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Composition, Fluorescence, and Circular Dichroism of Rat Lysozyme†

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ABSTRACT: The fluorescence and circular dichroism of rat lysozyme have been investigated. The fluorescence emission spectrum has λ_{max} 328 nm and Q = 0.034, at pH 7.5. These values are almost identical with those of human lysozyme. The effects of the inhibitor tetrasaccharide $(AcGlcN)_4$ and pH on the rat lysozyme fluorescence were also determined. The results parallel corresponding studies with human lysozyme and suggest that the fluorescence of the rat enzyme is also dominated by Trp-109 (108). The near- and far-ultraviolet circular dichroism (CD) spectra of rat lysozyme are described and compared to those of human lysozyme. A striking similarity is observed, also, between the CD spectra of these two proteins. In particular, the near-ultraviolet CD spectrum of rat lysozyme develops a negative band near 313 nm at alkaline pH. The amino acid composition of rat lysozyme was determined, the tyrosine and tryptophan contents being established by a spectrophotometric method. These data are compared to the compositions of human and mouse lysozyme. Discussion of the spectral properties of rat lysozyme is based upon previous studies with human lysozyme and on the known sequences of the human and mouse enzymes.

Since the determination of the three-dimensional structure of hen egg-white lysozyme (Blake *et al.*, 1965) considerable interest has been shown in the homologous lysozymes and α-lactalbumins. In particular, the chemical and physical properties of human lysozyme and bovine α-lactalbumin have been examined in a number of laboratories. Constructive analysis of these properties is greatly facilitated by comparison of the homologous sequences and by reference to the hen egg-white lysozyme structure (see, for example, Sommers *et al.*, 1973; Mulvey *et al.*, 1973, 1974). The validity of the approach rests on the assumption of a high degree of similarity in tertiary structures among homologous proteins. With respect to the lysozymes, the assumption is, for the most part, supported by X-ray crystallographic studies of human lysozyme (Blake and Swan, 1971; Banyard *et al.*, 1973).

Recently the nearly complete sequence of mouse lysozyme has been described (Riblet, 1974). We considered it worthwhile, therefore, to examine some of the spectroscopic properties of the closely related rat lysozyme. Here, we report the amino acid composition of rat lysozyme, including a spectrophotometric determination of tryptophan and tyrosine content, and compare it with the composition of the mouse enzyme. The circular dichroism and fluorescence emission spectra have been determined, as well as the effects of pH and inhibitor binding on the latter.

Experimental Section

Materials. Three times crystallized hen egg-white lysozyme was purchased from Pentex and used without further purification. Human and rat lysozymes were a generous gift of Dr. E. F. Osserman. The human lysozyme was prepared as described previously (Mulvey *et al.*, 1973). The rat enzyme had been isolated from the urine of rats bearing a transplantable Shay chloroleukemia by elution from bentonite in 5% aqueous pyridine at pH 5. This material was received in a lyophilized state, and was further purified on a CM-32 cellulose column and finally deionized on Bio-Rad AG 501-X8 mixed bed resin. The enzyme had a tendency to precipitate on the deionizing resin, and after lyophilization its solution showed considerable light scattering at long wavelengths (350–400 nm). Since no other proteins were detectable by either chromatography or electrophoresis on polyacrylamide gels the light scattering was probably caused by partially denatured aggregates of lysozyme. Stock solutions of rat lysozyme were routinely passed through Millipore filters before use.

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Guanidine hydrochloride (Schwarz/Mann, Ultra Pure) was found to have negligible absorption at wavelengths longer than 280 nm in a 6 M solution. The absorption between 240 and 280 nm was small (less than 0.03) and this material was used without further purification. DL-Tryptophan was purchased from Eastman Kodel Co. and the tetramer of *N*-acetylglucosamine, $(AcGlcN)_4$, was prepared from chitin by the method of Rupley (1964).

