A Transition State Analog for Lysozyme*

(Received for publication, March 2, 1972)

ISAAC I. SECEMSKI,‡ SHERWIN S. LEHRER, AND GUSTAV E. LIENHARD‡

From the Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138; the Department of Muscle Research, Boston Biomedical Research Institute, and Department of Neurology, Harvard Medical School, Boston, Massachusetts 02114, 02115

SUMMARY

The δ-lactone derived from tetra-N-acetylchitotetraose (TACL) has been prepared by oxidation of the tetra-N-acetylchitotetraose with iodine. The binding of TACL to lysozyme has been investigated by its inhibition of the lysozyme-catalyzed lysis of Micrococcus lysodeikticus cells and by its perturbation of the tryptophyl fluorescence spectrum of lysozyme. At pH 6.2, the concentration of TACL that is required for 50% inhibition of the rate of lysis is 0.7 μM, which is 1/110 of the concentration of the unmodified tetrasaccharide that is required for such inhibition. The association constants for the binding of TACL to lysozyme over the pH range from 2 to 8 were obtained by fluorescence measurements. Their pH dependence shows that TACL binds most strongly to the species of lysozyme in which the carboxyl group of glutamate 35 is dissociated. In agreement with this result, the fluorescence-pH profile of the TACL-lysozyme complex indicates that the pK of glutamate 35 is about 4.7 in the complex, whereas the pK of glutamate 35 in the enzyme alone is about 6.0. The value of the association constant for the binding of TACL to lysozyme at pH 5.0 and 25° is 3.3 × 10⁶ M⁻¹, which is 32 times larger than that for the binding of the unmodified tetrasaccharide under the same conditions. On the basis of these results and of the similarity between the known conformation of the lactone ring and the proposed conformation of the transition state for lysozyme-catalyzed reactions of the half-chair types, we conclude that TACL is a transition state analog for lysozyme. Furthermore, with these results we can estimate that the affinity of Subsite D of lysozyme for the half-chair conformation of the pyranose ring of N-acetylglucosamine is greater by a factor of 6 × 10³ than its affinity for the chair conformation and thus contributes this factor to catalysis.
Since the conformation of crystalline n-gluconic acid δ-lactone is known to be a slightly distorted half-chair (12), TACL should be an analog of the transition state conformation. The lactone was prepared from the tetrasaccharide in order to satisfy the specificity of lysozyme, which has three binding subsites referred to as A, B, and C, that bind three β-(1 → 4)-linked N-acetylglucosamine residues. These subsites are contiguous with the Subsite D, at which the N-acetylglosamine or N-acetylmuramic acid residue that contains the glycosidic linkage to be cleaved binds (7).

The perturbation of the tryptophyl fluorescence of lysozyme was used to determine the binding constants for TACL because of the fluoresence changes observed with GlcNAc inhibitors (13, 14) and the sensitivity of the technique at the low enzyme concentrations required. This technique has, in addition, yielded information regarding some interactions between the analog and the enzyme. It is our hope that the binding interactions in the crystal will ultimately be determined by x-ray studies of the complex.

While this work was in progress, δ-lactones related to the corresponding substrates were reported to be potent inhibitors of a number of glycosyl-transferring enzymes and surmised to be transition state analogs (15–18).

**EXPERIMENTAL PROCEDURE**

**Materials**

Preparation and Chemical Properties of TACL—A typical preparation was carried out in the following way.

One hundred milliliters of tetra-N-acetylchitotetraose (0.005 m) were oxidized to the corresponding acid (TACA) with iodine (0.01 m) in KI (0.05 m)–K₂CO₃ (0.045 m) at 4° in the dark (19). After 3½ hours, when titration of an acidified aliquot with sodium thiosulfate (19) showed that 1 mole of iodine per mole of sugar had been reduced, the reaction mixture was adjusted to pH 2 with 5 N H₂SO₄ and the excess iodine was extracted with benzene. The extracted aqueous solution was adjusted to pH 7 with 1 N KOH, concentrated to 15 ml on a rotary evaporator, and chromatographed on a column (135 × 5.7 cm²) of Bio-Gel P-2, 200 to 400 mesh, with water as the eluant (20). This chromatography separated the potassium salt of TACA from the inorganic ions. The 25-ml fractions from the Bio-Gel column were monitored for TACA by measuring the absorbance at 215 nm, which is due to the amide function, and also by boiling 20-μl aliquots with 1 ml of 12 N HCl for 2 hours and testing the neutralized mide-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-D-glucuronic acid; TACL, the δ-lactone of TACA; MurNAc, 2-acetamido-2-deoxy-D-glucuronic acid (Bio-Rad, AG50W-X-8, H+ form), removing the water on the rotary evaporator, and drying the solid in an evacuated desiccator over P₂O₅ for a day. The yield on the basis of the amount of tetra-N-acetyltetrasaccharose was 80%. Colorimetric tests for reducing sugar (21) with GlcNAc as a standard and for amine (22) with D-glucosamine and δ-glucosaminic acid as standards showed that the potassium TACA contained 2 mole % unreduced sugar and 5 mole % monodeacetylated product.

