The Stereospecificity of Human, Hen, and Papaya Lysozymes*

F. W. Dahlquist,† C. L. Borders, Jr.,‡ G. Jacobson, and M. A. Raftery

ABSTRACT: Lysozymes from human, hen egg white, and papaya sources have been studied in regard to the stereospecificity of their rupture of the β-(1–4)-linked glycosidic bonds of substrates. The human and hen enzymes catalyze extensive transglycosylation in addition to hydrolysis. Use has been made of this to quantitate the formation of methyl glycosaminide(s) during cleavage of ^{14}C-labeled chitobiose in the presence of methanol. In this manner it could be shown that human and hen lysozymes catalyze cleavage of the disaccharide with retention of configuration to an extent of at least 99.9 and 99.7%, respectively. Papaya lysozyme does not catalyze glycosyl transfer. In addition it differs from the human and hen enzymes in that inversion of configuration prevails in the products obtained from degradation of chitotetraose. The findings are discussed in relation to possible mechanistic pathways for catalysis by the three lysozymes.

Hen egg white lysozyme is the enzyme which, from a structural point of view, is at present best understood. Although several studies of the action of the enzyme on cell-wall oligosaccharides (Salton, 1964; Sharon, 1967; Chipman *et al.*, 1968) and on chitin oligosaccharides (Wenzel *et al.*, 1962; Rupley, 1964; Maksimov *et al.*, 1965; Dahlquist and Raftery, 1967; Rupley and Gates, 1967) have been reported little work has been done on the detailed mechanism of the bond cleavage. From crystallographic studies involving lysozyme–saccharide complexes Blake *et al.* (1965, 1967) have outlined a tentative scheme for the hydrolytic mechanism. This proposal involves catalytic production of a carbonium ion which is given steric and electrostatic stabilization by the enzyme. Other mechanistic schemes are also plausible (Raftery and Rand-Meir, 1969) for such an enzyme-catalyzed reaction and in this respect a knowledge of the stereochemistry of the products of lysozyme action is of importance.

The ability of lysozyme to act as a transferase has been previously recognized. Kravchenko (1967) has shown that lysozyme-catalyzed hydrolysis of chitotetraose resulted in production of insoluble chitin-like polymers. Rupley (1965) has also shown that glycosyl units could be transferred by lysozyme from chitotriose to 2-acetamido-2-deoxy-D-glucopyranose. The action of lysozyme on a cell-wall tetrasaccharide was shown to result in glycosyl transfer with production of higher molecular weight compounds, as judged by paper chromatography (Sharon, 1967; Chipman *et al.*, 1968) of reaction mixtures.

Since such higher molecular weight compounds formed by transglycosylation of cell-wall oligosaccharides have been shown to be susceptible to degradation by lysozyme it has been suggested that *all* of the glycosidic bonds in the compounds have the same stereochemistry as in the initial substrates (Sharon and Seifter, 1964; Sharon, 1964, 1967; Pollock *et al.*, 1967; Chipman *et al.*, 1968). There is, however, no direct chemical evidence bearing on the stereochemistry of the glycosidic bonds in these compounds. Furthermore, in the absence of evidence that lysozyme cannot hydrolyze α-linked glycosidic bonds or form them (to a greater or lesser extent) by transglycosylation, it is not clear that the enzymatic reaction proceeds with *complete* or *partial* retention or inversion of configuration.

The purpose of the present investigation was to carry out a *quantitative* study of the stereochemistry of the lysozyme-catalyzed hydrolysis and transfer of β-(1–4)-linked glycosidic bonds. In addition to the well-known lysozyme from hen egg white, lysozymes from a human source[1] and from papaya were also studied.

Experimental Section

Materials. Hen egg white lysozyme was purchased from Sigma Chemical Co. (Lot No. 96B-8572). Human lysozyme was a generous gift of Dr. Robert Canfield. Papaya lysozyme was isolated and chromatographed according to Howard and Glazer (1967). Chitobiose, chitotriose, and chitotetraose were isolated from acid hydrolysates of chitin by a gel filtration method (Raftery *et al.*, 1969). Chitobiose octaacetate was prepared by acetylation of chitobiose with pyridine–acetic anhydride (1:2, v/v) as described by Zechmeister and Toth (1931).