Activity. Enzyme activity was measured at 26° by the turbidimetric assay of Gorin *et al.* (1971). An aqueous solution of lysozyme (0.1 ml) (20–100 μg/per ml) was rapidly mixed with 3 ml of a *Micrococcus lysodeikticus* cell suspension (A_{570} = 0.75) in a cuvet. The time taken for the A_{570} to drop from 0.7 to 0.65 ($t_{0.05}$) was measured with a Beckman DU2 spectrophotometer. A graph of $(t_{0.05})^{-1}$ against the final concentration of lysozyme in the cuvet gives a linear plot.

Amino Acid Composition. Approximately 0.4 mg of rat lysozyme was hydrolyzed in 2 ml of 6 N HCl for 18 hr, at 110° *in vacuo*. The hydrolysate was cooled and evaporated to dryness, and the residue was dissolved in 0.5 ml of citrate buffer (pH 2.21). The composition of small aliquots of this solution was analyzed on a Beckman 120 C amino acid analyzer.

Spectrophotometric Determination of Tyrosine and Tryptophan. The tyrosine–tryptophan content of rat lysozyme was determined spectrophotometrically following the procedure of Edelhoch (1967). This measurement was repeated on human lysozyme so that the precision of the measurement could be gauged.

Rat lysozyme was dissolved in 0.035 M potassium phosphate buffer at pH 7.9, to an absorbancy (A_{280}) of 1.2. Guanidine hydrochloride (5.73 g) was added to 5.75 ml of the lysozyme solution giving 10 ml of solution at pH 6.4 and 6 M guanidine hydrochloride. The near-ultraviolet (uv) absorption spectrum of 3 ml of this solution was measured on a Cary 14 spectrophotometer against a reference of 3 ml of 6 M guanidine hydrochloride in buffer. Aqueous KOH (0.25 ml; 5 M) was then added to both sample and reference solution raising the pH to 12.51. Again the absorption spectrum of the protein was measured.

The high pH spectrum showed a time-dependent increase in A_{295}. The spectrum was measured at 3-min intervals (starting 0.5 min after the addition of KOH) and the A_{295} was extrapolated to time zero.

Fluorescence Spectra. Fluorescence emission spectra and quantum yields were determined on a Perkin-Elmer MPF 3 spectrofluorimeter as described previously (Mulvey *et al.*, 1973).

Circular Dichroism (CD) Spectra. A Cary 60 spectropolarimeter with a CD attachment was used to record circular dichroism spectra. For the near-ultraviolet CD spectra protein concentrations of approximately 0.05% and a pen deflection range of 0.04° were used. Cell path lengths were 1 cm from 240 to 300 nm and 2 cm above 300 mn. Far-ultraviolet CD spectra (200–250 nm) were recorded in a cell of 0.1-cm path with protein concentrations close to 0.03%. On the 0.04° range the signal-to-noise ratio, measured at the spectral extrema, varied from 5:1 to 12:1. For far-ultraviolet CD measurements utilizing the 0.1° range, this ratio was considerably better: 16:1 to 100:1.

Results

Enzyme Activity. The activity of the rat lysozyme toward a suspension of killed cells of *M. lysodeikticus* was determined by the method described above, over a concentration range 0–2.8 μg/ml of enzyme. At pH 6.2 in phosphate buffer of ionic strength 0.14, the activity of rat lysozyme is intermediate between those of hen egg-white and human lysozyme. Human and rat lysozymes are respectively 4.6 and 2.1 times more active than hen egg-white lysozyme.

Amino Acid Composition. The composition of rat lysozyme, calculated on the basis of 130 amino acids in the polypeptide chain, is shown in Table I. These data agree closely with the composition previously determined by G. A. Mross and A. C. Wilson (personal communication). The data shown for mouse lysozyme were determined by R. J. Riblet (personal communication) and those for human lysozyme are taken from Imoto *et al.* (1972). The rat and mouse lysozymes differ in the content of at leave five amino acids (Lys, Glx, Pro, Gly, and Ala) and perhaps also in Asx. However, the composition of human lysozyme differs from that of either rodent enzyme in the content of several more amino acids (12 with rat and 13 with mouse lysozyme).

