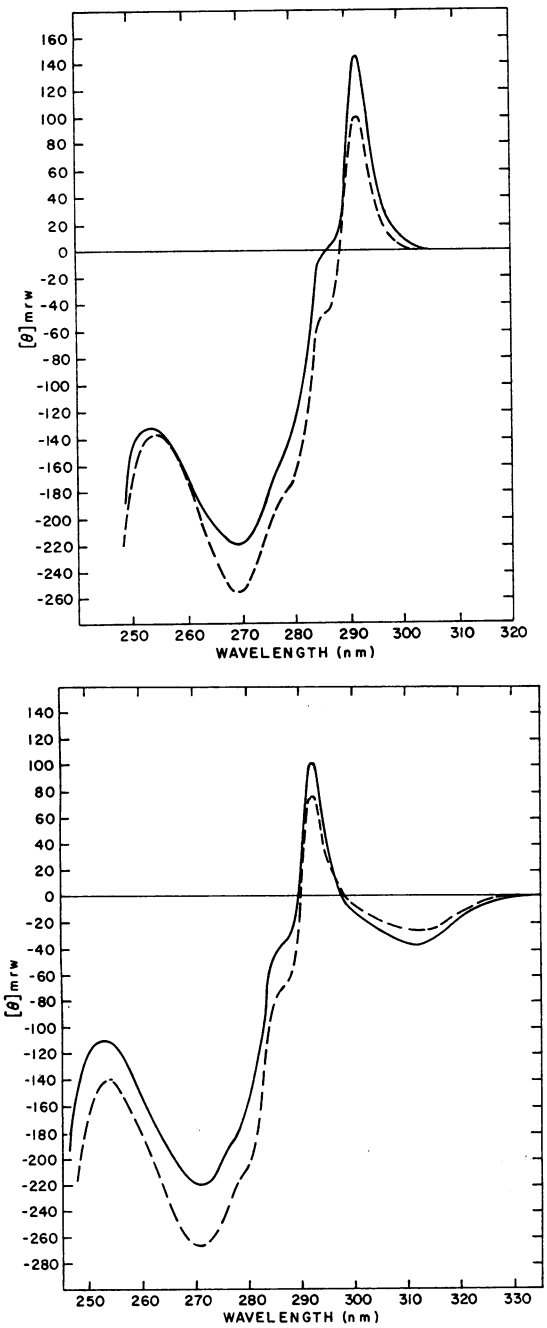

FIG. 5. (*Above*) Effect of added D-GlcNAc on CD spectrum of human lysozyme at pH 5.8. (- -), no added D-GlcNAc; (—), 0.25 M D-GlcNAc.

(*Below*) Effect of added D-GlcNAc on CD spectrum of human lysozyme at pH 9.13. (- -), no added D-GlcNAc; (—), 0.25 M D-GlcNAc.

Fig. 5 (*above*) and (*below*) illustrates the effect of added *N*-acetyl-D-glucosamine (D-GlcNAc) on the CD spectra of human lysozyme at pH 5.8 and 9.13, respectively. No profound changes in shape or numbers of bands and shoulders occur, but intensity alterations occur at both pH values. The positive peak at 292 nm becomes more positive, as do the ellipticities of the negative shoulders and the band at 270 nm. Two additional changes are observed at pH 9.13: there is a slight intensification of the 313-nm band and a small change towards positive values at 253 nm. A spectrum in the presence of D-GlcNAc at pH 9.80 was also recorded. It is identical to that at 9.13 except for slight, but further, increases in the intensities of the bands at 254 and 313 nm. The changes at 254 nm are shown in Fig. 4, and it is seen that the points are now displaced to lower pH, compared to the results in the absence of D-GlcNAc.

Curve resolutions were performed to ascertain whether the D-GlcNAc effect was restricted to certain of the underlying bands. Resolutions of such complex spectra, even when successful in matching the observed curves to a sum of Gaussians, are usually not unique. In the present case, it was only possible to decide that the largest change is associated with the intensity of a negative resolved band at 270 nm, which diminishes by 20–30% in the presence of D-GlcNAc. In addition, a resolved positive band near 292 nm increases by 20% at pH 5.8, but is largely unchanged at pH 9.13, although this is not evident in the observed spectra. Smaller intensity changes, including increased positive ellipticity in a band near 285 nm, occur in a number of the other bands required to fit the full spectra. Changes in position are not excluded, but it was possible to fit all of the data in the presence of D-GlcNAc without introducing such alterations.

Far-UV region

The far-UV CD spectra of human lysozyme are shown in Fig. 6. The shaded area in the figure includes curves for a number of pH values; no clear trend was observed within this shaded area, which probably defines the experimental uncertainty.