The synthesis of *N,N'*-diacetyl-^{14}C-chitobiose was accomplished, as outlined in Figure 1, through first treating chitobiose octaacetate (I) with triethyloxonium fluoroborate (Hanessian, 1967) to obtain 3,4,6-tri-*O*-acetyl-2-deoxy-2-amino-D-glucopyranosyl-β-(1–4)-1′,3′,-6′-tri-*O*-acetyl-2-deoxy-2-amino-D-glucopyranose hydrofluoroborate (II). Acetylation of this material in pyridine and acetyl-1-^{14}C chloride (Volk Radiochemical Co., Lot No. MA 1093) gave octaacetylchitobiose (III),

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† National Institutes of Health trainee.

‡ National Institutes of Health predoctoral fellow.

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with ^{14}C-labeled acetamido groups. Deacetylation in methanol (containing 0.1 N sodium methoxide) gave the desired *N,N'*-diacetyl-^{14}C-chitobiose with a specific activity of 50 μCi/mmole. This material was characterized by its melting point of 260–262° (7) and its proton magnetic resonance spectrum (Dahlquist and Raftery, 1969).

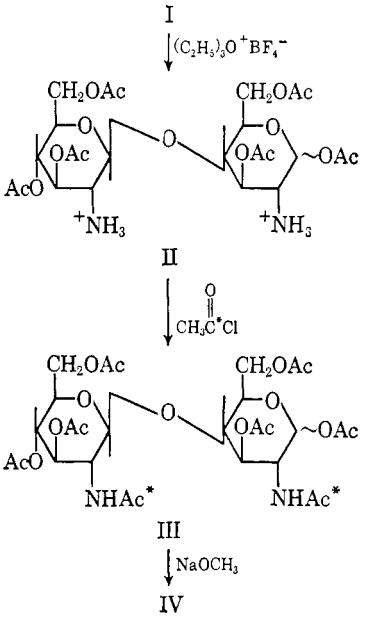

FIGURE 1: Outline of scheme for the synthesis of chitobiose-*N,N'*-^{14}C from octaacetylchitiose.

A mixture of α- and β-methyl *N*-acetylglucosaminides was prepared by acetylation (Horton, 1966) of glucosamine with acetic anhydride-^{3}H (Volk Radiochemical Co., Lot No. 1314-31-39), followed by reaction with methanol, as previously described (Zilliken *et al.*, 1955). The specific activity of the glycoside mixture was 1 mCi/mmole. The relative concentration of α- to β-methyl-2-acetamido-2-deoxy-D-glucopyranose in this mixture was determined by integration of the methoxyl resonances obtained in a 60-MHz proton magnetic resonance spectrum of the sample. The ratio of α- to β-methyl 2-acetamido-2-deoxy-D-glucopyranose was found to be 1.9:1.0.

Methods. Hydrolysis of chitotriose by hen egg-white or human lysozymes was conducted under the following conditions. The trisaccharide (10^{-2} M) was incubated with the enzyme (3×10^{-3} M) in 0.1 M citrate buffer (pH 5.5) for 1.5 hr at 40°. Total volume of such incubation mixtures was 250 μl. Following incubation the mixture was subjected to gel filtration on a column (0.9 × 100 cm) of Bio-Gel P-2 (200–400 mesh) which was equilibrated with 25% acetic acid. The eluting solvent was 25% acetic acid and fractions of 1–2 ml were collected at a flow rate of 30 ml/hr. Reducing sugars in the eluted fractions were determined by analysis of an aliquot (200 μg) by the ferricyanide method (Park and Johnson, 1949).

Hydrolysis of chitobiose was attempted using a solution which contained the disaccharide (1.2×10^{-1} M) and lysozyme (hen egg-white or human enzymes) (3×10^{-3} M) in the buffer used for hydrolysis of the trisaccharide. The mixture was incubated at 40° for 15 hr and then chromatographed on Bio-Gel P-2 as already described.

The hydrolysis of chitin oligosaccharides by papaya lysozyme was carried out in 0.1 M citrate buffer (pH 4.7). The concentration of enzyme used was in all cases equal to 8×10^{-5} M. Chitobiose (1.1×10^{-2} M) was hydrolyzed at 40° for 17 hr and chitotriose (1.1×10^{-2} M) for 10 min at 25°.