The potassium salt of TACA was converted to a mixture of TACA and TACL by passing it through a cation exchange resin (Bio-Rad, AG50W-X-8, H+ form), removing the water on the rotary evaporator, and drying the solid in an evacuated desiccator over P₂O₅ for a day. The yield on the basis of the amount of tetra-N-acetyltetrasaccharose was 80%. Colorimetric tests for reducing sugar (21) and amine (22) showed that the mixture contained 1.5 mole % reducing sugar and 1.0 mole % monodeacetylated product. The lactone content and equivalent weight were determined by rapid titration of a freshly prepared solution to pH 5.5 (2 moles of NaOH consumed in neutralizing TACA), at which pH the half-time for hydrolysis of TACL is greater than 60 min, followed by titration from pH 5.5 to 9 (y moles of NaOH consumed in hydrolyzing TACL), at which pH the half-time for hydrolysis of TACL is less than 1 min. The mixture contained 23 mole % TACL (100 x/z + y) and had an equivalent weight of 850 (weight of mixture/z + y), theoretical value 840). Acid titration of the sodium salt of TACA, which was prepared from the mixture of TACL and TACA by neutralization with NaOH, gave an acid dissociation constant of 3.5 for TACA. Thin layer chromatography of the potassium TACA and of the TACA-TACL mixture after hydrolysis of the lactone in carbonate buffer showed a single spot in each case (see below for details).

The concentration of TACL was routinely measured with the neutral hydroxyamine-ferric chloride test, with N₃S-diacectylthioglycollate as the standard (23, 24). The solution of lactone was added to an equal volume of a 1:1 mixture of 4 M hydroxyamine hydrochloride and 3.5 M sodium hydroxide. After 5 min, 4 volumes of 0.46 M ferric chloride in 0.46 M HCl were added and the absorbance at 540 nm was read. The test was standardized by use of a solution of the TACA-TACL mixture which had had its lactone content determined by titration. The color yield of TACL is 1.05 times that of N₃S-diacectylthioglycollate.

The equilibrium between TACL and TACA was established by allowing solutions of potassium TACA and of the mixture of solid TACA and TACL, adjusted to pH 2.1 to 2.3 with HCl, to equilibrate at 23–25°. The equilibration process, which required about 20 hours, was followed by assaying for lactone with the hydroxyamine-ferric chloride test. Tests for reducing sugar and amine (21, 22) showed that during this period there was no hydrolysis of the glycosidic or amide linkages. At equilibrium there is 14 ± 1 mole % TACL.

The results of an investigation of the kinetics of the hydrolysis of the lactone function of TACL are summarized in Table I. In the presence of an equimolar amount of lysozyme, under which conditions TACL is entirely in complex with lysozyme at these concentrations (see “Results”), the rate of equilibration between the lactone and acid is accelerated approximately 20-fold.

Other Materials—The β-(1 → 4)-linked oligosaccharides of GlcNAc were prepared from chitin by the method of Rupley.
Table I

Rates of hydrolysis of lactone group of TACL

The rates of hydrolysis of TACL were measured by removing aliquots of the reaction mixture and analyzing for lactone with the hydroxylamine-ferric chloride test. The kinetics were first order, since the plots of \( \log (A_t - A_0) \), where \( A_t \) and \( A_0 \) are the absorbances at time \( t \) and after complete reaction, respectively, against time were linear. \( T, 30^\circ \).

| Buffer          | Concentration | TAC \(^a\) | pH \(^b\) | \( t^d \) |
|-----------------|---------------|-----------|-----------|---------|
| NH₄ acetate     | 0.16          | 0.10      | 1.5       | 4.7     | 65      |
| Sodium phosphate| 0.07          | 0.11      | 1.8       | 6.2     | 18      |
| Sodium phosphate| 0.07          | 0.11      | 1.8       | 0.2     | 17\*    |
| Sodium citrate  | 0.01          | 0.22      | 0.9       | 4.9     | 105     |
| Sodium citrate  | 0.01          | 0.22      | 0.9       | 4.9     | 105     |
| Sodium citrate  | 0.01          | 0.23      | 1.1       | 4.9     | 105     |
| Sodium citrate  | 0.01          | 0.20      | 1.1       | 5.8     | 75\*    |

* Ionic strength, made up with NaCl.
† Added as the mixture of TACA and TACL. The TACA was neutralized by including an equivalent of NaOH in the reaction mixture.
* Initial pH. The concentration of buffer was enough to keep the pH from dropping by 0.2 unit or less.
† From the first order plots.
* 0.12 nm lysozyme present in the reaction mixture.
† The reaction mixtures contained lysozyme at a concentration equimolar with that of TACL.
* Not accurately determined due to inappropriate sampling times.
* At 25\°.
† In this case the first order plot showed considerable scatter and was only approximately linear.

(25) and were further purified by the procedure of Raftery et al. (20). A mixture of 2-acetamido-2-deoxy-D-glucionic acid and its \( \gamma \)- and \( \delta \)-lactones was prepared from \( \delta \)-glucosaminic acid by acetylation with acetic anhydride according to the method of Cross. The mixture contained about 60 mole % lactone on the basis of titration (see above), and paper chromatography showed that both the \( \gamma \)- and \( \delta \)-lactones were present (24). Other materials were purchased from the following sources: twice crystallized, salt-free, hen egg white lysozyme from Worthington Biochemical Corp.; four times crystallized, salt-free, hen egg white lysozyme from Miles Chemical Co.; dried Micrococcus lysodeikticus cells from Sigma Chemical Co.; GlcNAc from Aldrich Chemical Co.; \( \delta \)-glucosaminic acid from Mann Research Laboratories.

