Further Studies on the Circular Dichroism of Human Lysozyme. Implications for Structure and Comparison of Predicted Secondary Structures in Homologous Lysozymes†

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ABSTRACT: The near-ultraviolet circular dichroism (CD) spectrum of the human lysozyme–inhibitor complex and its dependence on pH have been investigated. It was found that the inhibitors $(AcGlcN)_3$ and $(AcGlcN)_4$ alter both the intensity and the wavelength position of the negative band at 313 nm and that they lower the apparent pK of its titration. Since it has been shown previously that the pK_{app} of the binding site residue Tyr-63 is lowered by inhibitor, we believe that the unusual CD band is associated with this side chain. It is probable that this activity arises from an interaction between Trp-64 and the ionized form of Tyr-63. The far-ultraviolet CD spectra of hen egg-white, human, and rat lysozymes are used in calculations of the helix contents of these proteins. According to the method adopted estimations of helix content range from 18 to 25%. Though these are considerably lower than the helix content deduced from the crystallographic structure of hen egg-white lysozyme they do suggest that the homologous lysozymes have similar helix contents. The examination of secondary structure is extended to the location of helical and β-sheet regions in the sequences of three lysozymes by the empirical method of T. T. Wu and E. A. Kabat ((1971), *Proc. Nat. Acad. Sci. U. S. 68,* 1501). This method is based upon the effects that nearest neighbor interactions have on secondary structure, as determined by a statistical analysis of the locations of amino acids in proteins of known structure. The secondary structures of hen egg-white, human, and mouse lysozymes, determined by this method, show remarkably good agreement with the known structure of hen egg-white lysozyme. Since the same structure is arrived at in proteins of different sequence, it is concluded that residue substitutions among the lysozymes occur in a conservative manner with respect to the effects of their nearest neighbor interactions on secondary structure.

The availability of human lysozyme isolated from the urine of patients with monocytic and monomyelocytic leukemia (Osserman and Lawlor, 1966) has permitted extensive examination of its physical properties. The absorption, fluorescence, and circular dichroism (CD) spectra and their dependence on pH and inhibitor binding have been reported (Latovitzki *et al.*, 1971; Halper *et al.*, 1971; Ikeda *et al.*, 1972, Teichberg *et al.*, 1972; Mulvey *et al.*, 1973). The work of Halper *et al.* (1971) established the existence of a negative band centered at 313 nm in the CD spectrum of human lysozyme at pH above 9. These authors suggested that this unusual band might be associated with the binding site residues Tyr-63 and Trp-64. In hen egg-white lysozyme the tyrosine is replaced by Trp-62 and the enzyme shows no comparable optical activity near 313 nm. It has also been shown that the CD spectrum of rat lysozyme possesses the "313-nm band" and this enzyme, like human lysozyme, has a tyrosine residue at position 63 (Mulvey *et al.*, 1974). In an attempt to establish the assignment of this band the alkaline titration of the near-ultraviolet CD spectrum of human lysozyme has been examined in the presence of inhibitors and is reported here.

Despite frequently extensive substitution of residues, homologous proteins appear to have common secondary structures. This is certainly the case with myoglobin and the hemoglobins (Perutz *et al.*, 1968) as well as with the serine proteinases (Hartley and Shotton, 1971). The secondary structure of the lysozymes is investigated here in two ways. First, the far-ultraviolet CD spectra are analyzed by the methods of Greenfield and Fasman (1969) and Chen *et al.* (1972) to estimate their relative helix contents. Second, with the development of increasingly reliable methods for predicting the absence of certain residues from periodic segments of secondary structure when sequences are known, it is possible to compare the likely positions of periodic structures in the homologous enzymes. To this end the sequences of hen egg-white, human, and mouse lysozyme were analyzed by the method of Wu and Kabat (1971) and Kabat and Wu (1973a). The positions of α helix and β sheet were compared with the known positions in hen egg-white lysozyme. The empirical procedure employed here is of importance since it is based on the nearest neighbor influences on a residue's ϕ,ψ angles. It should be possible, then, to determine whether the same local influences on secondary structure are maintained in the different lysozymes.

Experimental Section

Materials. Human lysozyme, isolated from the urine of patients with acute monocytic and monomyelocytic leukemia, was a gift from Dr. E. F. Osserman. It was prepared as described previously (Osserman and Lawlor, 1966; Mulvey *et al.*, 1973). The inhibitors $(AcGlcN)_3$ and $(AcGlcN)_4$[1] were prepared from chitin by the method of Rupley (1964).