Hydrolysis of Chitobiose in the Presence of Methanol. A solution containing 40 mg of lysozyme and 40 mg of *N,N'*-diacetyl-^{14}C-chitobiose in 800 μl of 0.1 M citrate buffer (pH 5.5) which was 8 M in methanol was incubated for 20 hr at 38°. A drop of toluene was added to inhibit fungal and bacterial contamination. After incubation the solution was diluted to 50 ml with water. To this was added 0.4 mg each of methyl 2-acetamido-2-deoxy-α-D-glucopyranoside and methyl 2-acetamido-2-deoxy-β-D-glucopyranoside, and 5×10^{5} dpm of the mixture of methyl 2-acetamido-2-deoxy-α-D-glucopyranoside and methyl 2-acetamido-2-deoxy-β-D-glucopyranoside. This solution was passed through an Aminex UM-1 ultrafiltration membrane (Amicon Corp.) to remove the enzyme. The filtrate was treated with Amberlite MB-1 ion-exchange resin to remove salts and most of the free sugars (the hemiacetal is a weak acid). The sample was lyophilized, taken up in a minimum of water, streaked on Whatman No. 3MM paper as a 7-cm wide band, and developed in descending chromatography for a distance of 60 in. using the upper phase of pyridine–ethyl acetate–water (1:2:2, v/v). Following development, the chromatogram was cut in half lengthwise. The sugars were located on one strip using a modification (W. J. Dreyer, personal communication) of the peptide-bond spray (Mazur *et al.*, 1962). Strips (2 cm) were cut out from the second half of the chromatogram and these were eluted with water. An aliquot of each eluted fraction was removed, and after addition to 15.0 ml of Bray's solution (Bray, 1960), the ratio of ^{3}H to ^{14}C was determined by liquid scintillation in a Packard Model 3324 Tri-Carb liquid scintillation spectrometer.

Mutarotation of 2-Acetamido-2-deoxy-α-D-glucopyranoside. A fresh solution of the sugar (1.2×10^{-2} M) in 0.1 M citrate (pH 4.7) was placed in a 10-cm jacketed cell (1.0-ml capacity) and the mutarotation at 25° followed as a function of time in a Perkin-Elmer Model 141 polarimeter at 578 mμ.

Mutarotation of the Products of Lysozyme Action on Chitotetraose. Papaya lysozyme (1×10^{-4} M) and chitotetraose (1.2×10^{-2} M) at mutarotation equilibrium were incubated at 25° in a polarimeter tube (1.0-ml capacity, 10-cm path length) and changes in rotation were observed as a function of time. Product analysis was done on samples of a mixture similarly incubated for 10 min and 30 min. The method employed was gel filtration of aliquots on columns (0.9 × 100 cm) of Bio-Gel P-2 as already described for determination of ratios of chitotetraose, chitotriose, chitobiose, and *N*-acetylglucosamine.

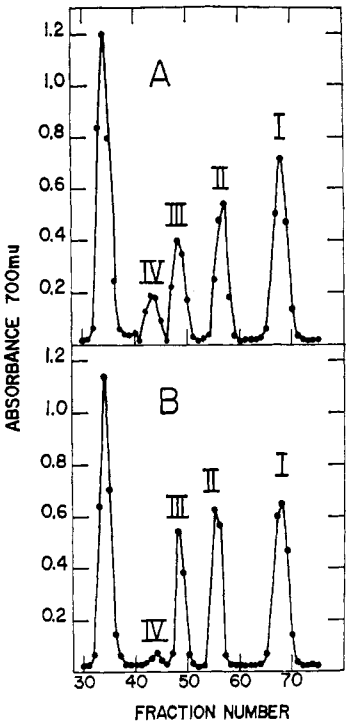

FIGURE 2: Gel filtration of lysozyme digests of chitotriose. (A) Human lysozyme. (B) Hen egg-white lysozyme. In each case the peak eluted between fractions 30 and 40 was enzyme. Numerals I, II, III, and IV refer to 2-acetamido-2-deoxy-D-glucopyranose, chitobiose, chitotriose, and chitotetraose. Conditions employed and analytical determinations are explained in the Experimental Section.