Methods

Rates of Cell Lysis (25) — About 2.9 ml of a suspension of \( M. \) lysodeikticus cells in buffer were prepared in a 3-ml cuvette from stock solutions of cells and buffer at 30\°. A small volume of the inhibitor solution was then added, followed within 15 s by the addition of a small volume of a solution of Worthington lysozyme. The final volume was 3.0 ml; and the final concentrations of lysozyme and cells were 0.11 \( \mu \)g and 53 \( \mu \)g per ml, respectively. The decrease in absorbance at 450 nm with time due to lysis was followed using either a Zeiss PMQ II spectrophotometer or Gilford 240 spectrophotometer with recorder, both with cell compartments thermostatted at 30\°. The change in absorbance with time was linear for at least the period between 0.1 and 1 \( \mu \) min after initiation, and the rate of lysis was arbitrarily taken to be the slope of the plot of absorbance versus time for this period.

Fluorescence Measurements — Stock solutions of the Miles lysozyme in buffer were prepared, and the concentration of lysozyme was determined from the absorbance at 280 nm by use of the molar absorption coefficient of \( 3.8 \times 10^4 \) M\(^{-1}\) cm\(^{-1}\) (27). These solutions were diluted with the same buffer and passed through 0.45 \( \mu \)m Millipore filters in order to remove any light-scattering particles. The first 25 ml of filtrate were discarded because it contained some fluorescent substance from the filter. The stock solutions of the TACL-TACA mixture for the fluorescence measurements were prepared in 0.005 N HCl and left at room temperature for 20 hours in order to allow equilibration of the acid and lactone forms. The concentration of TACL in the equilibrated mixture was determined with the hydroxylamine-ferric chloride test. The stock solutions of TACA were prepared by hydrolyzing the TACA-TACL mixture with dilute NaOH and adjusting the hydrolysate to pH 7 with HCl. The following procedure was used in making the fluorescence measurements for determining the association constants. About 1.9 ml of lysozyme in buffer was temperature equilibrated in a 3-ml, 1-cm cuvette at 25.0 ± 0.1\°. When necessary, sufficient NaOH or HCl was added to the lysozyme-buffer solution so that the subsequent addition of the TACL-TACA mixture or of TACA would bring the pH back to that of the lysozyme in buffer alone. A small aliquot (0.10 ml or less) of a stock solution of TACL-TACA or of TACA was added to the lysozyme solution, and the fluorescence intensity was measured within 15 s. The recording fluorometer employs two Jarrell-Ash 3-micrometer monochromators, an EMI 9601B photomultiplier, and an Osram 150 watt high pressure Xenon lamp. The excitation and emission band widths were 7 nm. The exciting wave length was 280 nm, at which the absorbance of the solutions of lysozyme used varied between 0.005 and 0.024. The wave length at which the fluorescence intensity was recorded was chosen so that the intensity of the free enzyme fluorescence and the enzyme-inhibitor fluorescence differed markedly. This wave length varied with pH. The change in fluorescence was complete by the time of the first measurement, and the fluorescence was constant for at least 1 min (40 s at pH 7.84 due to lactone hydrolysis) thereafter. Before and after each such measurement the fluorescence of a standard solution of lysozyme was recorded. The small variations in the fluorescence which occurred during a series of measurements as the result of fluctuations in the intensity of the light source were minimized by normalizing all the intensities to a value for the lysozyme standard.

The procedure for obtaining the fluorescence spectrum of lysozyme and of its complexes was the same as that just described, with the exception that 1 to 2 min were required for recording the spectrum over the range between 290 and 400 nm. A small contribution of the buffer to the spectrum was subtracted from the spectrum. The inhibitors themselves exhibited no significant fluorescence. Normalization of the spectra against the fluorescence of a standard solution of lysozyme, whose fluorescence was monitored at intervals during the measure-
ment of each series of spectra, corrected for small changes in the lamp intensity.

A control experiment was carried out to test for any hydrolysis of the glycosidic linkage of TACL or TACA under the conditions of the fluorescence measurements. Aliquots of a reaction mixture at 30° that contained 0.54 mM TACL, 3.0 mM TACA, and 0.11 mM lysozyme in 0.01 M sodium citrate buffer, pH 5.8, at 0.2 M ionic strength with NaCl were analyzed for reducing sugar (21). The increase in the concentration of reducing sugar was less than 0.01 mm after 20 min, 0.10 mm after 50 min, and 0.45 mm after 80 min. Thus, the extent of hydrolysis was negligible during the fluorescence measurements. The rates of hydrolysis of the lactone function in the presence and absence of lysozyme (Table I) show that during the fluorescence measurements the extent of this reaction was also negligible.

