Oxindolealanine-62 Lysozyme: Equilibrium, Calorimetric, and Kinetic Studies of the Reaction with *N*-Acetylglucosamine Oligosaccharides†

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ABSTRACT: Oxindolealanine-62 lysozyme, formed by reaction with *N*-bromosuccinimide and purified by using affinity chromatography, was examined in its binding of homologous oligosaccharides of *N*-acetylglucosamine and in its catalysis of hydrolysis and of transglycosylation of the hexasaccharide of *N*-acetylglucosamine. Equilibrium binding constants were determined by changes in absorbance or fluorescence associated with ligand binding. Enthalpies of binding were measured calorimetrically. The pattern of variation of $\Delta G°$ and $\Delta H°$ of binding with ligand chain length and pH was different for oxindolealanine-62 lysozyme compared with native lysozyme. These results indicate that the interactions of the ABC region of the active site with substrates are substantially altered by Trp-62 oxidation, more than expected for loss only of the Trp-62 interactions. The partitioning of the glycosyl enzyme intermediate between reaction with a saccharide acceptor (transglycosylation) and reaction with water is unaffected by oxidation of Trp-62. Similarly, the pattern of cleavage of the hexasaccharide, predominantly to tetra- and disaccharide, is unaffected by oxidation of Trp-62. The 2000-fold slower rate of catalytic reaction (relative to free enzyme and substrate) apparently reflects a sterically hindered fit of the substrate into the active site of the modified enzyme and not a special catalytic importance of Trp-62. The geometry of the transition state for oligosaccharide hydrolysis is inferred to be the same for native and oxidized enzymes.

Because of the considerable body of information on the properties of hen egg white lysozyme in the crystal and in solution (Imoto et al., 1972), studies aimed at elucidating details of the enzymatic mechanism are of particular interest. Tryptophan residue 62 is on the surface of the protein (Shrake & Rupley, 1973), at the edge of the active-site cleft. *N*-Bromosuccinimide specifically oxidizes residue 62 to oxindolealanine (Hayashi et al., 1965) at low molar ratios of oxidant to protein. The side chain of residue 62 does not participate in intramolecular interactions (Blake et al., 1967), and thus its oxidation is not expected to perturb the protein structure. This expectation has been confirmed by comparison of the solution properties of the native and oxindolealanine-62 proteins. Takahashi et al. (1965) and Tanaka et al. (1975) investigated the circular dichroic properties of native lysozyme and oxidized derivatives and concluded that Oxa-62[1] lysozyme has essentially the same conformation as intact lysozyme. Glickson et al. (1971), using ^{1}H NMR, and Norton & Allerhand (1976), using ^{13}C NMR, came to the same conclusion; only the resonances of the ^{1}H and ^{13}C nuclei of Trp-62 and the adjacent residue Trp-63 were modified on oxidation.

Various studies of Oxa-62 lysozyme have been carried out with the intent of using this derivative to probe the chemistry of the active site. Analyses of the optical properties of native lysozyme and its saccharide complexes have relied on comparisons of the Oxa-62 and Oxa-108 derivatives with the native enzyme [fluorescence, Formoso & Forster (1975); CD, Tanaka et al. (1975); absorbance, Imoto et al. (1975)]. Oxa-62 lysozyme does not undergo self-association (Banerjee et al., 1975b), and its formation of hybrids with the native enzyme served to establish the mechanism of self-association. Cooper (1974) measured $\Delta G°$ and $\Delta H°$ for binding of the α and β anomers of GlcNAc and the mutarotated mixtures to the Oxa-62 derivative. Imoto et al. (1974) compared the enzymatic activities of Oxa-62 and native enzyme toward glycol chitin and bacterial cell wall and inferred that Trp-62 participates not only in the binding of substrate but also more directly in the catalytic process by straining or aligning the substrate.

The aim of the studies described here is to examine the catalysis and substrate binding properties of Oxa-62 lysozyme, in order to evaluate the propriety of using this derivative in comparisons aimed at understanding the properties of the native enzyme. A particularly important point is the description of the participation of residue 62 in the catalytic events. In this regard, Trp-62 and adjacent elements of the active site move slightly when substrates bind (Blake et al., 1967).