Circular Dichroism Spectroscopy. A Cary 60 spectropolarimeter with a CD attachment was used to record all circular dichroism spectra. The protein concentration and cell path

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[1]Abbreviation used is: AcGlcN, *N*-acetylglucosamine.

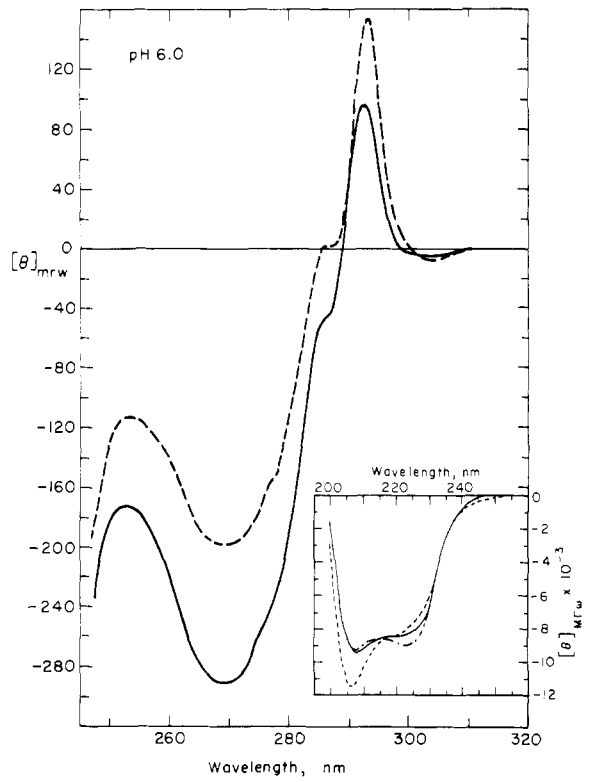

FIGURE 1: Near-ultraviolet CD spectra of human lysozyme (——) and the human lysozyme-$(AcGlcN)_3$ complex (- - - -) (pH 6.0). Portein concentration 0.046%; $(AcGlcN)_3$, 2.1×10^{-3} M. Inset: Far-ultraviolet CD spectra of hen egg-white (- - - -), human (——), and rat lysozyme (-·-·-); taken from Mulvey *et al.* (1974).

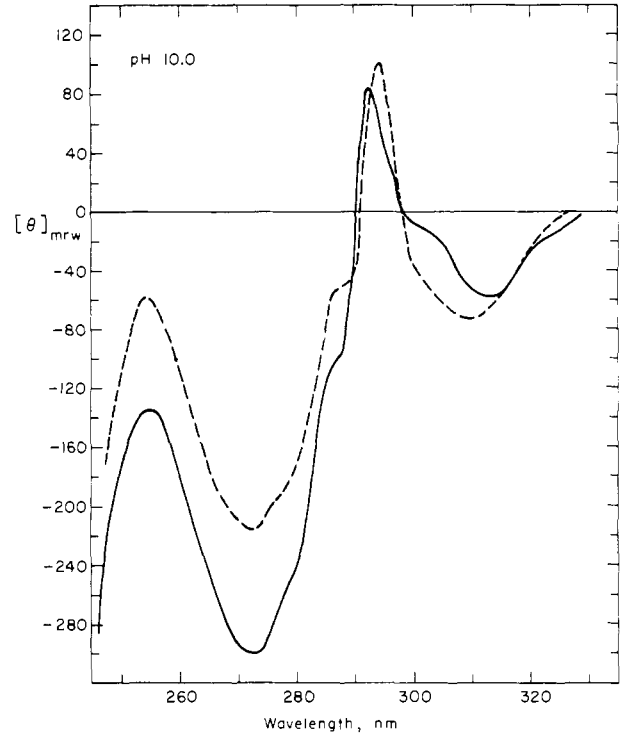

FIGURE 2: Near-ultraviolet CD spectra of human lysozyme (——) and human lysozyme-$(AcGlcN)_3$ complex (pH 10.0). Concentrations as in Figure 1.

lengths were as described in Mulvey *et al.* (1974). When recording the CD spectra of the complexes between human lysozyme and $(AcGlcN)_3$ or $(AcGlcN)_4$, the inhibitor concentration was 2.08×10^{-3} M. The ultraviolet absorption and CD spectra of these oligosaccharides were examined and found to be negligible at wavelengths longer than 250 nm. For this reason they were not included in the buffer solution when recording the base line.

Alkaline Titration of CD Spectra. The CD spectra of human lysozyme and its $(AcGlcN)_3$ and $(AcGlcN)_4$ complexes were recorded over the pH range 6–11.5. The ionic strength of buffers was 0.1 and the following systems were used: pH 6–8, potassium phosphate buffer; pH 8.5–10.5, glycine-KOH; pH 11.0–12.0, KH_2PO_4-KOH.