TABLE I: Amino Acid Compositions of Mammalian Lysozymes.

	Rat[a]	Mouse[b]	Human[c]
Lys	6.1	3.9	5
His	2.0	1.9	1
Arg	12.4	12.0	14
Asx	17.6	18.7	18
Thr	5.9	5.8	5
Ser	6.2	5.7	6
Glx	12.4	10.8	9
Pro	5.7	2.8	2
Gly	10.8	8.8	11
Ala	11.0	13.9	14
Half-Cys	6.6	6	8
Val	6.5	6.8	9
Met	1.2	0.9	2
Ile	6.5	6.2	5
Leu	6.2	5.8	8
Tyr	7.5	7.4	6
Phe	2.0	2.0	2

[a] Average of two determinations. [b] R. J. Riblet, personal communication. [c] See Imoto *et al.* (1972).

In the spectrophotometric determination of the aromatic residues tyrosine may be estimated independently of the others using the following equation (Edelhoch, 1967)

$$N_{Tyr} = \Delta\epsilon_{295}/2480 \quad (1)$$

where N_{Tyr} is the number of tyrosine residues, $\Delta\epsilon_{295}$ is the difference in the extinction coefficient of the protein at neutral and alkaline pH, and $\Delta\epsilon_{295}$ = 2480 for the ionization of a single tyrosine in 6 M guanidine hydrochloride.

The tryptophan content may be obtained from two simultaneous equations using the extinction coefficients of the protein at different wavelengths (Edelhoch, 1967)

$$\epsilon_{288} = N_{Trp}4815 + N_{Tyr}385 \quad (2)$$

$$\epsilon_{280} = N_{Trp}5690 + N_{Tyr}1280 \quad (3)$$

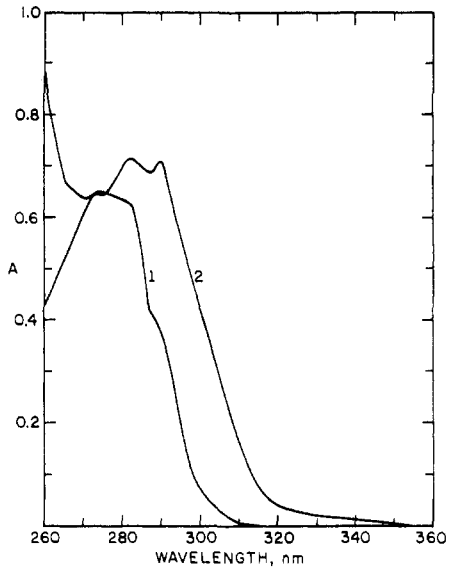

FIGURE 1: Absorption spectrum of rat lysozyme. Approximately 0.03% protein in 6 M guanidine hydrochloride: (1) pH 6.4; (2) pH 12.5. Spectrum 2 was measured 0.5 min after the addition of KOH (see Methods) and corrected for the resulting dilution.

Therefore

$$N_{Trp} = \frac{\epsilon_{288}}{3103} - \frac{\epsilon_{280}}{10,318} \quad (4)$$

The numbers on the right-hand side of eq 2 and 3 are the extinction coefficients of tryptophan and tyrosine, in 6 M guanidine hydrochloride, at the appropriate wavelengths. These equations should be corrected for the small contribution of cystine absorption. However, in the absence of an independently determined extinction coefficient for rat lysozyme this correction is not possible. Further, only the ratio of tyrosine and tryptophan contents can be obtained. ϵ_λ in eq 1 and 4 may be replaced by A_λ/c, where c is the protein concentration in moles liter^{-1} and A is the absorbancy. Combination of the two resulting equations eliminates the concentration term.