Experimental Section

Materials. Twice-crystallized hen egg white lysozyme (Lot No. LY9FA and LY0EA) and *Micrococcus lysodeikticus* cell wall were purchased from Worthington. The GlcNAc oligosaccharides were prepared from chitin as described previously (Rupley, 1964). Some oligosaccharide preparations contained small amounts of impurities that absorbed in the near-UV and were fluorescent. Chromatography using Dowex 50 W-X2 (Holler et al., 1975) removed these impurities. β-Me-GlcNAc was prepared in this laboratory by Dr. A. Pogolotti.

Preparation of Oxa-62 Lysozyme. Trp-62 of lysozyme is selectively oxidized by SucNBr (Hayashi et al., 1965). We confirmed that titration of the fluorescence of lysozyme with SucNBr is linear and biphasic with a break at about unit mole ratio SucNBr/enzyme. In a typical reaction, 3.10 mL of 6.83 $\times 10^{-3}$ M SucNBr (0.90 mol/mol of protein) was added slowly with stirring to 55 mL of 4.29 $\times 10^{-4}$ M lysozyme[2] in 0.1 M sodium acetate–HCl buffer of pH 5.3, which had been cooled to 0 °C. Less than unit mole ratio SucNBr/protein was chosen

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[1] Abbreviations used: Oxa, oxindolealanine; $(GlcNAc)_n$, β1→4-linked *n*-mer of *N*-acetyl-D-glucosamine; β-Me-GlcNAc, β-methyl-*N*-acetyl-D-glucosaminide; SucNBr, *N*-bromosuccinimide.

[2] Protein concentrations were determined spectrophotometrically by using values of 25.5 (Imoto et al., 1973) and 22.7 (Imoto & Rupley, 1973) for $E_{280}^{1\%}$ of native and Oxa-62 lysozymes, respectively.

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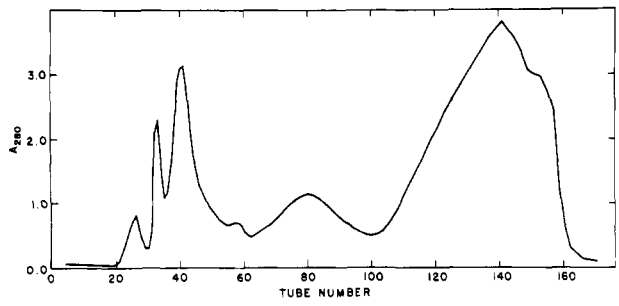

FIGURE 1: Chromatographic separation of lysozyme reacted with *N*-bromosuccinimide. Product (~270 mg) was applied to a 1.5 × 76 cm Bio-Rex 70 column equilibrated with 0.05 M $Na_2B_4O_7$ buffer of pH 10.0 and was eluted with a linear gradient over 600 mL of total volume to 0.04 M NaCl in the same buffer. The flow rate was 0.40 mL/min. The fraction volume was 2.4 mL. The curve gives A_{280} of the effluent.

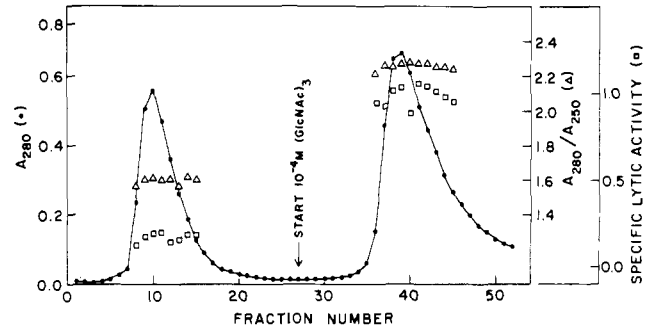

FIGURE 2: Affinity chromatographic separation of the last eluting fraction from Bio-Rex 70 chromatography. Protein (10 mg) was applied to a 1 × 15 cm $(GlcNAc)_3$–Sepharose affinity column, equilibrated with 0.1 M ammonium acetate buffer of pH 7.0. Oxa-62 lysozyme eluted with this buffer. Native lysozyme was eluted with buffer containing ~10^{-4} M $(GlcNAc)_3$. The solid curve (●) gives A_{280} of the effluent. Activity (□) is reported as the fraction of the specific lytic activity of native lysozyme. The 280 nm/250 nm absorbance ratio (△) was determined at pH 7.0.