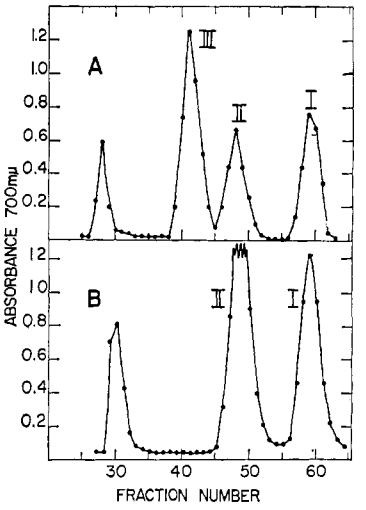

FIGURE 3: Gel filtration diagrams of papaya lysozyme digests of chitotriose (A) and chitobiose (B). The peak eluted at approximately fraction 30 was enzyme. Numerals I, II, and III refer to 2-acetamido-2-deoxy-D-glucopyranose, chitobiose, and chitotriose. Conditions employed and analytical methods used are explained in the Experimental Section.

Results

Hydrolysis of Chitotriose. Both human and hen egg white lysozymes effected hydrolysis of chitotriose over short time periods (1.5 hr) in the present experiments. Figure 2 shows the product distribution obtained in each case. Extensive degradation of the trisaccharide occurred with concomitant formation of chitobiose and 2-acetamido-2-deoxy-D-glucopyranose. The other feature of interest in the chromatographic patterns obtained is that transglycosylation was effected by both enzymes with formation of small amounts of material of molecular weight comparable with chitotetraose. Therefore the suitability of both lysozymes for studies of their reaction mechanisms employing this transglycosylation reaction was evident.

Treatment of a solution of chitotriose with papaya lysozyme for 10 min resulted in extensive degradation of the trisaccharide, as shown in Figure 3A, to give chitobiose and *N*-acetylglucosamine. In contrast to the patterns obtained using the hen egg-white and human enzymes formation of higher molecular weight oligosaccharides was not observed.

Hydrolysis of Chitobiose. Treatment of the disaccharide with hen egg-white or human lysozyme for extended time periods (15 hr) resulted in extensive degradation to form the monosaccharide, 2-acetamido-2-deoxy-D-glucopyranose. The results obtained from product analysis are shown in Figure 4. It was evident that transglycosylation also occurred in this reaction to form products with molecular weights corresponding to the tri- and tetrasaccharides of *N*-acetylglucosamine. This result confirmed the suitability of this system for study of the transglycosylation reaction.

Hydrolysis of chitobiose was also effected by papaya lysozyme. A separation of the products of such a hydrolysis is depicted in Figure 3B. Formation of *N*-acetylglucosamine was evident. However, no traces of chitotriose or chitotetraose appeared and this was suggestive that transglycosylation had not occurred.

Methanol Partitioning during Hydrolysis of Chitobiose by Hen Egg White Lysozyme. Figure 5 shows the results obtained. Good yields of methyl 2-acetamido-2-deoxy-β-D-glycopyranose were achieved. There was a small amount of a compound which chromatographed with an R_F value almost equal to that of methyl 2-acetamido-2-deoxy-α-D-glucopyranoside, and was found to appear in each of three separate experiments. However, this compound was not methyl 2-acetamido-2-deoxy-α-D-glucopyranoside since its peak at fractions 20 and 21 did not coincide with the tritiated methyl 2-acetamido-2-deoxy-α-D-glucopyranoside carrier peak. It is possible, however, that this apparent impurity could mask a low level of methyl 2-acetamido-2-deoxy-α-D-glucopyranoside. Table I shows the $^3H/^{14}C$ ratios of the fractions corresponding to the methyl 2-acetamido-2-deoxy-β-D-glucopyranoside and methyl 2-acetamido-2-deoxy-α-D-glucopyranoside peaks. These figures allow a comparison of both the constancy of the ratios across each peak and also of the relative amounts of methyl 2-acetamido-2-deoxy-β-D-glucopyranoside to methyl 2-acetamido-2-deoxy-α-D-glucopyranoside formed during the transglycosylation. By comparing the $^3H/^{14}C$ ratio of the methyl 2-acetamido-2-deoxy-α-D-glucopyranoside to that of the methyl 2-acetamido-2-deoxy-β-D-glucopyran-

oside peak, it was found that the ^{14}C content of the methyl 2-acetamido-2-deoxy-α-D-glucopyranoside area was 0.3% that of the methyl 2-acetamido-2-deoxy-β-D-glucopyranoside peak. At least some, if not all, of this ^{14}C was due to the faster running impurity. These results indicate that the transfer reaction catalyzed by hen egg white lysozyme forms the β product with a very high level of stereospecificity. Since the glycosidic linkage in *N,N'*-diacetylchitobiose is β-(1–4) the reaction therefore involves retention of configuration.