Secondary Structure Calculations. The sequences of hen egg-white, human, and mouse lysozymes were analyzed in an attempt to locate helical and β-sheet regions. The method employed was that of Wu and Kabat (1971). This procedure focuses on the influence of the two nearest neighbor residues on the ϕ,ψ angles of the residue between them. Using the ϕ,ψ angles of several known protein structures these authors have compiled a 20 × 20 table of every possible pair of $(n - 1)$ and $(n + 1)$ residues. Each occurrence of a particular pair about a residue with helical ϕ,ψ angles is entered into the table. Similarly, those occurrences in tripeptides with β-structure angles, or random chain angles, are also entered separately. Thus, from a 20 × 20 table compiled from 11 proteins of known structure (Kabat and Wu, 1973a) the frequencies of occurrence of the pair (Gly)-(x)-(Cys) in tripeptides with ϕ,ψ angles at residue "x" of α helix, β sheet, or random chain are 0, 3, and 3, respectively. Out of six observations of this pair none are found in helices and these residues will have a helix-breaking influence on the middle residue. On the other hand, the tripeptide (Ala)-(n)-(Leu) has a "score" of 10, 1, 3 and will be permissively helical at residue "n."

The lysozyme sequences were analyzed to locate helix and β-sheet breakers. Following the criteria of Kabat and Wu (1973b), a residue, n, was considered a helix breaker if the ratio of occurrences of the $(n - 1)$ and $(n + 1)$ pair in helical and nonhelical regions was less than or equal to 1:5. Therefore, a sequence of four or more residues uninterrupted by a helix breaker was permissively helical. β-Sheet breakers were defined as those residues whose $(n - 1)$ and $(n + 1)$ pair had at least three occurrences in the 20 × 20 table, none of which were in regular β structures.

Results

Near-Ultraviolet CD Spectrum. The near-ultraviolet CD spectra of human lysozyme, shown in Figures 1 and 2, are in excellent agreement with the spectra reported previously from this laboratory (Halper *et al.*, 1971) and by Ikeda *et al.* (1972). The spectrum at pH 6.0 is dominated by a large negative band at 268 nm and a positive band at 292.5 nm. The negative band displays two shoulders, one at 286 nm and a less pronounced shoulder between 275 and 285 nm. A negative band of very low intensity is also observed at 305 nm, in accord with the results of Ikeda *et al.* (1972). The alkaline spectrum of human lysozyme (Figure 2) shows a number of pH dependent changes, the most distinctive being the development of negative ellipticity at 313 nm. This band has a shoulder near 303 nm presumably reflecting the presence of the small negative band seen at pH 6.0. The position of the positive band at 292.5 nm remains unchanged at high pH though its intensity is diminished. Between 265 and 290 nm the negative ellipticity of human lysozyme is slightly enhanced at pH 10 and the 268-nm peak is red-shifted to 272 nm.

As noted by the authors cited above, the major effect of bound inhibitor at pH 6.0 is to change the ellipticity of human lysozyme in a positive direction at all wavelengths between 250

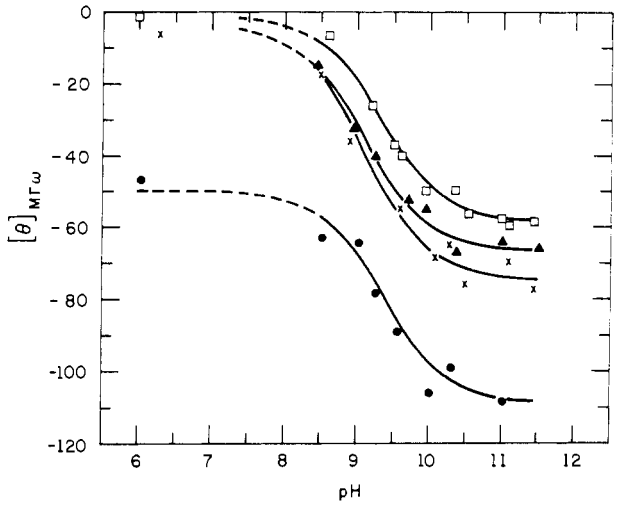

FIGURE 3: Variation with pH of the mean residue ellipticity of human lysozyme and its inhibitor complexes: (●) human lysozyme, 286 nm; (□) human lysozyme, 313 nm; (x) $(AcGlcN)_3$ complex, 313 nm; (▲) $(AcGlcN)_4$ complex, 313 nm. Concentrations as in Figure 1.