in order to minimize side reactions. After reaction for 30 min at 0 °C with stirring, pH was brought to 10 with NaOH. This solution was condensed to ~10 mL by ultrafiltration and applied to a 1.5 × 76 cm column of Bio-Rex 70 equilibrated with 0.05 M $Na_2B_4O_7$ buffer at pH 10.0. Elution was with a linear gradient over 600 mL of total volume from the equilibration buffer to a solution of 0.05 M $Na_2B_4O_7$ and 0.04 M NaCl of pH 10.0. Figure 1 gives a typical elution pattern. The Oxa-62 lysozyme and native lysozyme peaks eluted last, the latter appearing as a trailing shoulder. The fractions that contained Oxa-62 lysozyme (tubes 102–159 of Figure 1) were combined immediately after collection and adjusted to pH 7 by using glacial acetic acid. This solution was condensed to ~5 mL by ultrafiltration, clarified by centrifugation, and applied to a 1.6 × 27 cm $(GlcNAc)_3$–Sepharose affinity column (Cornelius et al., 1974), which had been equilibrated with 0.1 M ammonium acetate buffer of pH 7.0. Elution was with the same buffer. Figure 2 illustrates the separation of Oxa-62 and native lysozymes on an affinity column. The native protein (tubes 36–50 of Figure 2) is bound more tightly to the resin than Oxa-62 lysozyme (tubes 8–15 of Figure 2). Oxa-62 lysozyme fractions were condensed and desalted by ultrafiltration, clarified by centrifugation, and lyophilized to give 135 mg of protein.

The following results indicate that the Oxa-62 protein as isolated above is homogeneous. The ratios (A_{280}/A_{250}) for the Oxa-62 and native lysozyme fractions from the affinity separation were constant through each peak (values of 1.60 and 2.25, respectively; Figure 2). The specific lytic activity (Rupley, 1967) was also constant through each peak (with values of 18 and 100%, respectively, relative to the specific activity of untreated lysozyme; Figure 2). Disc gel electrophoresis at pH 4.5 showed a single band for Oxa-62 lysozyme, which moved similarly to the native enzyme. Comparison of tryptic digests of Oxa-62 and native lysozymes, using gel filtration (Hartdegen & Rupley, 1973), showed that complete conversion of Trp-62 to Oxa-62 had occurred.

Norton & Allerhand (1976) have shown that SucNBr oxidation of lysozyme in acetate buffer produces an acetylated Trp-62 derivative (δ_1-acetoxytryptophan), which converts slowly to the stable oxindolealanine product. The method of preparation used in this work gives the oxindolealanine derivative, as expected, owing to the long period of time required for purification and as demonstrated by the UV spectrum of the product.

pH. pH was measured at room temperature by using a Beckman Model 76 instrument, standardized at pH 4.01 and pH 6.50. pH was controlled by using 0.1 M sodium acetate–HCl buffer of ionic strength 0.1 for pH 5.3 or a solution of 0.01 M HCl and 0.09 M NaCl for pH 2.3.

Absorbance Difference Spectra. Absorbance differences resulting from binding of saccharide were measured with a Cary Model 15 spectrophotometer equipped with a thermostated cell holder. Two matched and stoppered cylindrical cells of 1-cm path length were used for all UV difference spectrum measurements, except those for the binding of β-Me-GlcNAc, for which rectangular cells of 1-mm path length were used in order to conserve this weakly binding ligand. The protein concentration was typically 6 × 10^{-5} M; it was 10-fold more concentrated for measurements with β-Me-GlcNAc. Spectra were corrected for any absorbance of substrate. Whenever possible, the range of saturation covered in an experiment was 20–90%. The measurements for binding of $(GlcNAc)_6$ to Oxa-62 lysozyme at pH 2.3 were made within 15 min of the addition of enzyme to the substrate solution; thus, no correction for enzymatic cleavage of $(GlcNAc)_6$ was required. Difference spectra obtained with β-Me-GlcNAc required correction for nonspecific perturbation by ligand (Banerjee et al., 1974), owing to the high concentration of β-Me-GlcNAc necessary for obtaining high saturation.