$$\frac{N_{Trp}}{N_{Tyr}} = \frac{1}{\Delta A_{295}}[A_{288}(2480/3103) - A_{280}(2480/10,318)] \quad (5)$$

Figure 1 shows the absorption spectra of rat lysozyme in guanidine hydrochloride. The low value of λ_{max} at neutral pH (~274 nm) suggests a low N_{Trp}/N_{Tyr} ratio. The data calculated according to the above equations are shown in Table II. The results from a single determination on human lysozyme show fairly close agreement with the known content of five tryptophan and six tyrosine residues. The N_{Tyr}, when calculated from the N_{Trp}/N_{Tyr} ratio and the known number of tryptophans, is overestimated at 6.5 tyrosines.

TABLE II: Tyrosine–Tryptophan Content of Rat Lysozyme.

Lysozyme	N_{Tyr}	N_{Trp}	N_{Tyr}/N_{Trp}	Assumed N_{Trp}	Calcd N_{Tyr}	Calcd ϵ_M
Human	5.84	4.6	0.772	5	6.5	35,550
Rat			0.470	3	6.4	24,472
				4	8.5	32,430
				5	10.6	40,388

The N_{Trp}/N_{Tyr} ratio for rat lysozyme (0.47) is the average of two separate determinations. Assuming various values for N_{Trp} the corresponding values for N_{Tyr} can be obtained. For example, with 3 and 4 tryptophans per molecule the ratio gives 6.4 and 8.5 tyrosines, respectively. Unfortunately, the value of N_{Tyr} determined in the amino acid composition is 7.5 (Table I) and this does not distinguish between these tyrosine–tryptophan contents. It is possible, however, to calculate the protein extinction coefficient from the compositions listed in Table II. The only residues that absorb at 280 nm are cystine, tryptophan, and tyrosine. The molar extinction coefficients of these amino acids and several of their derivatives have been reported in a number of publications: Beaven and Holiday (1952); Schellman and Schellman (1964); Edelhoch (1967); Donovan (1969); Strickland *et al.* (1969); Barth *et al.*, (1972). The average values for the extinction coefficients reported between 279 and 281 nm are: Trp, 5480; Tyr, 1180; Cys, 120. Assuming four cystine residues per molecule the possible extinction coefficients for rat lysozyme are listed in the last column of Table II. A very approximate determination of the enzyme's extinction coefficient was made during the preparation of a stock solution. Protein (1.7 mg) was dissolved in 10 ml of water. This solution was filtered and its A_{280} was 0.35. Assuming the same molecular weight as human lysozyme (*i.e.*, mol wt 14,600) this corresponds to a molar extinction coefficient of 30,100. It should be emphasized that this is not a dry weight determination and is probably a low estimate due to the presence of water in the protein preparation and to the removal of traces of insoluble material by filtering. Even so, the determined value readily distinguishes between the possible tyrosine–tryptophan contents suggested in Table II. Only the values of $N_{Trp} = 4$ and $N_{Tyr} = 8.5$ are reasonable. If, as with human lysozyme, the value of N_{Tyr} is slightly overestimated the tyrosine and tryptophan content of rat lysozyme is, respectively, 8 and 4 residues.

In measuring the enzyme's activity and circular dichroism spectra the protein concentrations were estimated from an extinction coefficient calculated as indicated above. The composition of four Cys, eight Tyr, and four Trp residues gives $\epsilon_{280} = 31,840$ (or $E^{1\%}_{280} = 21.8$, assuming a mol wt of 14,600). If the extinction coefficients of hen egg-white and human lysozymes are calculated in the same way the resulting values agree within 3% of the experimentally determined values (Mulvey, 1973).