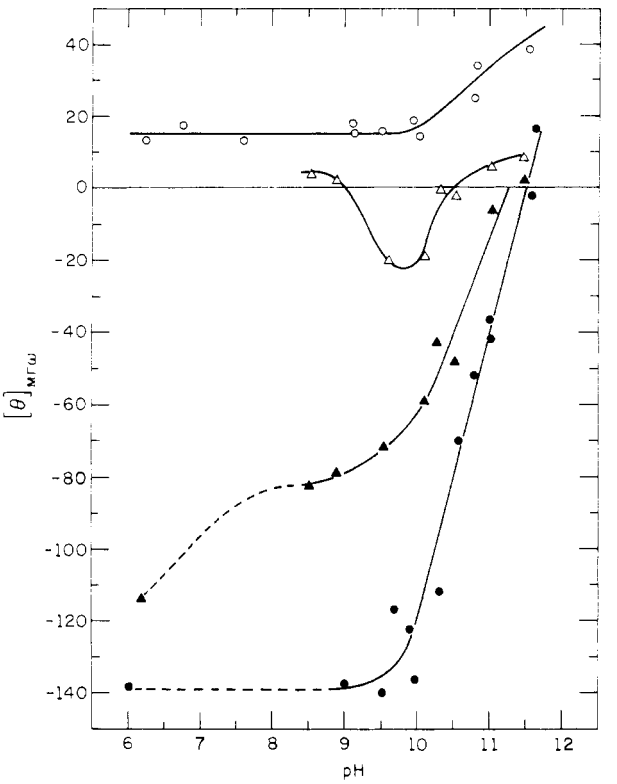

FIGURE 4: Variation with pH of the mean residue ellipticity of human lysozyme and its inhibitor complex at 254 and 298 nm: human lysozyme, 254 nm (●) and 298 nm (○); $(AcGlcN)_3$ complex at 254 nm (▲) and 298 nm (△). Concentrations as in Figure 1.

and 300 nm. The effect of triNAG (Figure 1) on the wavelength positions of the peaks and shoulders is not clear, except at the positive peak at 292.5 nm and the shoulder at 286 nm. These are red-shifted approximately 2 nm when triNAG binds to the enzyme. At pH 10.0 the CD spectrum of the human lysozyme–triNAG complex has a broad negative envelope from 300 to 325 nm. The negative ellipticity of the shoulder near 305 nm is markedly enhanced relative to the free enzyme. The effects of bound inhibitor on the 313-nm band is difficult to separate from the changes in this shoulder. However, it seems that the peak is slightly blue-shifted and its intensity is enhanced.

The near-ultraviolet CD spectra of human lysozyme and its $(AcGlcN)_3$ and $(AcGlcN)_4$ complexes were recorded at several pH values between 6.0 and 11.5. The pH dependent changes observed can be separated into two groups: those that begin near pH 8.5 and are essentially complete by 10.5, and those changes that start above pH 10. The titrations of the band at 313 nm and the shoulder at 286 nm are among the first group (Figure 3). The curves in Figure 3 are theoretical and have been fitted to the data. They were calculated as follows. The degree of ionization, α, of a titratable group may be related to

$$\alpha = ([\theta]_\lambda - [\theta]_{\lambda,u})/([\theta]_{\lambda,i} - [\theta]_{\lambda,u}) \quad (1)$$

the change in ellipticity. $[\theta]_{\lambda,u}$ is the mean residue ellipticity before, and $[\theta]_{\lambda,i}$ the ellipticity after ionization. The titration curve is then calculated using eq 2. The titration at 286 nm

$$\log [\alpha/(1 + \alpha)] = \text{pH} - \text{p}K_{app} \quad (2)$$

(Figure 3) was calculated assuming $pK_{app} = 9.35$, and the curve provides a reasonably good fit to the data. In human lysozyme this value corresponds to an intrinsic pK of 9.76 (Latovitzki *et al.*, 1971) which suggests that the ionization of a normal tyrosine residue is involved. Similarly the titration of $[\theta]_{313}$ in the absence of bound inhibitor has a pK_{app} of 9.1. Some uncertainty in positioning the top and bottom of these curves (*i.e.*, in determining $[\theta]_{\lambda,u}$ and $[\theta]_{\lambda,i}$) will add to the error in estimating the apparent pK's. However, it is clear that the inhibitors cause a depression of the pK_{app} in the titration of the 313-nm band. Figure 3 also shows that near pH 11.0 the inhibitors enhance the negative ellipticity of this band. $(AcGlcN)_3$ appears to give a greater enhancement than $(AcGlcN)_4$. There is some doubt as to the reality of this difference since the titration of the two inhibitor–enzyme complexes was performed on separate lysozyme preparations.