Fluorescence. Fluorescence measurements were made with an Aminco-Bowman spectrofluorometer equipped with a thermostated cell holder and a Sargent Model SR recorder. Excitation was at 280 or 285 nm [the latter for measurements with $(GlcNAc)_2$]. Fluorescence change at 330 nm was used for determination of binding parameters. Small corrections were applied as needed for absorbance and fluorescence of saccharide. Protein concentrations were typically 6 × 10^{-6} M, and the range of saturation was 20–90%. Fluorescence measurements for binding of $(GlcNAc)_6$ to Oxa-62 lysozyme at pH 5.3 were performed within 10 min of mixing enzyme and substrate solutions.

Equilibrium Constants. Association constants were obtained through fitting the Scatchard equation to the absorbance and relative fluorescence data by using a nonlinear least-squares fitting procedure (Williamson, 1968), in which the experimental points were weighted according to the estimated error in both ΔX and $\Delta X/(S)$ [ΔX is the change in F or A, and (S) is the free substrate concentration]. We assume an error of ±0.002 in ΔA, an error of ±2 units in ΔF and no error in substrate concentration.

Heats of Binding. Heats of binding of saccharides to Oxa-62 lysozyme were measured at 30.0 ± 0.1 °C by using an LKB batch microcalorimeter monitored by a Sargent Model SRB recorder equipped with an integrator. The ex-

Table I: Binding of Saccharides to Oxa-62 Lysozyme at Ionic Strength 0.1[a]

ligand	pH	temp (°C)	log K^{assoc}	K^{assoc} (M^{-1})[b]	ΔX_{max}[b,c]	$-\Delta H°$[d] (kcal/mol)
β-Me-GlcNAc	5.3	30	0.48	3	1290	
$(GlcNAc)_2$	5.3	29.5	3.10	1300	(55)	7.4
$(GlcNAc)_3$	5.3	30	2.88	770	1690	5.4
$(GlcNAc)_3$	5.3	38.2	2.78	600	(45)	
$(GlcNAc)_3$	2.3	30	2.90	800	870	3.6
$(GlcNAc)_4$	5.3	29.5	3.62	4100	(40)	3.6
$(GlcNAc)_6$	5.3	11.1	4.00	9900	(15)	
$(GlcNAc)_6$	5.3	24.8	3.73	5400	(30)	
$(GlcNAc)_6$	5.3	38.2	3.53	3400	(45)	
$(GlcNAc)_6$	2.3	17.5	4.09	12000	810	
$(GlcNAc)_6$	2.3					1.6

[a] Measured in 0.1 M sodium acetate–HCl buffer solution of pH 5.3 or in 0.01 M HCl with 0.09 M NaCl. [b] Measured by UV difference spectra or by fluorescence changes; nonparenthetical and parenthetical ΔX_{max} values indicate former and latter methods, respectively. [c] Value of change in absorbance, $\Delta\epsilon_{max}$, or in fluorescence, ΔF_{max} (in parentheses), for saturating Oxa-62 lysozyme with ligand. $\Delta\epsilon_{max}$ in M^{-1} cm^{-1} was measured as 294–290 nm peak – trough differences and ΔF for ~6×10^{-6} M enzyme was measured at 330 nm with excitation at 280 nm [at 285 nm for $(GlcNAc)_2$]. [d] Measured calorimetrically at 30 °C.

perimental procedures have been described by Banerjee et al. (1974).

Kinetic Measurements. The kinetic parameters for enzymatic cleavage of $(GlcNAc)_6$ were obtained by using chromatographic analyses of the products of reaction of lysozyme with $(GlcNAc)_6$ in the presence of excess GlcNAc (Banerjee et al., 1973). Data were corrected for competitive inhibition by GlcNAc, which was important for Oxa-62 lysozyme but not for native lysozyme, due to the weak binding of substrate by the former.