Thin Layer Chromatography of Compounds and Reaction Mixtures—TACL, TACA, and the oligosaccharides of GlcNAc were chromatographed on Eastman chromagram cellulose sheets No. 6065 in the solvent system, pyridine-2-pentanol-water (1:1:1 by volume). The compounds were detected with a colorimetric test for the amide function as follows: spray with 0.25% NaOCl; dry; spray with 95% ethanol; dry; spray with 0.2% starch in 0.1 M KI. The limit of detection by this procedure is about 0.2 µg of di-N-acetylatedihiose. The RF values were 0.25 ± 0.1 for TACA, 0.5 ± 0.05 for tetra-N-acetylatedohexasaccharose, and 0.75 ± 0.1 for di-N-acetylatedihiose.

This chromatographic procedure was used to search for lysozyme-catalyzed transglycosylation (28) under the conditions of the fluorescence measurements. In the experiment with TACA, aliquots of a reaction mixture that contained 7.7 mM TACA and 0.53 mM lysozyme in 0.01 M sodium citrate buffer, pH 5.3, at 0.2 M ionic strength with NaCl were removed at time intervals and put into Na2CO3 buffer, pH 10.1, in order to stop the enzymatic reaction. One microliter (2.2 µg of TACA and 2.6 µg of lysozyme) of the quenched samples was chromatographed. The samples taken at 1 and 5 min of reaction showed strong spots at the origin (lysozyme) and at RF 0.25 (TACA) and a very faint spot at RF 0.48, which we have shown to arise from the lysozyme under these conditions. The five samples that were taken subsequently over the period from 14 to 93 min showed the development of elongation of the spot at RF 0.25 and a slight increase in the intensity of the spot at RF 0.48. In the experiment with TACL, TACA was first removed from the TACL-TACA mixture by quickly passing a freshly prepared TACL-TACA mixture (by colorimetric assay), 1.10 mM lysozyme, and 0.04 mM sodium acetate buffer, pH 5.0. Aliquots of the reaction mixture were quenched in Na2CO3 buffer, and samples that contained 4.2 µg of TACA and 32 µg of lysozyme were chromatographed. The samples that were taken at 1 and 3 min of reaction showed only a very large spot at the origin (lysozyme) and a spot at RF 0.2 (TACA).

The 12-, 19-, and 26-min samples showed the development of additional spots at RF 0.45 and 0.71. Samples after 37 and 47 min showed marked trailing with the spots at RF 0.2 (TACA) and RF 0.4 of approximately equal intensity. Thus, although

M. Raftery, personal communication.

RESULTS

Effect of TACL upon Rates of Cell Lysis by Lysozyme—The inhibition of lysozyme-catalyzed cell lysis in the presence of the TACL-TACA mixture, of TACA alone, and of tetra-N-acetylatedohexasaccharose are shown in Fig. 1. It can be seen that although it required 700 µM TACA and 80 µM tetra-N-acetylatedohexasaccharose for 50% inhibition, only 0.7 µM TACL (3.6 µM in the TACL-TACA mixture) was required. Similar values were obtained for the TACL-TACA mixture and the tetrasaccharide when the rates were measured in weaker buffer (0.01 M sodium phosphate, pH 6.2, at ionic strength 0.11 M with NaCl). A mixture of 2-acetamido-2-deoxy-d-glucose and its γ- and δ-lactones that contained 2.3 mM total lactone did not inhibit the rate of lysis in 0.07 M sodium phosphate buffer, pH 6.2, at ionic strength 0.11 M with NaCl.

Association Constants for Binding of TACA and TACL to Lysozyme—The formation of complexes between lysozyme and TACA or TACL is accompanied by substantial changes in the fluorescence spectrum of lysozyme (Fig. 2). The fluorescence intensity changes, at a constant wave length and concentration of lysozyme, produced by changes in the concentration of the inhibitor were used to obtain the association constants (13, 14).

Equations 1 and 2, which apply to the cases in which the fraction of inhibitor bound is small and large, respectively, were used to analyze the results with TACA and TACL, respectively. In Equation 1, F0, F0, and F are the fluorescence intensities of

![Fig. 1. Initial rates of lysozyme-catalyzed lysis of Micrococcus lysodeikticus in the presence of inhibitors. A mixture of TACA and TACL (X), TACA (O), tetra-N-acetylatedohexasaccharose (C). The experimental conditions are given under "Methods," except for the buffer which was 0.07 M sodium phosphate, pH 6.2, at ionic strength 0.11 M with NaCl. The TACA-TACL mixture contained 10% mole % TACL. The TACA was prepared by hydrolysis of this mixture in 0.1 M sodium phosphate, pH 6.0, for 4 hours. The rates of lysis are not corrected for a very slow rate (0.0040 per min) that occurs in the absence of lysozyme.](http://www.jbc.org/)

lysozyme catalyzes glycosyl transfer reactions of TACA and TACL, these reactions are sufficiently slow so that they do not interfere with the fluorescence and cell lysis measurements.
lysozyme in the absence of TACA, in the presence of saturating amounts of TACA, and in the presence of TACL at a total concentration, $[\text{TACL}]_0$; $K_A$ is the association constant for TACA binding. The symbols in Equation 2 are the same, with the exception that the subscript $L$ applies to TACL and $C_R$ is the total lysozyme concentration. In the case of TACL, the plots of $1/(F - F_E)$ against $1/[\text{TACL}]_0$ were linear, as expected from Equation 1; and the ratios of intercept to slope yielded the values of $K_A$ given in Table II.