The major changes in ellipticity above pH 10 can be followed at 254 and 298 nm (Figure 4). The growth in positive ellipticity with increasing pH has been directly ascribed to the ionization of tyrosine residues (Halper *et al.*, 1971). It was not possible to complete these titrations since there is some unfolding of the protein above pH 11.7 as the last two tyrosine residues ionize (Latovitzki *et al.*, 1971). At 254 nm $(AcGlcN)_3$ reduces the negative ellipticity, but does not necessarily affect the titration at this wavelength. The titration of the enzyme–complex spectrum at 298 nm is biphasic. Between pH 8.5 and 9.8 the ellipticity falls to negative values and above pH 10 it rises again. This complicated behavior may result from the blue shift of the 313-nm band caused by bound inhibitor. In this case, the first part of the titration curve at 298 nm would represent the growth of the 313-nm band while the second part would reflect the same tyrosine ionization that is observed in the free enzyme. Again, bound inhibitor does not necessarily affect the latter ionization.

Secondary Structure Calculations. Analysis of the lysozyme sequences for the location of periodic structure was first performed using a 20 × 20 table assembled by Kabat and Wu (1973a) from the structures of 11 proteins. This procedure locates residues which interrupt helical regions. There is no implication insofar as this method is concerned that a helix exists between two helix breaking tripeptides. Accordingly, the intermediate regions have been termed permissively helical. A total of 73% of the hen egg-white lysozyme sequence was found to be permissively helical. This high value may stem from the fact that three of the sequences used in compiling the 20 × 20 table were of myoglobin and the α and β chains of hemoglobin. There is some evidence that these proteins may form a special group with regard to their high helix content (Yip *et al.*, 1972).

TABLE I: Calculation of Permissively Helical Residues.[a]

HEWL			
X-ray α[b]	Permissively α	Human L	Mouse L
5–15	2–14	2–10	2–10
	17–20	16–19	16–20
24–34	27–38	27–36	25–29
			31–36
[60–63]			
		74–79	
80–85		81–84	81–84
88–96	91–98	87–104	87–?[c]
[97–101]			?–105
[109–115]	104–115	108–117	108–120
[119–123]	117–122	119–122	
45%	43%	50%	

[a] All sequence numbers in this table correspond to the hen egg-white lysozyme (HEWL) numbering. [b] Imoto *et al.* (1972). Bracketed residues are distorted helices. [c] Sequence data in this region are incomplete.

Their inclusion might, therefore, weight this calculation of secondary structure toward the prediction of permissively helical residues. The procedure was repeated with an eight protein table which excluded the myoglobin and hemoglobin data. The results of this calculation are shown in Table I. The correspondence between permissively helical residues and the known α helices of hen egg-white lysozyme (Imoto *et al.*, 1972), though not perfect, is remarkably good. A short helix was allowed at residues 17–20 where none actually occurs. In addition, the helices 60–63 and 80–85 were missed. It is known that the 60–63 turn is distorted from the regular α-helical conformation. The overall content of permissively helical residues (43%) agrees well with the value of 45% estimated from the crystal structure.

It should be noted that this analysis was based on a 20 × 20 table which itself included data from the crystal structure of hen egg-white lysozyme. This may prejudice the analysis toward favorable results. However, repetition of the calculation with a table which excluded the lysozyme data gave results almost identical with those of Table I.

All the helical regions in the hen egg-white lysozyme crystal structure are predicted to be helical in human lysozyme except for the single turn at 60–63. The region 80–101 shows excellent correspondence. Residues 16–19 and 74–79 in human lysozyme are also permissively helical and these partially account for the higher calculated helix content of this enzyme (50%). The results for mouse lysozyme are very close to those of human lysozyme. However, the sequence of mouse lysozyme is incomplete with respect to a few residues in the C-terminal half of the molecule (Riblet, 1974, and personal communication). A complete analysis for secondary structure of this enzyme was therefore not possible.

Extension of the 20 × 20 table to include data from 12 proteins and the successful use of this table in locating the β structure of concanavalin A (Kabat and Wu, 1973b) encouraged a similar analysis of the lysozyme sequences. β-Sheet breaking residues were located using the revised 20 × 20 table and the criteria described under Methods. The resulting stretches of "permissively β sheet" are shown in Table II. For hen egg-white lysozyme the analysis is not very discriminating. Many

TABLE II: Calculation of Permissively β-Structure Residues.