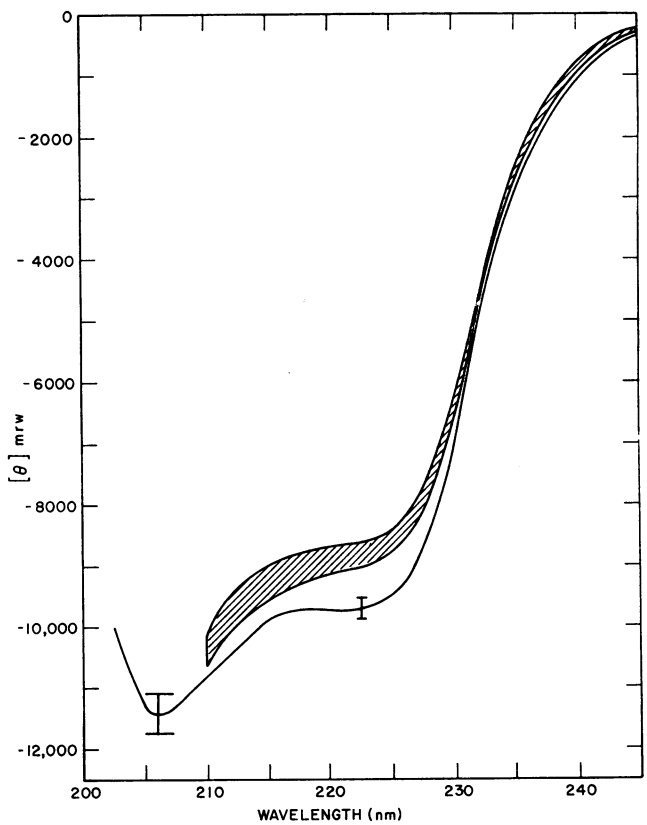

FIG. 6. Far-UV CD spectra of human lysozyme. The solid line is at pH 5.8. The shaded area represents curves measured at various pH values between 9.1 and 11.7.

The acid curve is reproducibly different from those at higher pH, as indicated. The spectrum of egg-white lysozyme at pH 5.8 is not shown. Our results were virtually identical with those presented by Greenfield and Fasman (14) and quite similar to those of Cowburn *et al.* (15). At 220 nm, the ellipticity is 9050 ± 200 deg cm²/decimole, and at 207 nm, the value is −12,400 ± 400 deg cm²/decimole.

DISCUSSION

In the complex, and quite different, near-UV CD spectra of human and hen egg-white lysozymes, the least equivocal assignments are of the 292-nm band in human lysozyme and the 294-nm band in hen egg-white lysozyme at pH 5.8. Both bands may confidently be assigned to tryptophan residues. At higher pH, when account is taken of a small band at 296–298 nm due to ionized tyrosine residues (Fig. 4), the 292–294 nm band in both proteins is observed to change very little or not at all, and binding of inhibitor increases the intensity of the band. Furthermore, numerous small peptides (22–24) that contain tryptophan, as well as poly(L-tryptophan) (25), show sharply peaked bands at 289–292 nm. Usually the band is accompanied by another, especially at low temperature in small peptides, of the same sign, at about 7 nm toward the blue, which represents the same electronic transition between different vibrational states (23, 24). This doublet is present in hen egg-white lysozyme. In human lysozyme, the doublet is not apparent in the unresolved spectra, but curve resolution brings out the shorter wavelength band. The total intensity of these bands is substantially greater in the human lysozyme spectra than in the egg-white lysozyme spectra, despite the larger number of tryptophan residues in the latter.

There is an experimental basis for believing that the lesser net intensity of the tryptophan bands in hen egg-white lysozyme may arise from cancellation of opposite sign contributions. Teichberg *et al.* (16) have recently shown that selective oxidation of Trp 108 in egg-white lysozyme abolishes the positive ellipticity, with the appearance of negative bands only between 265 and 300 nm. This oxidation interrupts coupling of transitions in residue 108 with identical transitions in Trp residues 63 and 111, which are close, and possibly in 28 and 62 as well. In bovine α-lactalbumin, positions corresponding to residues 62 and 111 are not occupied by tryptophans (26), although the homologous tryptophan corresponding to position 108 is present and only negative bands are observed in the near-UV CD spectrum (15, 27).

A theoretical analysis of the differences in tryptophan optical activity in these two proteins is in progress. For example, one of the structural features that is absent in human lysozyme and present in hen egg-white lysozyme is the adjacent pair of Trp residues, 62 and 63, in the active site. This pair can generate an exciton couple. The main spectral peak at 292 nm is due to a transition moment inclined at 40° to the long axis bisecting the indole nucleus (28). When the phases of the coupled moments in the two residues are such that the interaction energy between them is positive, the vectors form a right-handed screw. Thus the long-wavelength band of the exciton couple would be negative and the short-wavelength one positive (29).

The dominant difference, at neutral pH, between the CD spectra of the two proteins at shorter wavelength—255 to 285 nm—is accounted for by a large negative band in the human enzyme centered near 270 nm. The CD of human lysozyme near 270 nm resembles that of bovine α-lactalbumin much more than that of hen egg-white lysozyme (15).