\[
\frac{1}{F - F_E} = \frac{1}{F_E - F_E} + \frac{1}{[\text{TACL}]_0 (F_E - F_E)} \tag{1}
\]

\[
[\text{TACL}]_0 \left( \frac{F_E - F}{F_F - F_E} \right) = C_R \left( \frac{F_E - F}{F_F - F_E} \right) + \frac{1}{K_L} \tag{2}
\]

The determination of the association constants for the binding of TACL to lysozyme was complicated somewhat by the fact that TACL was added to the enzyme as part of an equilibrium mixture of TACL (14 mole %) with TACA (86 mole %). Preliminary experiments revealed that at pH values above 4.5 the concentrations of TACL alone that were required to cause fluorescence intensity changes similar to those observed with the TACL-TACA mixture were 30 times or more greater than the concentrations of free lysozyme, TACL-lysozyme complex, and TACL-TACA mixture. Consequently, at pH values above 4.5, the contribution of the TACL-lysozyme complex to the fluorescence changes that were observed upon addition of the TACL-TACA mixture to lysozyme are negligible. At these pH values the association constants for the formation of the TACL-lysozyme complex, $K_L$, were obtained by use of Equation 2. Three typical plots made according to this equation are given in Fig. 3. The values of $K_L$ that were obtained from the intercepts of such plots are presented in Table II.

Below pH 4.5 the binding of TACL to lysozyme is comparable to the binding of TACL, and significant amounts of both complexes form in the solutions of lysozyme, TACA, and TACL. By use of the following relationships (Equations 3 to 6), it was possible to separate the two binding constants, $K_A$ and $K_L$, from the data at low pH. In these equations, $f_C$, $C_{EL}$, and $C_{EA}$ are the concentrations of free lysozyme, TACL-lysozyme complex, and TACL-TACA complex, respectively; $f$ is the mole fraction of TACL in the inhibitor mixture (0.14); the $F$'s refer to the fluorescence of the various species; and $C_R^E$ and $C_R^T$ are the total concentrations of lysozyme and the TACL-TACA mixture, respectively.

\[
\frac{C_{EL}}{C_{EA}} = \frac{f_K A}{(1 - f)K_A} \tag{3}
\]

\[
F = \frac{C_E}{C_R^E} + F_{EL} \frac{C_{EL}}{C_R^T} + F_{EA} \frac{C_{EA}}{C_R^T} \tag{4}
\]

\[
K_{obs} = \frac{C_{EL} + C_{EA}}{C_E \cdot C_R^T} - K_L f + K_A (1 - f) \tag{5}
\]
FIG. 3. Determination of the association constants for the binding of TACL to lysozyme at pH 6.44 (○), 5.85 (○), and 4.95 (×). The experimental conditions are given in Table II.

Combination of Equations 3 to 5 yields the following

\[
\frac{1}{F - F_E} = \frac{\alpha}{\beta} + \frac{\alpha}{K_{a1}(\beta - aF_E)C_f}\]

where

\[\alpha = 1 + \frac{K_{a1}}{K_{a1}(1 - f)}\]
\[\beta = F_{EA} + F_{EL} \frac{K_{a1}}{K_{a1}(1 - f)}\]

In the derivation of Equation 6, the concentrations of uncomplexed TACL and TACA are taken to be equal to the total concentrations of these species. Sufficiently low lysozyme concentrations were used below pH 4.5 to allow this approximation (see Table II). The slopes of 1/(F - F_E) against 1/C_f were linear and yielded values of \( K_{obs} \). Values of \( K_L \) (see Table II) were calculated from the values of \( K_{obs} \) by use of Equation 5 and the values of \( K_A \) that were determined separately under the same conditions. A comparison of the pH dependence of the association constants of TACL, TACA, and tri-N-acetylchitotriose is presented in Fig. 4.

Fluorescence Spectra—Fluorescence spectra of lysozyme and of lysozyme in the presence of saturating concentrations of the TACL-TACA mixture, of TACA, and of tri-N-acetylchitotriose at pH values over the range from 2 to 7.9 were obtained. The wave lengths of maximum fluorescence intensity do not change markedly with pH: for lysozyme alone \( \lambda_{max} \) falls between 335 and 338 nm, and for all the lysozyme-inhibitor complexes \( \lambda_{max} \) is 325 to 328 nm. In order to separate the contribution of the fluorescence intensity of the TACL-lysozyme complex, the observed fluorescence intensities in the presence of the TACL-TACA mixtures were corrected for the contribution due to the TACA-lysozyme complex. Equations 3 and 4 yield Equation 7, which allows the fluorescence of the TACL-lysozyme complex, \( F_{EL} \), to be obtained from the fluorescence at saturation of lysozyme with the TACA-TACL mixture, \( F_S \), and the fluorescence of the TACA-lysozyme complex, \( F_{EA} \), which was measured separately.

FIG. 4. The dependence of the association constants for the binding of TACL (●, left ordinate), tri-N-acetylchitotriose (○, right ordinate) and TACA (□, right ordinate) upon pH, at 25° and 0.2 M ionic strength. The data for the trisaccharide is taken from Reference 13.