HEWL		Human L
X-ray β[a]	Permissively β	Permissively β
	2–10	
	21–26	
	32–40	
42–46	43–46	42–46
50–54	50–60	51–59
57–60		
	64–71	64–76
	77–80	78–87
	82–94	
	96–104	
	115–120	
	122–126	

[a] Imoto *et al.* (1972).

regions that are known to be aperiodic or α helical are, apparently, also permissively β sheet. However, the β-sheet region of the hen egg-white lysozyme structure (residues 42–60) is found to be permissively β sheet in both the hen egg-white and human lysozyme sequences. The sequences of human and mouse lysozyme differ by only two residues in this region. The analysis for mouse lysozyme is identical with that of human lysozyme.

Discussion

Near-Ultraviolet CD Spectrum. The two ultraviolet absorption bands of tyrosine are red-shifted on ionization of the phenol hydroxyl to approximately 240 and 295 nm, respectively. In proteins ionization of tyrosine residues is often signalled by the growth of positive ellipticity in the CD spectrum near 254 nm. Such is the case with hen egg-white lysozyme. The titration of $[\theta]_{254}$ parallels the spectrophotometric titrations of the first two tyrosines exactly (Ikeda and Hamaguchi, 1969). The titration of $[\theta]_{298}$ corresponds to the ionization of a tyrosine which possesses a normal pK. Moreover the growth of positive ellipticity at 298 nm is accompanied by a reduction of positive ellipticity at 283 nm (Halper *et al.*, 1971). This suggests that a positive tyrosine band centered close to 283 nm moves to longer wavelengths on ionization.

In parallel with the titration of $[\theta]_{283}$ in hen egg-white lysozyme, $[\theta]_{286}$ of human lysozyme becomes increasingly negative over the pH range 8.5–10.5 (pK_{app} = 9.35). However, this is not accompanied by any changes at 298 nm. The titrations of both $[\theta]_{254}$ and $[\theta]_{298}$ do not begin until the pH is above 10 (Figure 4). At this pH an average of 2.4 tyrosines have already ionized (Latovitzki *et al.*, 1971). It would seem that those tyrosines with normal pK's in human lysozyme do not contribute directly to the CD spectrum in their phenolate forms.

The unusual negative band of human lysozyme at 313 nm titrates between pH 8.5 and 10.5 (Halper *et al.*, 1971). Its apparent pK, estimated from the titration curve of Figure 4, is 9.35. Though the ionization of a tyrosine residue is clearly implicated in the development of this band, its very long wavelength position suggests that it might not arise from tyrosine alone. It is possible that this optical activity results from the interaction of an ionized tyrosine residue with another chromophore. Kahn (1972) has noted that some highly strained, cyclic disulfide compounds (*e.g.*, lipoic acid) can have CD bands at wavelengths longer than 300 nm. Therefore gross distortion of a cystine residue (at both the dihedral and sulfur bond angles) could lead to a long wavelength disulfide band. However, it is

TABLE III: Helix Content Estimated from CD Spectra.[a]

	$[\theta]_{208}$	H[b]	$[\theta]_{222}$	H[c]
Hen egg-white	11,400	24.5	8,000	19.0
Human	9,300	18.6	8,400	20.0
Rat	9,200	18.0	8,900	21.6

[a] CD data taken from Mulvey *et al.* (1974). [b] Method of Greenfield and Fasman (1969). [c] Method of Chen *et al.* (1972).

extremely unlikely that the ionization of a tyrosine residue in human lysozyme could result in such a drastic conformational change.

Another possibility for the origin of the 313-nm band is a highly perturbed vibronic component of a tryptophan transition. Small absorption and CD bands have been found at wavelengths longer than 300 nm (*i.e.*, near 305 nm) in several indole containing compounds and proteins (Strickland *et al.*, 1972; Ikeda and Hamaguchi, 1972; Cowburn *et al.*, 1972). It is possible therefore that the 313-nm band arises from a tryptophan residue closely coupled with an ionized tyrosine. Examination of the hen egg-white lysozyme structure shows that Trp-64 of human lysozyme could be less than 6 Å from Tyr-63. There is reason to believe that this same pair of residues exists in rat lysozyme which also possesses a titratable CD band at 313 nm (Mulvey *et al.*, 1974).