Methanol Partitioning during Hydrolysis of Chitobiose by Human Lysozyme. In the partitioning reaction the results obtained using the human enzyme were quite similar to those observed for the hen egg-white enzyme. The results are presented in Table I and show that methyl 2-acetamido-2-deoxy-β-D-glucopyranoside was produced. Comparison of the $^{3}H/^{14}C$ ratio of the methyl 2-acetamido-2-deoxy-α-D-glucopyranoside peak with that of the methyl 2-acetamido-2-deoxy-β-D-glucopyranoside peak showed that the amount of ^{14}C-labeled methyl 2-acetamido-2-deoxy-α-D-glucopyranoside was only 0.1% that of similarly labeled methyl 2-acetamido-2-deoxy-β-D-glycopyranoside. Again the faster running impurity was observed, although in the present case the separation obtained was superior. It is most probable that all of the ^{14}C counts in the methyl 2-acetamido-2-deoxy-α-D-glucopyranoside peak area were due to this impurity. Therefore it is evident that in the case of human lysozyme catalyzed cleavage of glycosidic bonds the configuration of the anomeric carbon undergoing rupture is retained to an extent of at least 99.9%. Thus the stereospecificity of the human enzyme is the same as that of the hen egg-white enzyme.

Methanol Partitioning during Hydrolysis of Chitobiose by Papaya Lysozyme. Treatment of chitobiose with papaya lysozyme for extended periods of time gave no detectable levels of methyl 2-acetamido-2-deoxy-β-D-glucopyranoside or methyl 2-acetamido-2-deoxy-α-D-glucopyranoside. This result confirmed those described earlier for the hydrolysis of chitotriose and chitobiose

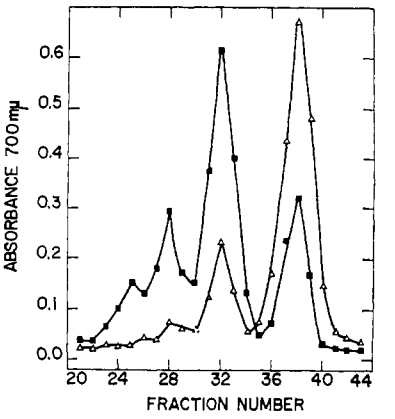

FIGURE 4: Gel filtration patterns obtained from mixtures of chitobiose and lysozyme. (■—■) Hen lysozyme; (△—△) human lysozyme. Numerals I–IV represent 2-acetamido-2-deoxy-D-glucopyranose, chitobiose, chitotriose, and chitotetraose.

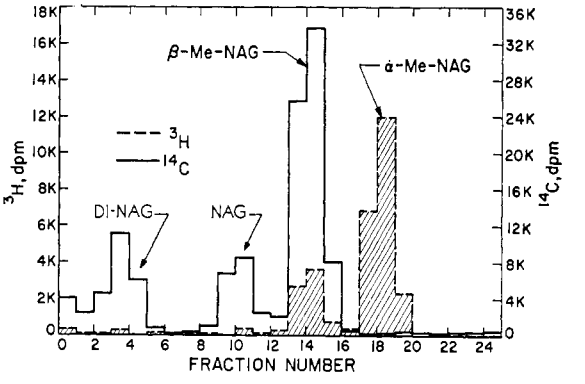

FIGURE 5: Nomogram of radioactivity measurements of fractions eluted from paper chromatogram employed to separate the products of lysozyme action on chitobiose. (—) ^{14}C disintegrations of residual chitobiose-*N,N'*-^{14}C and of products of hydrolysis and transglycosylation catalyzed by lysozyme; (---) (enclosing shaded area) denote ^{3}H disintegrations of added methyl 2-acetamido-2-deoxy-β-D-glucopyranoside and methyl 2-acetamido-2-deoxy-α-D-glucopyranoside.

TABLE I

Lysozyme	Compound	Fraction	dpm Ratio $^{3}H/^{14}C$
Hen	Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside	14	0.104
		15	0.107
		16	0.089
	Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside	18	63.4
		19	60.8
		20	6.47
Human	Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside	61	0.471
		62	0.498
		63	0.501
	Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside	67	490
		68	914
		69	273

where no evidence was obtained for transglycosylation. Such a finding is of interest since it suggests a difference in the mechanism of the reaction catalyzed by papaya lysozyme when compared with enzymes from human and hen egg white sources.