The dependence of the transglycosylation reaction on acceptor concentration can be used to characterize the interaction between acceptor and glycosyl enzyme. The following mechanism describes the competition between water and an acceptor such as GlcNAc:

$$E + S \rightarrow\rightarrow\rightarrow P_1 + EP_2' \xrightarrow{H_2O,\ k_{H_2O}} E + P_2 \qquad EP_2' \xrightarrow{A,\ k_A} E + P_2\text{–}A \qquad (1)$$

The rate-determining step in lysozyme-catalyzed reactions of oligosaccharides occurs prior to the formation of the glycosyl-enzyme intermediate EP_2'. Reaction of $(GlcNAc)_6$ has been shown (Rupley, 1967) to give predominantly tetrasaccharide as P_2 and disaccharide as P_1.

The fraction of the cleavages that results in transfer can be expressed as

$$\frac{(P_2\text{–}A)}{(P_2) + (P_2\text{–}A)} = \frac{K^*_A(A)}{1 + K_A^*(A)} \qquad (2)$$

where

$$K^*_A = \frac{k_A}{k_{H_2O}(H_2O)} \qquad (3)$$

The dependence of the extent of transglycosylation on acceptor concentration defines the value of K^*_A. In the present experiments the acceptor A was GlcNAc and the products P_2–A and P_2 were $(GlcNAc)_5$ and $(GlcNAc)_4$, respectively.

The constant K^*_A is an apparent equilibrium constant for the interconversion of the transition states for hydrolysis and glycosyl transfer:

$$[EP_2'\cdots H_2O]^\ddagger + A \rightleftharpoons [EP_2'\cdots A]^\ddagger \qquad (3a)$$

Furthermore, the rate constant k_A (or k_{H_2O}) can be viewed as the product of an equilibrium constant for binding of acceptor (or water) to EP_2' and a rate constant for reaction of the glycosyl enzyme-bound acceptor (or water) to give the product P_2–A (or P_2). Because the rate constants for reaction of alcohols and for reaction of water with the glycosyl enzyme are the same (Rupley et al., 1968), K^*_A can be viewed also as an apparent equilibrium constant for binding of acceptor to EP_2'. This formalism has been used to compare various compounds in binding to the EF region[3] of the lysozyme active site (Imoto et al., 1972).

Results

Absorbance and Fluorescence Changes Associated with Saccharide Binding. The UV difference spectrum measured for binding $(GlcNAc)_3$ to Oxa-62 lysozyme at pH 5.3 is given in Figure 3. Difference spectra measured for β-Me-GlcNAc at pH 5.3 and for $(GlcNAc)_3$ and $(GlcNAc)_6$ at pH 2.3 were similar in shape although different in intensity. There are positive extrema at 294 and 286 nm, a shoulder at 297 nm, and a trough at 290 nm. These features are similar to those present in saccharide binding difference spectra of the native protein. The absorbance properties of lysozyme have been analyzed (Imoto et al., 1975).

Table I gives the changes in extinction ($\Delta\epsilon_{max}$) associated with saturation of the Oxa-62 enzyme with various ligands. Change in oligosaccharide size (comparison of monomer, trimer, and hexamer) similarly affects $\Delta\epsilon_{max}$ for Oxa-62 lysozyme and native lysozyme (Imoto et al., 1975).

The fluorescence emission maxima for the complexes of enzyme with all saccharides studied in this work are at 340 nm at pH 5.3, compared with the emission maximum of 345 nm for the unliganded Oxa-62 protein. The quantum yields for the complexes were greater than that for the free Oxa-62 enzyme. These results are in agreement with previous observations (Imoto et al., 1972).

Figure 4 gives the fluorescence changes at 330 nm measured for binding of $(GlcNAc)_3$ and $(GlcNAc)_6$ at pH 5.3 and 38.2 °C. Table I gives the relative fluorescence changes at enzyme

[3] The six regions of the enzyme active site interacting with monosaccharide elements of $(GlcNAc)_6$ have been designated A–F, in order from the nonreducing end of the substrate (Imoto et al., 1972).