Effects of Bound Inhibitors. Despite the large differences in the near-ultraviolet CD spectra of hen egg-white and human lysozymes the effects of the inhibitors $(AcGlcN)_3$ and $(AcGlcN)_4$, are quite similar. In both cases the ellipticity at all wavelengths between 250 and 300 nm is shifted to more positive values. In agreement with the results of Ikeda *et al.* (1972), the major change in the spectrum of human lysozyme is observed at 270 nm where inhibitor binding reduces the ellipticity of this strongly negative band. The inhibitor also red-shifts the position of both the 292.5-nm band and the 286-nm shoulder. Therefore, while their positions change their separation remains constant. This lends support to the suggestion by Halper *et al.* (1971) that the 286-nm shoulder represents a positive band superimposed on a larger negative ellipticity. Thus the tryptophan ^1L_b doublet seen in the hen egg-white lysozyme spectrum at 288 and 294 nm is also present in human lysozyme at approximately 286 and 292.5 nm. In this respect it is significant that the red shift of a tryptophan ^1L_b transition due to inhibitor binding is also observed in the absorption difference spectra (Mulvey *et al.*, 1973). If Imoto *et al.* (1972) are correct in their assertion that the contribution of Trp-108 to the spectrum of hen egg-white lysozyme is small and negative near 290 nm, then the positive doublet must originate, at least partially, in Trp-63 (64 in human lysozyme).

Those pH dependent changes in the human lysozyme CD spectrum which may be directly attributed to the ionization of tyrosine residues (*i.e.*, the titrations of $[\theta]_{254}$ and $[\theta]_{298}$) do not seem to be affected by bound inhibitor. The inhibitors do alter the titration of the 313-nm band. The pK_{app} of this titration is lowered from 9.35 in the free enzyme to 9.1 in the enzyme-inhibitor complex. The negative ellipticity of this band is enhanced and its wavelength position is blue-shifted by inhibitor. C. C. F. Blake (personal communication) has pointed out that the conformational changes observed with the binding of $(AcGlcN)_3$ to hen egg-white lysozyme are not seen in the human lysozyme complex. The effects of inhibitor on the 313-nm band strongly suggest, therefore, that the optical activity is associated with Tyr-63 of the binding site. It has been shown (Mulvey *et al.*, 1973) that the pK_{app} of this residue is lowered by bound inhibitor. In the free enzyme its pK_{int} was estimated as 10.4 (±0.3) (Mulvey, 1973). The pK_{app} of the titration of the 313-nm band is 9.35, which in human lysozyme corresponds to an intrinsic pK of approximately 9.75. The agreement between these intrinsic pK's is not good. However, given the uncertainty in establishing the values of these pK's, the weight of the evidence favors the assignment of the 313-nm band to the residues Tyr-63 and Trp-64.

Lysozyme Secondary Structure. Two classes of procedures are increasingly used for estimating secondary structure of proteins in solution and both are being continuously refined and calibrated as new X-ray crystallographic data appear. The methods may be grouped into those based on optical activity of proteins and those involving empirical predictions of the occurrence of helices and β sheets from sequence data. Representative approaches of both kinds have been employed here to evaluate the secondary structure of hen egg-white, human, and rodent lysozymes.

The far-ultraviolet CD spectra of hen egg-white, human, and rat lysozymes have been reported previously (Mulvey *et al.*, 1974) and are reproduced in Figure 1. The spectra have been analyzed by the methods of Greenfield and Fasman (1969) and Chen *et al.* (1972). Both methods give similar results (Table III), with somewhat lower values for helix content than the 29% of residues in regular α helices and significantly lower values than the 45% of residues in regular and distorted helices (Imoto *et al.*, 1972).

At present the reason behind the low estimations of helix content from the lysozyme CD spectra is unclear (for a review, see Sears and Beychok (1973)). However, the overall shape of these spectra and particularly the helix calculations based on $[\theta]_{222}$ suggest that the homologous lysozymes have closely similar secondary structures.

The analysis of the lysozyme sequences by the method of Wu and Kabat (1971) produced some interesting results with regard to their secondary structures. The location of helical residues in the sequence of hen egg-white lysozyme corresponds well with the known structure of that enzyme. Further, those regions shown to be permissively helical in hen egg-white lysozyme are also permissively helical in human and mouse lysozymes. Perhaps the most striking result is obtained in the region of residues 42–60, which forms the β sheet in hen egg-white lysozyme. With each lysozyme this region is not permissive for helix but is permissive for β sheet. Therefore, both the calculations based on the far-ultraviolet CD spectra and the empirical location of periodic structure point to the similarity of secondary structure among the homologous lysozymes. This result is in complete accord with the X-ray crystallographic study of human lysozymes by Blake and Swan (1971) and Banyard *et al.* (1974).