Fluorescence Spectra. Figure 2A shows the fluorescence emission spectra of rat, human, and hen egg-white lysozymes. The human and hen egg-white lysozyme spectra have been compared previously (Teichberg *et al.*, 1972; Mulvey *et al.*, 1973). The rat lysozyme emission spectrum closely resembles that of human lysozyme, with λ_{max} 328 nm and the quantum yield, $Q = 0.034$. The corresponding values for human lysozyme are λ_{max} 330 nm and $Q = 0.035$. The distinct shoulder at 310 nm on the rat lysozyme emission spectrum reflects the high Tyr/Trp ratio in the enzyme.

The fluorescence of rat lysozyme shows the same variation with pH in the acid region (Figure 2B) as has been described for human lysozyme (Mulvey *et al.*, 1973, 1974). This includes the quenching of fluorescence between pH 5 and 7.5, where Glu-35 ionizes. The effect of bound $(AcGlcN)_4$,[1] shown in the

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

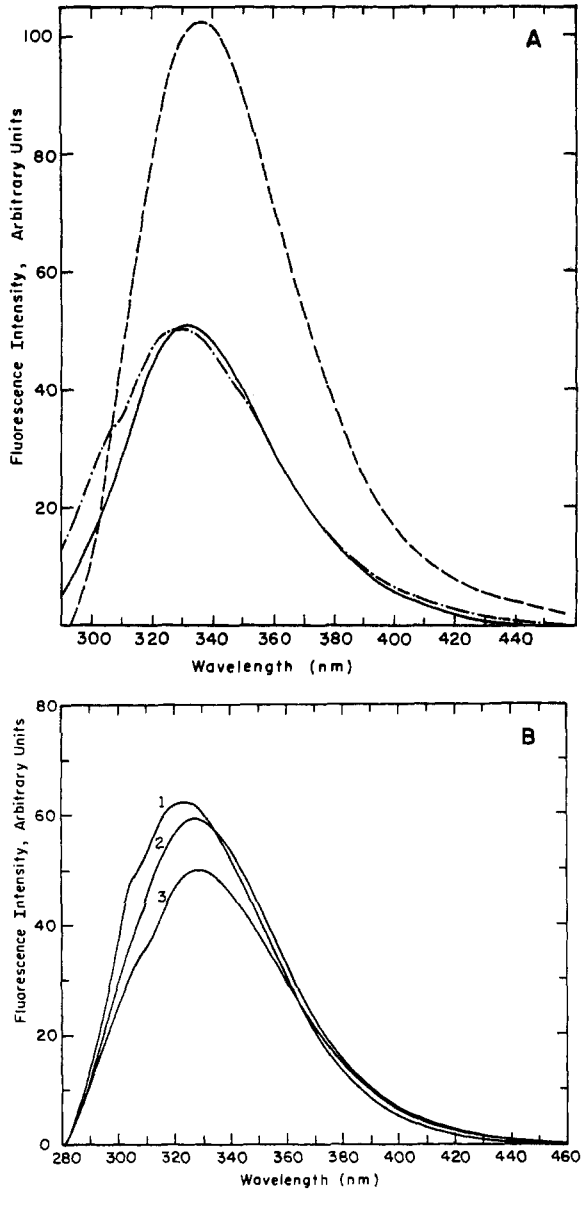

FIGURE 2: Fluorescence emission spectra of lysozymes: (A) (- - -) hen egg-white lysozyme; (—) human lysozyme; (– · –) rat lysozyme; phosphate buffer (pH 7.5); μ = 0.1; excitation wavelength 280 nm; room temperature; A_{280} = 0.1; (B) rat lysozyme; (1) acetate buffer (pH 2.9); (2) acetate buffer (pH 5.5); (3) phosphate buffer (pH 7.5).

difference fluorescence spectra (Figure 3), is entirely analogous to the effects of this inhibitor on the fluorescence of human lysozyme. At pH 7.5 the inhibitor strongly dequenches the fluorescence of rat lysozyme and shortens its emission maximum by some 6 nm. At lower pH (5.5 and 2.9) the dequenching effect is lost but the shorter emission maximum persists. As with human lysozyme the difference fluorescence spectrum of the rat enzyme does not change with pH below 5. This is in marked contrast to the same spectrum of hen egg-white lysozyme which indicates marked quenching of fluorescence by the inhibitor at low pH (Lehrer and Fasman, 1967).