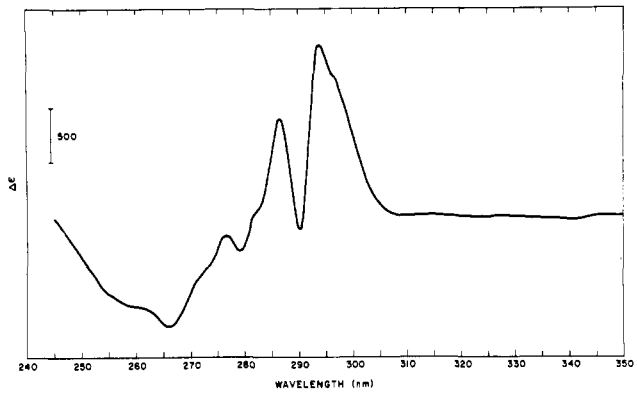

FIGURE 3: Difference spectrum for $(GlcNAc)_3$ binding at saturation to Oxa-62 lysozyme at 30 °C, pH 5.3, and 0.1 ionic strength; $\Delta\epsilon$ in M^{-1} cm^{-1}.

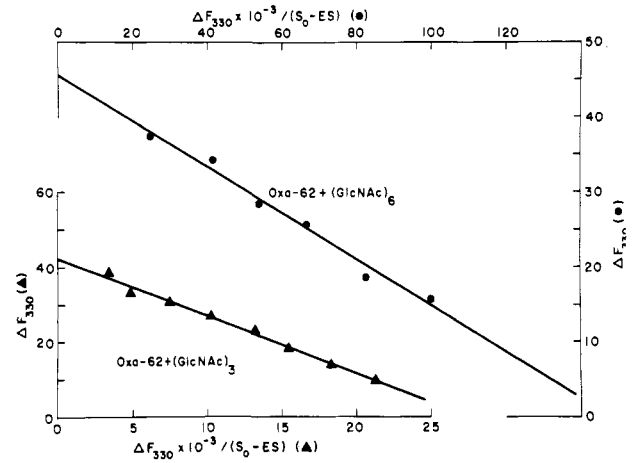

FIGURE 4: Scatchard plots of relative fluorescence changes for binding $(GlcNAc)_3$ and $(GlcNAc)_6$ to Oxa-62 lysozyme (6.5×10^{-6} M enzyme) at 38.2 °C, pH 5.3, and 0.1 ionic strength. Excitation was at 280 nm and emission was measured at 330 nm.

saturation (ΔF_{max}) for various saccharides and reaction conditions.

Equilibrium and Calorimetric Results. Table I gives association constants determined from the dependence of the absorbance or fluorescence of Oxa-62 lysozyme on saccharide concentration. Table I also gives heats of binding determined calorimetrically. These heats were measured in buffers with negligible heats of protonation and therefore need not be corrected for any proton uptake or release. The reaction of $(GlcNAc)_6$ at pH 5.3 had to be examined by van't Hoff analysis (Figure 5), owing to enzymatic hydrolysis of the substrate. The pair of values measured at 11.1 °C agree poorly, due to the large experimental error in K^{assoc} associated with the small values of ΔF found for these conditions (Table I).

The thermodynamic values of Table II were calculated by using the values for K^{assoc} and $\Delta H°$ given in Table I and Figure 5. These values are compared with those for native lysozyme in Figure 6. The following are the most significant relationships among these results. (a) The dependence of $\Delta G°$ and $\Delta H°$ on saccharide size is much different for the Oxa-62 and native proteins. Specifically, the values of $\Delta G°$ and $\Delta H°$ for binding $(GlcNAc)_2$ to Oxa-62 lysozyme at pH 5–5.3 are more negative than for binding $(GlcNAc)_3$, and the value of $\Delta H°$ for binding $(GlcNAc)_6$ is more negative than the value for binding $(GlcNAc)_3$. This behavior is opposite to that of

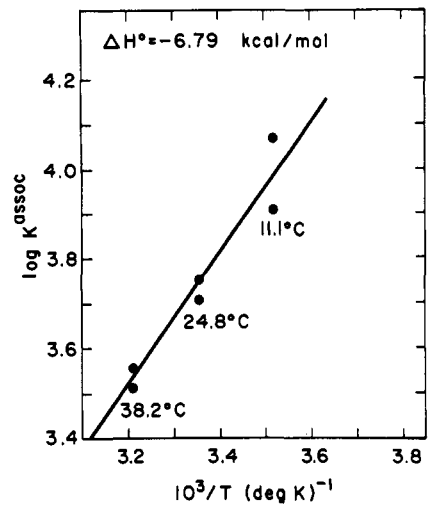

FIGURE 5: van't Hoff plot of association constants for Oxa-62 lysozyme and $(GlcNAc)_6$ at pH 5.3 and 0.1 ionic strength. The line represents a least-squares analysis (data were weighted according to the square of the error in the abscissa and the square of the error in the ordinate). The error in K^{assoc} was the standard deviation obtained from the least-squares analysis of the Scatchard plot, and the error in T was ±1 K. The resulting value of $\Delta H°$ is −6.8 kcal/mol with ±1.5 kcal/mol estimated uncertainty.