In view of the tryptophan contributions at longer wavelengths and of the effect of inhibitor on the spectrum near 270 nm, it is probable that much of the intensity near 270 nm derives from the ^1L_a transition of tryptophan residues. The CD bands associated with this transition have greater rotational strengths than the ^1L_b transitions in the same compounds (22–25). Arguments similar to those advanced above suggest that cancellation is greater in egg-white than in human lysozyme. Differences due to tyrosine contributions, at neutral pH, do not seem likely to be responsible for the great discrepancy near 270 nm. From resolved spectra and comparison of computed differences at pH 5.8 and 11.2, one may conclude that accessible tyrosine residues contribute only small positive bands near 280 nm, in both proteins. Ionization of these tyrosine residues leads to a diminution in positive ellipticity near 280 nm and the change is greater in human lysozyme, in accord with the observation that more tyrosine residues become ionized between pH 5.8 and 11.2. The small positive peak at 280 nm in egg-white lysozyme and the shoulder at 280 nm in human lysozyme thus arise, at least partly, from ionizable tyrosine residues.

An alternative or additional explanation of the difference near 270 nm may be sought in terms of cystine optical activity. In their model of bovine α-lactalbumin, based on the main chain conformation egg-white lysozyme, Browne *et al.* (30) make the interesting observation that disulfide bridge 6—127 is most probably of right-handed chirality in bovine α-lactalbumin, and of left-handed chirality in egg-white lysozyme. In examining stereo models (31), we have noted that the deletion in human lysozyme of the four residues corresponding to residues 122 through 125 of egg-white lysozyme might be accommodated with minimum structural changes by a change in the screw sense of cystine 6—127. It is interesting, then, to inquire whether the difference in screw sense might explain the great similarity in the spectra of human lysozyme and bovine α-lactalbumin in the spectral region 250–280 nm, and their dissimilarity with hen egg-white lysozyme between 260 and 280 nm.

Straight-chain, or large-ring, disulfides characteristically generate a CD band between 249 and 260 nm (9). In addition, the disulfide can generate another band at 270–280 nm, as was observed with oxytocin derivatives (32) and neurophysin (33). The three proteins exhibit about the same ellipticity at 250 nm. To the extent that chirality itself governs the 270-nm band, the direction of the change in both human lysozyme and bovine α-lactalbumin is opposite to what one might expect, since this would be the longest-wavelength disulfide band, and a right-handed disulfide should generate a positive band if due to dissymmetry. However, if the band is due predominantly to asymmetric perturbation, the contribution could be of either sign, and changing from one screw sense to the other would change the sign of the bands if the perturbant is held in a fixed position, since disulfides follow a quadrant rule (32, 34). On balance, we are slightly disposed to favor aromatic interactions as providing the major part of the intensity of the large 270-nm band. Computational and experimental tests should allow this question to be resolved.

We turn, finally, to the highly unusual band at 313 nm observed at alkaline pH in human lysozyme. The band could arise from a bound low molecular weight substance carried

through the isolation. There is no evidence for this; extensive purification, including gel filtration and recrystallization, had no effect on this new CD band.

Human lysozyme from other tissues has not yet been investigated by this technique. It would also be useful to know whether bovine α-lactalbumin exhibits such a band at high pH.

Since the possibility of a bound extrinsic chromophore seems remote, it is necessary to explore other possibilities. The CD band appears over a pH interval in which three tyrosine residues titrate with normal intrinsic pK values. If the CD band represents an ionized tyrosine absorption, then it is at a wavelength 18 nm displaced from its usual position. Tryptophan-residue CD bands have been observed at wavelengths as long as 302 nm in aqueous solutions (35). The proximity of Tyr 63 and Trp 64 (or 62 and 63 with hen egg-white numbering) in the active site of human lysozyme seemed a possible source of a special interaction not observed in hen egg-white lysozyme. Our preliminary, but still weak, evidence on this gives conflicting results. In this view, Tyr 63 would have to be one of the three normally ionizing residues. The binding of D-GlcNAc should have some effect on the pK of the group and some effect on the anomalous band at 313 nm. Titration experiments thus far show no difference between pH 7 and 9.6 when D-GlcNAc is present. The CD band at 313 nm is, however, slightly altered in intensity in the presence of D-GlcNAc. A possible complication is that D-GlcNAc may be bound differently at neutral and alkaline pH, as evidenced by the different effect of D-GlcNAc on the 292-nm band at pH 5.8 and 9.1. The binding of D-GlcNAc is weak and the experiments are performed at 0.25 M inhibitor. More definitive results should emerge with larger inhibitors that bind at lower concentrations.

Note Added in Proof. Shortly after this paper was communicated, Dr. D. A. Cowburn made available to us his Ph.D. dissertation (U. of London, 1970), which included CD spectra of human lysozyme similar to several reported here, as well as comparative studies of other lysozymes that we have not investigated.

We are grateful to Drs. Robert E. Canfield and Elliott F. Osserman for generously providing the preparations of human lysozyme used in this work and for many valuable discussions. This work was supported in part by grants from the National Science Foundation (GB 7128) and the National Institutes of Health (GM 10576).

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