Circular Dichroism. Like the fluorescence spectra the circular dichroism (CD) spectra of rat lysozyme closely resemble those of human lysozyme. The far-ultraviolet CD spectra of the two proteins (Figure 4) are virtually identical, and suggest very similar secondary structures. A detailed discussion of these spectra is given in a forthcoming paper (R. S. Mulvey and S. Beychok, manuscript in preparation) on lysozyme circular dichroism and secondary structure.

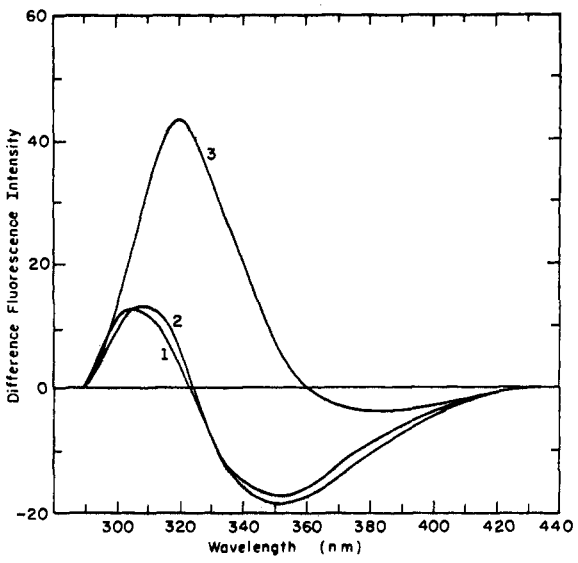

FIGURE 3: Calculated fluorescence difference spectra (rat lysozyme–$(AcGlcN)_4$ complex) – (rat lysozyme). Conditions as for Figure 2, except: (1) pH 2.9; (2) pH 5.5; (3) pH 7.5; $(AcGlcN)_4$, 4.6×10^{-3} M.

The near-ultraviolet CD spectrum of rat lysozyme is compared with the human lysozyme spectrum in Figures 5A and 5B. The correspondence of the peak and shoulder positions of these spectra is very close. At pH 7.1 both show the tryptophan bands at 292.5 and 305 nm, as well as the broad negative envelope centered near 268 nm. What appears as a shoulder at 286 nm in the human lysozyme spectrum is a more pronounced peak in the rat lysozyme spectrum. Finally, at pH 10.4 the negative band at 313 nm is apparent in the rat lysozyme spectrum. In general, the intensity of the rat lysozyme ellipticity is less than that of human lysozyme. In the case of the band at 313 nm the lower intensity with rat lysozyme might have one of two sources. Either this band has less intrinsic intensity in the rat enzyme or at pH 10.4 is not fully titrated. The second possibility would require that the band in rat lysozyme has a considerable higher pK_{app} than found with human lysozyme.

Discussion

Seventy-eight per cent of the sequences of human and mouse lysozymes are identical (Riblet, 1973). Their amino acid com-

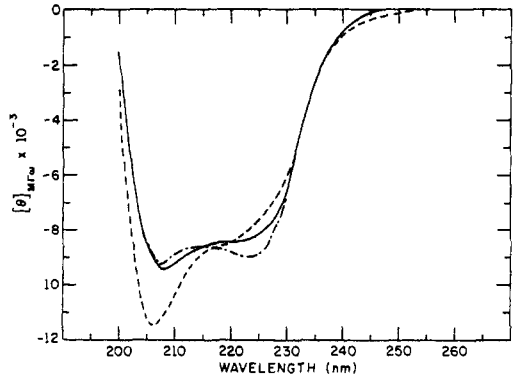

FIGURE 4: Far-ultraviolet CD spectra of lysozymes; phosphate buffer (pH 7.0); μ = 0.1: (- - -) hen egg-white lysozyme; (—) human lysozyme; (– · –) rat lysozyme.