Mutarotation of the Products of Papaya Lysozyme Action on Chitotetraose. Since transglycosylation to form compounds of known stereochemistry could not be used to investigate the mechanism of glycosidic bond cleavage by papaya lysozyme other methods had to be sought. A method commonly used in the past to study the stereochemistry of glycosidic bond rupture by enzymatic means was to follow the changes in optical rotation as the products of the reaction came to mutarotation equilibrium. This technique has been used to advantage in studies of amylases (Freeman and Hopkins, 1936; Thoma and Koshland, 1960; Thoma *et al.*, 1963) and other glycosidases (Ono *et al.*, 1965; Hamauzu *et al.*,

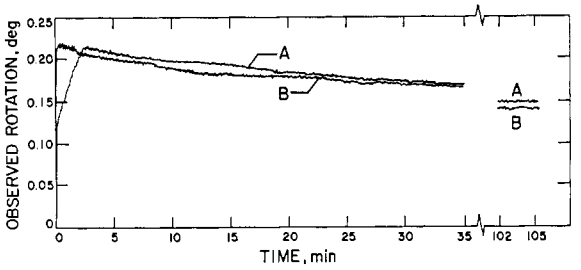

FIGURE 6: Mutarotation studies. (A) The mutarotation of the products of papaya lysozyme hydrolysis of chitotetraose. Recording was started 4 min after addition of the enzyme to a solution of the tetrasaccharide at mutarotation equilibrium. Conditions used are explained in the Experimental Section. (B) Mutarotation of a sample of 2-acetamido-2-deoxy-β-D-glucopyranoside. Conditions used are described in the Experimental Section. All rotations are positive.

1965). Such studies were not possible with hen egg white and human lysozymes due to the slow rates at which they hydrolyze oligosaccharide substrates and the rapidity with which 2-acetamido-2-deoxy-D-glucopyranose anomers mutarotate to equilibrium. It has been recently shown, however, that papaya lysozyme degrades chitotetraose four hundred times faster than hen egg-white enzyme (Howard and Glazer, 1967). It seemed possible that this rapid rate of cleavage of the tetrasaccharide to lower molecular weight products could allow investigation, by polarimetry, of their anomeric forms. Furthermore, the hydrolysis was, as shown in the present investigation, uncomplicated by transglycosylation. It was further shown by means of nuclear magnetic resonance at 220 MHz that the relative proportions of α- and β-anomeric forms of chitobiose and chitotetraose at equilibrium, in 0.1 M sodium citrate pD 5.0, were essentially the same. We first showed by gel filtration of reaction mixtures that incubation of chitotetraose with papaya lysozyme for 10 min resulted in almost complete degradation of the tetrasaccharide, at 25°, to chitobiose and *N*-acetylglucosamine (ratios of chitotetraose:chitotriose:chitobiose:*N*-acetylglucosamine = 0.4:0.6:10:3) while the radios of the same products after incubation for 30 min changed very little (--0.6:10:4). Observation of the changes in rotation at 578 mμ which accompanied the hydrolysis of chitotetraose under these conditions are shown in Figure 6A. Initially an increase in rotation was observed. This was due to the formation of the lower molecular weight oligomers of *N*-acetyl-D-glucosamine since it has been shown (Barker *et al.*, 1958) that these have rotations which are progressively more positive as their molecular weight decreases. After about 10 min from the time when the enzyme was added this increase in positive rotation ceased and was followed by a slow decrease in positive rotation, until a final value had been reached after approximately 100 min. Such a decrease in rotation was due to the mutarotation of the products of hydrolysis of chitotetraose. Figure 6 compares this process with the mutarotation of *N*-acetyl-α-D-glucosamine in the same buffer and at the same temperature. It seems reasonable to conclude that the hydrolysis products of papaya lysozyme action on chitotetraose mutarotate from their α-anomeric forms to their equilibrium mixtures. Such a result establishes a clear difference in the stereochemistry of the papaya lysozyme-catalyzed hydrolysis β-linked glycosidic bonds compared with that of the same reaction catalyzed by lysozymes from hen egg white and human sources.