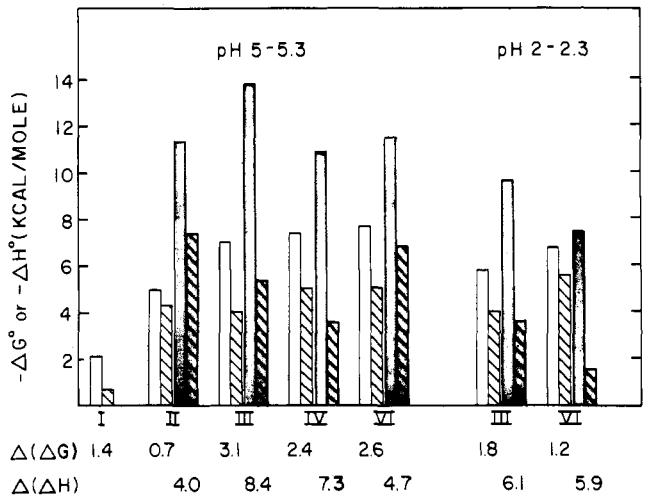

FIGURE 6: Comparison of thermodynamic values for binding of saccharides (unshaded bars for $\Delta G°$ and shaded bars for $\Delta H°$) to Oxa-62 (crosshatched bars) and native (uncrosshatched bars) lysozymes at pH 5–5.3 (left-hand side) and pH 2–2.3 (right-hand side). Differences between the values (Oxa-62 − native) are given below the representations. Values for native lysozyme are from Banerjee et al. (1975a) and references cited therein. Roman numerals give the chain length of the oligosaccharide.

Table II: Thermodynamics of Saccharide Binding to Oxa-62 Lysozyme at 30 °C and 0.1 Ionic Strength[a]

ligand	pH	$-\Delta G°$ (kcal/mol)	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (cal/deg mol)
β-Me-GlcNAc	5.3	0.7		
$(GlcNAc)_2$	5.3	4.32	7.4	10
$(GlcNAc)_3$	5.3	4.00	5.4	4.6
	2.3	4.03	3.6	−1
$(GlcNAc)_4$	5.3	5.01	3.6	−4.6
$(GlcNAc)_6$	5.3	5.08	6.8	5.6
	2.3	5.60[b]	1.6	−13

[a] Measured in a 0.10 M sodium acetate–HCl buffer solution of pH 5.3 or in 0.01 M HCl with 0.09 M NaCl. [b] Computed by using the values of $\Delta H°$ measured at 30 °C and K^{assoc} at 17.5 °C.

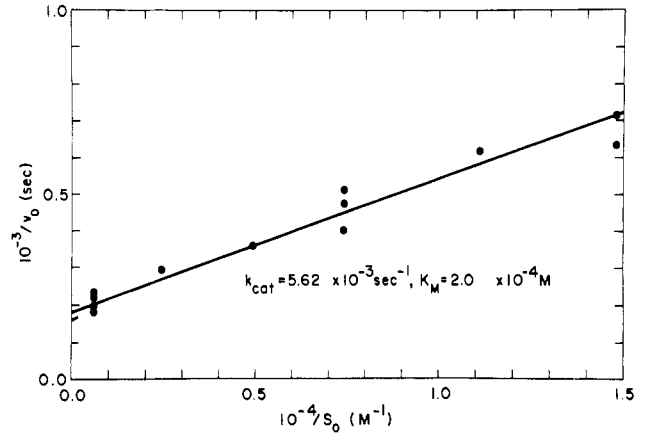

FIGURE 7: Lineweaver–Burk plot for the Oxa-62 lysozyme catalyzed hydrolysis of $(GlcNAc)_6$ at pH 5, 40 °C, and 0.1 ionic strength. The line represents a weighted $(1/v_0)^4$ least-squares fit to the data, which gave $K_m = (2.0 \pm 0.3) \times 10^{-4}$ M and $k_{cat} = (5.62 \pm 0.26) \times 10^{-3}$ s^{-1}. The uncertainties given are standard deviations.