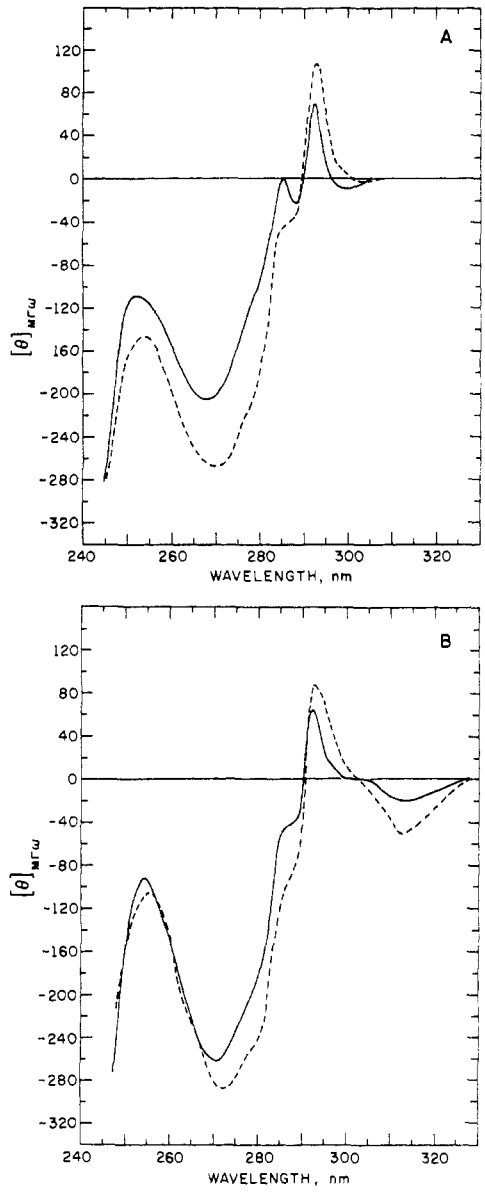

FIGURE 5: Near-ultraviolet CD spectrum of rat lysozyme: (—) rat lysozyme; (- - -) human lysozyme; (A) phosphate buffer (pH 7.1); μ = 0.1; (B) glycine–KOH buffer (pH 10.4); μ = 0.1.

positions are, however, quite distinct. Rat and mouse lysozymes have similar but not identical compositions. It is reasonable to expect a considerably higher degree of homology between these two enzymes. At least seven lysozymes have been sequenced: hen egg-white, Canfield (1963); human, Canfield *et al.* (1971); guinea-hen, Jolles *et al.* (1972); duck II, Herman and Jolles (1970); mouse, Riblet (1974). All have four common tryptophans: 28, 63, 108, and 111 (using hen egg-white lysozyme numbering). In mouse lysozyme these are the only four and it would seem probable that the tryptophans of rat lysozyme are also in these positions. Like the rat enzyme, mouse lysozyme may have as many as eight tyrosines, though only seven have been identified in the sequence so far. Finally, both lysozymes have just two phenylalanyl residues.

Hen egg-white lysozyme has a total of six tryptophan residues (28, 62, 63, 108, 111, 123) and a fluorescence quantum yield of 0.06 (Lehrer and Fasman, 1967). Imoto *et al.* (1971) have shown that the fluorescence of the hen egg-white enzyme originates mainly from the residues 62 and 108. The remaining residues are strongly quenched. Sommers *et al.* (1973) suggest that part of the emission from Trp-108 is lost, by energy transfer, to Trp-63 which is quenched by the neighboring disulfides (64–80 and 76–94). Trp-62 is exposed to solvent and therefore emits at a long wavelength while Trp-108 is partly buried and emits at a shorter wavelength. The resulting emission maximum of hen egg-white lysozyme (338 nm) is intermediate between the emission maxima of its two fluorescent tryptophans. In human lysozyme, which contains five tryptophans (28, 34, 64 (63), 109 (108), 112 (111)[2]), Trp-62 is replaced by Tyr-63 (62). In consequence the quantum yield is smaller, 0.035, and the emission maximum is at a shorter wavelength, 330 nm (Teichberg *et al.*, 1972; Mulvey *et al.*, 1973, 1974). All the major differences between the fluorescence of hen egg-white and human lysozyme may be related to this single substitution at residue 63 (62).