Discussion

The results obtained in the experiments reported have clearly shown by means of a simple chromatographic procedure on Bio-Gel P-2 that human, hen egg white, and papaya lysozymes all hydrolyze chitotriose and chitobiose to form lower molecular weight sugars. In those instances where chitobiose and chitotriose were treated with the human and egg white enzymes it was evident that extensive transglycosylation occurred, with the formation of higher molecular weight oligosaccharides, in addition to hydrolysis. Such a transferring ability has previously been demonstrated for the hen egg white enzyme but not for the human one. The hydrolysis of the trisaccharide by the papaya lysozyme was more rapid than with the hen and human enzymes but did not result in any detectable transglycosylation. Similarly, no higher molecular weight oligosaccharides were found as a result of treatment of chitobiose with papaya lysozyme.

When methanol was used as an acceptor for enzymatic transfer of an *N*-acetyl-D-glucosamine residue from chitobiose no methyl glycosides were formed in the presence of the papaya enzyme. Both the hen and human enzymes yielded significant amounts of methyl 2-acetamido-2-deoxy-β-D-glucopyranoside and no significant amounts of the corresponding α-linked methyl glycoside were detected using ^{14}C-labeled substrate. Therefore, the transglycosylation reaction is an effective means of quantitatively studying the stereochemistry of glycosidic bond cleavage by these two enzymes. From the results obtained it can be stated that hen and human lysozyme catalyzed hydrolysis results in retention of configuration to extents of at least 99.7 and 99.9%, respectively. A statement to the effect that retention of configuration obtains for hen lysozyme with a small acceptor such as methanol has appeared (Rupley and Gates, 1967) but no data were given nor was there any report on whether such retention was predominant or quantitative.

We have previously shown (Raftery and Rand-Meir, 1969) that participation of an acetamido side chain of the substrate does not occur in hen lysozyme catalyzed hydrolysis of some oligomeric *N*-acetyl-D-glucosamine derivatives. Because of this, and the observation that retention of configuration occurs during hydrolysis, there are only two possible mechanistic pathways which are reasonable for the enzyme-catalyzed cleavage of such substrates (Raftery and Rand-Meir, 1969). The first is an even number of SN2 type displacements, the simplest of these being the "double-displacement" mechanism suggested by Koshland (1953) to account for retention of configuration during amylolysis of starch by α-amylase. The second explanation involves production of an

enzyme-bound carbonium ion, perhaps stabilized as an ion pair (Winstein and Robinson, 1958) by a propinquous anionic base, which is stereospecifically quenched by the solvent. The quantitative results described in the present communication show that retention of configuration is absolute during cleavage of chitobiose by hen and human lysozymes, and this does not distinguish between these two mechanisms. A small but significant amount of methyl 2-acetamido-2-deoxy-α-D-glucopyranoside in addition to the β-linked methyl glycoside formed in relatively large amounts would have constituted direct proof for a carbonium ion mechanism.

In this respect methanol as a transglycosylation acceptor is probably a better choice than a saccharide molecule such as those employed by Chipman *et al.* (1968) since such saccharides would be expected to bind identically with the original saccharide leaving group and therefore give rise to products of retained configuration.

The present finding that the anomeric form of 2-acetamido-2-deoxy-D-glucopyranose produced as a result of enzymatic rupture of a β-linked glycosidic bond by papaya lysozyme is of interest since it is at least preponderantly of configuration opposite to that observed using hen and human lysozymes. This is reminiscent of the α- and β-amylases in their action on amylose. There is unfortunately no method available at present which allows definition of the precise extent of inversion at the anomeric carbon of the bond undergoing rupture. At best we can estimate that the main product is one of inverted configuration. The present findings allow some speculation on the mechanistic pathway of substrate molecules during catalysis by papaya lysozyme. Clearly any mechanism involving a double displacement (Koshland, 1953) or anchimeric assistance by the acetamido side chain (Lowe, 1967; Lowe *et al.*, 1967; Piszkiewicz and Bruice, 1967, 1968), perhaps in conjunction with general base catalysis by the enzyme (Raftery and Rand-Meir, 1969), is not to be considered. The most likely pathways are (a) a single-displacement reaction involving a water molecule on the enzyme or (b) a carbonium ion mechanism. Mechanism a would lead to quantitative inversion of configuration while mechanism b would result in either quantitative or partial inversion depending upon whether the solvent could react with the carbonium ion intermediate from both above and/or below the ring.

In conclusion glycosidic bond rupture by hen and human lysozymes involves complete retention of configuration and is likely to be due to either a double displacement or carbonium ion mechanism for glucoside or 2-deoxyglucoside substrates with the possibility of acetamido group participation in 2-acetamido-2-deoxy-D-glycoside substrates. Glycosidic bond cleavage by papaya lysozyme results in predominant (or perhaps complete) inversion of configuration which can be explained mechanistically by a single displacement or carbonium ion mechanism. While experiments to distinguish these possibilities are in progress it will also be of interest to conduct studies, similar to those reported here, on other lysozymes from widely differing sources.