It is increasingly clear that the same secondary structure is preserved throughout the whole molecule of each lysozyme. The similarities are not confined to those regions of the polypeptide chain which are directly responsible for the conformation of the substrate binding site. It seems probable that there may be features of the lysozyme structure which have been conserved for reasons other than those concerned directly with the hydrolysis of the bacterial cell wall. For instance, while hen egg-white and human lysozymes are catalytically active over the pH range 5–8 and higher, they show remarkable stability over a different pH range. Thermal denaturation studies on hen egg-white lysozyme have demonstrated a very high transition temperature from pH 2 to 6 (Sophianopoulos and Weiss, 1964). Clearly the native structure can be maintained at a pH

where catalytic activity is prohibited. In this connection it is interesting to note that the lysozyme of mammalian cells is subject to a wide fluctuation of pH. Lysozyme is active in the phagocytic vacuoles of rabbit polymorphonuclear leukocytes. Bainton (1973) has demonstrated that the pH in the vacuoles can range from 7 to below 4.

The secondary structures of the lysozymes are similar despite a number of substitutions in regions of both periodic and aperiodic structure. The majority of substitutions are conservative with respect to the charge, hydrophobicity, and size of the side chains. This is particularly true of internal residues. The analysis of the lysozyme sequences by the method of Wu and Kabat (1971) suggests another criterion for the natural selection of substituted residues. The method of analysis is based upon the influence that nearest neighbors have upon a residue's θ,ψ angles. The success which this analysis achieves in locating the correct positions of periodic structure in a variety of lysozymes suggests that residues are substituted in a conservative manner with regard to their local influence on secondary structure.

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References

Bainton, D. F. (1973), *J. Cell Biol. 58,* 249.

Banyard, S. H., Blake, C. C. F., and Swan, I. D. A. (1974), *in* Lysozyme, Osserman, E. F., Canfield, R. E., and Beychok, S., Ed., New York, N. Y., Academic Press.

Blake, C. C. F., and Swan, I. D. A. (1971), *Nature (London) 232,* 12.

Chen, Y.-M., Yang, J. T., and Martinez, H. M. (1972), *Biochemistry 11,* 4120.

Cowburn, D. A., Brew, K., and Gratzer, W. B. (1972), *Biochemistry 11,* 1228.

Greenfield, N., and Fasman, G. D. (1969), *Biochemistry 8,* 4109.

Halper, J. P., Latovitzki, N., Bernstein, H., and Beychok, S. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 517.

Hartley, B. S., and Shotton, D. M. (1971), *Enzymes, 3rd Ed. 3,* 323.

Ikeda, K., and Hamaguchi, K. (1969), *J. Biochem. 66,* 513.

Ikeda, K., and Hamaguchi, K. (1972), *J. Biochem. 71,* 265.

Ikeda, K., Hamaguchi, K., Miwa, S., and Nishina, T. (1972), *J. Biochem. 71,* 371.

Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., and Rupley, J. A. (1972), *Enzymes, 3rd Ed. 7,* 665.

Kabat, E. A., and Wu, T. T. (1973a), *Biopolymers 12,* 751.

Kabat, E. A., and Wu, T. T. (1973b), *Proc. Nat. Acad. Sci. U. S. 70,* 1473.

Kahn, P. C. (1972), Ph.D. Dissertation, Columbia University.

Latovitzki, N., Halper, J. P., and Beychok, S. (1971), *J. Biol. Chem. 246,* 1457.

Mulvey, R. S. (1973), Ph.D. Thesis, Columbia University.

Mulvey, R. S., Gualtieri, R. J., and Beychok, S (1973), *Biochemistry 12,* 2683.

Mulvey, R. S., Gualtieri, R. J., and Beychok, S. (1974), *Biochemistry, 13,* 782.

Osserman, E., and Lawlor, D. P. (1966), *J. Exp. Med. 124,* 921.

Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G. (1968), *Nature (London) 219,* 131.

Riblet, R. J. (1974), *in* Lysozyme, Osserman, E. F., Canfield, R. E., and Beychok, S., Ed., New York, N. Y., Academic Press.

Rupley, J. A. (1964), *Biochim. Biophys. Acta 83,* 245.

Sears, D., and Beychok, S. (1973), *in* Physical Principles and Techniques of Protein Chemistry, Vol. 3, Leach, S. J., Ed., New York, N. Y., Academic Press.

Sophianopoulos, A. J., and Weiss, B. J. (1964), *Biochemistry 3,* 1920.

Strickland, E. H., Billups, C., and Kay, E. (1972), *Biochemistry 11,* 3657.

Teichberg, V. I., Plasse, T., Sorell, S., and Sharon, N. (1972), *Biochim. Biophys. Acta 278,* 250.

Wu, T. T., and Kabat, E. A. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 1501.

Yip, Y. K., Waks, M., and Beychok, S. (1972), *J. Biol. Chem. 247,* 7237.