The fluorescence of rat lysozyme is virtually identical with that of human lysozyme. This not only applies to the emission maxima and quantum yields but also to the behavior of the fluorescence with respect to pH charges and inhibitor binding. This strongly suggests that, as with the human lysozyme fluorescence, the emission spectrum of rat lysozyme is dominated by Trp-109 (108) and the remaining three tryptophans are quenched.

Interestingly, the unusual fluorimetric titration of human lysozyme is also exhibited by rat lysozyme. Specifically, with human lysozyme the ionization of Glu-35 quenches the protein's emission and it is presumed that this reflects an interaction between the carboxyl and Trp-109 (108) (Mulvey *et al.*, 1973, 1974). Model compound studies (White, 1959) lead one to expect the opposite effect, that is tryptophan fluorescence is quenched by protonated carboxyls. As pointed out above, rat lysozyme fluorescence is also quenched by increasing the pH from 2.9 to 7.5. Though the full fluorimetric titration curve has been determined, Figure 2B shows that the quenching is accompanied by a shift in the emission maximum to longer wavelengths. There is now evidence that energy transfer occurs from Trp-109 (108) to Trp-64 (63), which is strongly quenched (Imoto *et al.*, 1971; Sommers *et al.*, 1973). This complication might have some bearing on the anomalous fluorimetric titration of the mammalian lysozymes. Thus, any change which increases the efficiency of the energy transfer will also quench the protein fluorescence. It is conceivable that in hen egg-white lysozyme energy transfer to Trp-62 occurs. Since the residue is also an emitter, increased efficiency in the transfer need not lead to fluorescence quenching. In fact, a small dequenching is observed with the ionization of Glu-35 in hen egg-white lysozyme.

The similarity between the near-ultraviolet CD spectra of human and rat lysozymes is remarkable. There is possibly a number of reasons for the lower intensity of the rat lysozyme spectrum. First, the ellipticity was computed on the basis of a calculated extinction coefficient. It is possible, therefore, that this coefficient is underestimated, leading to lower ellipticity. As pointed out above any such error should be small. It is also possible that the replacement of Trp-34 in human lysozyme by a nonaromatic residue in mouse lysozyme also occurs with the rat enzyme. Thus, any contribution of this residue to the circular dichroism of human lysozyme would also be lost. Finally, the increased number of tyrosine residues in rat

[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.

lysozyme may have a small effect on the spectrum. Otherwise, the considerable similarity between the human and rat lysozyme spectra suggests that those residues responsible for the spectra of one are also present in much the same environment in the other enzyme.

One important feature of the rat lysozyme CD spectrum is the presence of a negative band at approximately 313 nm (Figure 5B). As with human lysozyme this long-wavelength band only appears in alkaline solution. From studies on human lysozyme we have tentatively assigned this band to an interaction between Tyr-63 (62) in the ionized state and Trp-64 (63). The occurrence of this band in the spectrum of rat lysozyme suggests that the same interaction also occurs in this enzyme. The complete titration of the 313-nm band of the rat lysozyme spectrum has not been attempted. Therefore, it is not possible to decide whether it has the same apparent pK as in human lysozyme (*i.e.*, 9.35; Mulvey, 1973) or whether the intensity is fully developed at pH 10.4.

The fluorescence and circular dichroism of human and rat lysozymes are in many respects identical. This, therefore, suggests a high degree of homology in the distribution of aromatic residues throughout their sequences.

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