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The Nature of Amino Acid Side Chains Which Are Critical for the Activity of Lysozyme*

S. M. Parsons,† L. Jao, F. W. Dahlquist,† C. L. Borders, Jr.,† T. Groff,‡ J. Racs, and M. A. Raftery

ABSTRACT: Chemical modification by use of selective reagents has been applied to the side chains of lysine, histidine, tyrosine, and carboxyl residues in lysozyme. The properties of the lysozyme derivatives have been monitored by measuring (a) their ability to bind chitotriose and (b) their catalytic efficiency for hydrolysis of chitotriose or *Micrococcus lysodeikticus* cells. Thus, effects of chemical modification on binding properties or catalytic properties have been distinguished. In this manner it has been shown that neither lysine, histidine, nor tyrosine residues play a role in catalysis by lysozyme. Modification of carboxyl groups, however, by various chemical treatments abolishes catalytic activity. A new reagent for preferential esterification of protein carboxyl groups has been employed which has made it possible to isolate two singly esterified lysozyme derivatives. One of these derivatives has been shown to contain a labile carboxyl ester which is most likely at or close to the strong binding site for chitotriose. The other derivative has been shown to contain a single carboxyl ester and to be essentially inactive against both *M. lysodeikticus* and chitotriose as substrates, while retaining the ability to effect good binding of the trisaccharide.

From knowledge of the three-dimensional structure of lysozyme at 2-Å resolution (Blake *et al.*, 1965, 1967 a,b; Phillips, 1967) and knowledge of the structures of lysozyme–inhibitor complexes (Johnson and Phillips, 1965; Blake *et al.*, 1967a) now available, it appears that we should for the first time be close to understanding in detail the mechanism of action of an enzyme. Based on details of a nonproductive lysozyme–chitotriose complex in the crystalline state, it has been suggested as a result of further model building (Blake *et al.*, 1967b; Phillips, 1967) that the amino acid side chains most likely to play an active role in catalysis by the enzyme are the β- and γ-carboxyl groups of aspartic acid residue 52 and glutamic acid residue 35 in the amino acid sequence.

Numerous attempts have been made to identify by chemical means the amino acid side chains which affect catalysis by lysozyme. The approaches used have been reviewed (Jollès, 1964, 1967). In summary it has been claimed that oxidation (Fraenkel-Conrat, 1950) or reduction (Churchisch, 1962; Imai *et al.*, 1963, Jollès *et al.*, 1964) of disulfide bonds, photooxidation of histidine and aromatic amino acids (Weil *et al.*, 1952), oxidation (Horinishi *et al.*, 1964), iodination (Hartdegen and Rupley, 1964, 1967), or ozonization of tryptophan (Previero *et al.*, 1966, 1967) residues, acetylation of amino groups (Fraenkel-Conrat, 1950; Geschwind and Li, 1957), and esterification with methanol–HCl of carboxyl groups (Fraenkel-Conrat, 1950) all can cause inactivation of the enzyme.

In this communication we present the results of chemical modification, by various treatments, of the side chains of a number of amino acid residues in lysozyme. We have sought methods which to the best of our knowledge do not seriously disrupt the secondary or tertiary structures of the enzyme. An important feature of the investigation was the determination of whether inactivation caused by chemical modification of lysozyme resulted from an effect on the binding properties or on the catalytic properties of the enzyme derivatives.

Experimental Section

Materials. Lysozyme (Lot No. 96B-8572) was purchased from Sigma Chemical Co. Acetic anhydride, iodoacetic acid, and hydroxylamine hydrochloride were purchased from Eastman Kodak Chemical Co. Iodoacetamide and acetylimidazole were purchased from Mann Research Laboratories. *O*-Methylisourea was obtained from the Aldrich Chemical Co. Ninhydrin was a product of the Pierce Chemical Co. *N*-Acetyl-D-glucosamine was obtained from the California Corp. for Biochemical Research. Chitotriose was prepared from a partial acid hydrolysate of chitin by a gel filtration procedure

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† National Institutes of Health Predoctoral Fellow, 1967–1968.

‡ Deceased, Feb. 19, 1967.