FIG. 5. Peak fluorescence intensity as a function of pH for lysozyme (X) and the complexes of lysozyme with TACL (●), TACA (△), and tri-N-acetylchitotriose (○). The reaction mixtures contained 0.98 mM lysozyme, 0.01 M buffer (these are given in Table II, sodium citrate between pH 6.2 and 6.5), NaCl to ionic strength 0.2 M, and, where present, either 1 mM tri-N-acetylchitotriose, 0.4 mM (pH 2.1 and 4.9) or 0.8 mM (pH 3.4-5.5) TACA, or 0.077 mM (pH 4.4 to 7.9), 0.14 mM (pH 3.9), or 0.38 mM (pH 2.1 to 3.1) TACL-TACA mixture. Temperature, 25°. The concentrations of the inhibitors are enough to convert 90% or more of lysozyme to the corresponding complex (Table II and Reference 29). See the text for the description of the calculation of the intensities of the TACL complex.
At pH 3.05 the fraction of lysozyme complexed with TACL and the fraction of $F_B$ due to this complex are maximal and have values of 0.38 and 0.41, respectively.

The pH dependence of the peak fluorescence intensities are presented in Fig. 3. The data for lysozyme and the tri-N-acetyltetrasaccharide-lysozyme complex are in good agreement with earlier studies (13, 29). Although the pH dependence of the fluorescence of the TACL complex is very similar to that of the triasaccharide complex, the fluorescence-pH profile of the TACL complex is shifted in the acid direction by about 1.7 pH units. Thus, the ionizable group that quenches the tryptophyl fluorescence in lysozyme-inhibitor complexes has a considerably lower pKₐ in the TACL complex.

DISCUSSION

Mode of Binding of TACL to Lysozyme—If TACL is a transition state analog for lysozyme, its four rings must bind in Subsites A through D in the cleft. The tryptophyl fluorescence changes reported here suggest this mode of binding. It has been shown that tri-N-acetyltetraose, in the crystal (30) and in solution (31), binds predominantly in Subsite A through C and interacts with tryptophanes 62, 63, and 108 through hydrogen and hydrophobic bonds. The relative values of the association constants for a number of oligosaccharides (14) and limited nuclear magnetic resonance information (31) suggest that this binding register is also the predominant one for tetra-N-acetyltetrasaccharose. Previous fluorescence studies have indicated that the blue shift in the fluorescence spectrum that is produced by binding of the GlcNAc trisaccharide is due to a change in the average tryptophyl environment to a less aqueous one (13, 32).

From the pH dependence of the peak fluorescence of the lysozyme-trisaccharide complex, it was suggested (13, 29) and verified (32) that a carboxyl group with a pKₐ of about 6.3 in the complex (probably glutamate 35) interacts with tryptophan 108 to quench its fluorescence. The present fluorescence results with the TACL-lysozyme complex show that the fluorescence intensity and the spectrum of the TACL and tri-N-acetyltetrasaccharose complexes are very similar at pH 2.0 to 3.0 and at pH 7.5 to 8.0 (same state of ionization of lysozyme), indicating similar interactions for both inhibitors at Subsites A through C. On the other hand, the different fluorescence pH dependence of the TACL-lysozyme complex compared to that of the triasaccharide-lysozyme complex, which is presumably due to the altered pKₐ of glutamate 35 (see Fig. 5 and below), is most easily explained by the additional binding of the lactone ring in Subsite D, which is adjacent to glutamate 35 (30).

The relative values of the association constants for the binding of TACL and tetra-N-acetyltetrasaccharose to lysozyme provide further evidence that TACL binds in the A through D mode. In agreement with the prediction that a transition state analog should bind much more tightly than the corresponding substrate (4–6), the association constant for TACL is 32 times greater than that for the unmodified tetrasaccharide at pH 5 (Table III). This relative strength of binding is also shown by the fact that TACL is 110 times more potent than the tetrasaccharide as an inhibitor of lysozyme-catalyzed cell lysis. Although definitive proof of the binding mode for TACL may have to await the results of crystallography, we tentatively conclude that TACL binds in Subsites A through D.

pH Dependence of Fluorescence and Association Constants—The increase in the association constant for TACL over the pH range from 2 to 6.2 and its constancy between pH 6.5 and 8.0 (Fig. 4) show that TACL binds more strongly to the species of the enzyme in which glutamate 35 (pKₐ 5.9 in the free enzyme, see below) is ionized than to the species in which it is protonated. Conversely, the pKₐ of glutamate 35 must be lower in the complex than in the free enzyme. In fact, this lowered pKₐ is evident in the pH dependence of the fluorescence intensity of the complex (Fig. 5), which suggests a pKₐ of about 4.7 for glutamate 35 in the complex. In contrast, tri-N-acetyltetrasaccharose binds less tightly to the enzyme in which glutamate 35 is ionized and the pKₐ of glutamate 35 in the complex is greater (Figs. 4 and 5). It is not possible to decide with confidence from the pH profile for $K_L$ whether the state of ionization of aspartate 52 (pKₐ 4.5 in the free enzyme, see below) also affects $K_L$.

We have attempted to analyze quantitatively the pH dependence of the fluorescence intensity of the TACL-lysozyme complex (Fig. 5) and the pH dependence of the association constant for its formation (Fig. 4) by adapting the equations that describe the effect of the ionization of a single acid group of the enzyme (33, 34) to these data. In such an analysis the pH dependence of the fluorescence yields values of the acid dissociation constant for the complex, and the pH dependence of the association constant yields values of the ionization constant of the acid group for both the complex and the free enzyme. We have found that in both cases the values of the acid ionization constants that were so obtained are not constant, but decrease by a factor of 3 to 4 as the pH increases. Consequently, the ionization of a single group of the enzyme does not adequately account for the pH dependencies. This conclusion is not surprising, since crystallography has shown that there are four dissociable groups in the region of Subsites A through D. These groups are aspartate 101 and 103, which may interact with the inhibitor at Subsites A and B, and glutamate 35 and aspartate 52, which may interact with the inhibitor at Subsite D (30).

| Oligosaccharide and binding mode | Association constant ($K_L$) |
|---------------------------------|-----------------------------|
| GlcNAc                          | $1.05 \times 10^6$          |
| GlcNAc                          | $2.8 \times 10^6$           |
| GlcNAc                          | $2.1 \times 10^6$           |
| GlcNAc                          | $2 \times 10^6$             |
| GlcNAc                          | $9 \times 10^5$             |
| GlcNAc                          | $12 \times 10^5$            |

* At 25°, 0.1 M ionic strength, pH 5.0, from Reference 39.

$^b$ At 25° and pH 5.4, from Reference 14.

$^c$ At 25° and pH 5.5, from Reference 13.

$^d$ Estimated, as described in the text.

$^e$ At pH 6.2 to 7.8, from Table II.
values of aspartates 101 and 103 have not been determined. A better fit of the pH dependencies of the fluorescence intensity and association constant can be made if one assumes the involvement of two ionizable groups. However, the data are not sufficiently extensive to warrant the inclusion of this analysis here.

It is interesting to note that in the transition state of lysozyme-catalyzed glycosyl transfer the carboxyl group of glutamate 35 is probably largely ionized due to almost complete proton transfer to the RO moiety that is leaving or attacking the glycosidic carbon atom (see the introduction). Thus, glutamate 35 is ionized in both the proposed transition state and in the tightest complex with TACL, although TACL lacks the positive charge that the alkoxy carbocation ion possesses. Crystallography of the TACL-lysozyme complex may reveal whether the extent of the ionization of glutamate 35 upon $K_L$ is due to a direct interaction between the carboxylate anion and the lactone function or due to the strengthening of other interactions between TACL and the protein, perhaps as the result of a change in conformation of the protein.

**Role of Tight Binding of Half-Chair Conformation in Lysozyme Catalysis**—The binding of a pyranose ring in Subsite D appears to be accompanied by distortion of the pyranose ring from its normal chair conformation (30) toward the half-chair conformation which is expected for the transition state (14, 30). The distortion occurs to prevent an unfavorable steric interaction between the 6 CH₂OH group of the pyranose ring and the enzyme without destroying the other favorable interactions between enzyme and substrate (30). Our results with TACL, which does not need to be distorted to bind in Subsite D, can be used to estimate how the tighter binding of the half-chair conformation contributes to catalysis.

We will assume that the most favorable conformations of the lactone ring of TACL and of the pyranose ring in the transition state are identical and that the strong binding of TACL to lysozyme is largely due to the half-chair conformation of the lactone ring rather than to a specific strong interaction between the lactone function itself and the protein. If these assumptions are correct, then the ratio of the binding constant for TACL to that for tetra-$N$-acetylchitotetraose bound in the A through D mode is an estimate of the factor by which the half-chair conformation of the transition state allows more favorable interactions with the enzyme than does the chair conformation of the substrate. This factor is equivalent to a factor in catalysis, since it represents a stabilization of the transition state relative to the substrate that does not occur in the corresponding nonenzymatic reaction.

Although tetra-$N$-acetylchitotetraose does not bind predominantly in Subsites A through D, an estimate of this ratio can be obtained from other data. It is known that the interaction of an MurNAc residue or its methyl ester with Subsite D at pH 5.5 reduces the binding constant for association of the tetrasaccharide GlcNAc-MurNAc-GlcNAc-MurNAc with lysozyme by a factor of 130, relative to that for formation of the complex with the trisaccharide, GlcNAc-MurNAc-GlcNAc (Table III). Since the hexasaccharides of GlcNAc and of GlcNAc-MurNAc, which bind 80% or more in the A through F mode, have approximately the same association constants and are cleaved at the same rate in the complex (7, 28, 37, 38), it seems likely that the lactyl side chain of a MurNAc residue bound in Subsite D does not interact strongly with the enzyme. In the crystal structure that has been proposed for the complex with the GlcNAc-MurNAc hexasaccharide, the lactyl side chain in Subsite D is directed away from the enzyme (30). Consequently, it seems probable that the introduction of a GlcNAc residue into Subsite D is also destabilizing by a factor of about 100, and thus we estimate on the basis of the association constant for tri-$N$-acetylchitotetraose at pH 5.5 (Table III) that the association constant for the tetra-$N$-acetylchitotetraose bound in the A through D mode is $2 \times 10^4$ M⁻¹. This association constant is smaller than the maximal value of the association constant for the binding of TACL (Table III) by a factor of $6 \times 10^4$. We take this factor to be an estimate of the contribution to catalysis that can be assigned to tighter binding of the transition state due to its half-chair conformation.

**REFERENCES**

1. Sekemski, I. I., and Lienhard, G. E. (1971) J. Amer. Chem. Soc. 93, 3549
2. Jencks, W. P. (1969) in *Catalysis in Chemistry and Enzymology* McGraw-Hill Book Co., New York
3. Koszland, D. E., Jr., and Netter, K. E. (1968) *Ann. Rev. Biochem.* 37, 359
4. Pauling, L. (1948) *Amer. Sci.* 36, 51
5. Wolfenden, R. (1972) *Acta. Chem. Res.* 5, 10
6. Lienhard, G. E., Sekemski, I. I., Kohler, K. A., and Lindquist, R. N. (1971) *Cold Spring Harbor Symp. Quant. Biol.* 36, 45
7. Johnson, L. N., Phillips, D. C., and Rupley, J. A. (1968) *Brookhaven Symp. Biol.* 31, 129
8. Rupley, J. A., Gates, V., and Bilhemei, R. (1968) *J. Amer. Chem. Soc.* 90, 5033
9. Rand-Meir, T., Dahlquist, F. W., and Raftery, M. A. (1969) *Biochemistry* 8, 4296
10. Dahlquist, F. W., Rand-Meir, T., and Raftery, M. A. (1969) *Biochemistry* 8, 4214
11. Ohl, G. A., and Sommer, J. (1968) *J. Amer. Chem. Soc.* 90, 4323
12. Hauck, M. L., and Jacobson, R. A. (1971) *Acta Crystallogr. Sect. B* 27, 263
13. Lehner, S. S., and Fasman, G. D. (1966) *Biochem. Biophys. Res. Commun.* 23, 133–138
14. Chipman, D. M., Grezara, V., and Sharon, N. (1967) *J. Biol. Chem.* 242, 4388–4394
15. Leiback, D. H. (1968) *Biochem. Biophys. Res. Commun.* 32, 1025
16. Tu, J-J., Jacobson, G. R., and Graves, D. J. (1971) *Biochemistry* 10, 1229
17. Reese, E. T., Parrish, F. W., and Ettlinger, M. (1971) *Carbohydr. Res.* 15, 381
18. Gold, A. M., Legrand, E., and Sánchez, G. R. (1971) *J. Biol. Chem.* 246, 5700–5706
19. Jeanloe, R. W., and Forchielli, E. (1951) *J. Biol. Chem.* 185, 561
20. Rupley, M. A., Rand-Meir, T., Dahlquist, F. W., Parsons, S. M., Borders, C. L., Jr., Wolcott, R. G., Beranek, W. Jr., and Jiao, L. (1969) *Anal. Biochem.* 30, 427
21. Park, J. T., and Johnson, M. J. (1949) *J. Biol. Chem.* 181, 149
22. Rupley, M. A., Rand-Meir, T., Dahlquist, F. W., Parsons, S. M., Borders, C. L., Jr., Wolcott, R. G., Beranek, W. Jr., and Jiao, L. (1969) *Anal. Biochem.* 30, 427
23. Rupley, J. A. (1964) *Biochim. Biophys. Acta* 35, 245–255
24. Corin, G., Wang, S.-F., and Papavlis, L. (1971) *Anal. Biochem.* 22, 305–306
25. Goodman, A. J., Rhodes, C. K., Holcomb, D. N., and Van Holde, K. E. (1962) *J. Biol. Chem.* 237, 1107–1112
26. Chipman, D. M., Pollock, J. J., and Sharon, N. (1968) *J. Biol. Chem.* 243, 487–490
29. Lehrer, S. S., and Pasman, G. D. (1967) J. Biol. Chem. 242, 4644–4651
30. Blake, C. C. F., Johnson, L. N., Mahr, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R. (1967) Proc. Roy. Soc. Ser. B Biol. Sci. 167, 378
31. Dahlquist, F. W., and Raftery, M. A. (1969) Biochemistry 8, 713
32. Teichberg, V. I., and Sharon, N. (1970) Fed. Eur. Biochem. Soc. Lett. 7, 171
33. Gold, V. (1956) in pH Measurements p. 90, John Wiley and Sons, New York
34. Dixon, M., and Webb, E. C. (1964) in Enzymes p. 135, Academic Press, New York
35. Parson, S. M., and Raftery, M. A. (1970) Biochem. Biophys. Res. Commun. 41, 45–49
36. Angyal, S. J. (1969) Angew. Chem. Int. Ed. Engl. 8, 157
37. Rossi, G. L., Holler, E., Kumar, S., Rupley, J. A., and Hess, G. P. (1969) Biochem. Biophys. Res. Commun. 31, 757–766
38. Chipman, D. M., and Sharon, N. (1969) Science 165, 454–455
39. Dahlquist, F. W., Jao, L., and Raftery, M. (1966) Proc. Nat. Acad. Sci. U. S. A. 56, 26
A Transition State Analog for Lysozyme
Isaac I. Secemski, Sherwin S. Lehrer and Gustav E. Lienhard

J. Biol. Chem. 1972, 247:4740-4748.

Access the most updated version of this article at http://www.jbc.org/content/247/15/4740

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/247/15/4740.full.html#ref-list-1