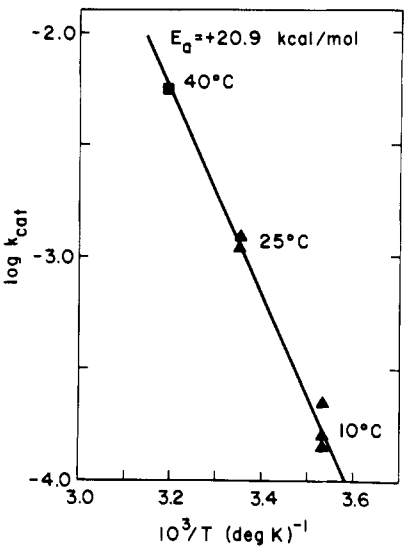

FIGURE 8: Arrhenius plot of k_{cat} values for the Oxa-62 lysozyme catalyzed hydrolysis of $(GlcNAc)_6$ at pH 5 and ionic strength 0.1. The 40 °C value (■) is the extrapolated value from the Lineweaver–Burk plot of Figure 7. The 25 and 10 °C values (▲) are for measurements at near saturation that were corrected for competitive inhibition of GlcNAc and nonsaturation with $(GlcNAc)_6$. The line represents a least-squares analysis (each point was weighted with the square of the error in the abscissa and the square of the error in the ordinate); the error in k_{cat} was estimated as ±15% and that in T was estimated as ±1 K. The error in k_{cat} comes primarily from the uncertainty in estimates of relative peak areas in the product analysis (Banerjee et al., 1973). The least-squares analysis gave E_a = 20.9 ± 1.9 kcal/mol; the uncertainty given is the standard deviation.

the native enzyme. (b) Native lysozyme shows a 1 kcal/mol weaker binding of $(GlcNAc)_3$ and higher oligomers at pH 2 than at pH 5. This behavior was not found for Oxa-62 lysozyme, for which change in pH from 5 to 2 has no effect [$(GlcNAc)_3$] or strengthens binding slightly [$(GlcNAc)_6$]. (c) Change in pH from 5 to 2 makes the enthalpy of $(GlcNAc)_3$ binding less negative by 1.8 kcal/mol for the Oxa-62 enzyme, compared with a difference of 4.1 kcal/mol for the native enzyme. The pH effects on $(GlcNAc)_6$ binding are opposite in direction, i.e., −0.5 kcal/mol for the Oxa-62 enzyme and +0.9 kcal/mol for the native enzyme. (d) The effect of Trp-62 oxidation on the thermodynamics of ligand binding is significantly different for the several ligands studied in this work. The ranges of the effects are 0.7–3.1 kcal/mol for $\Delta G°$ and 4.0–8.4 kcal/mol for $\Delta H°$ (Figure 6).

Cooper (1974) found the formation constant for binding the β anomer of GlcNAc to Oxa-62 lysozyme to be 11 M^{-1} at pH 5 and 5 °C and $\Delta H°$ to be −4.6 kcal/mol. Correction of this estimate of K^{assoc} to 30 °C gives a value twice that found in this work, which may reflect a difference between GlcNAc and β-Me-GlcNAc or the different procedures used for preparing the Oxa-62 protein. With regard to the latter possibility, Norton & Allerhand (1976) have suggested that the method of preparation used by Cooper (1974) could give predominantly the δ_1-acetoxytryptophan derivative.

Norton & Allerhand (1976) observed that SucNBr oxidation of Trp-62 produced equal amounts of the two C-3 diastereoisomers of oxindolealanine. Thus, thermodynamic parameters that are obtained from studies of ligand binding to this mixture represent a weighted average of the parameters for the pure isomers. It is therefore important that within experimental uncertainty the two isomers show identical affinities for the ligands studied, as demonstrated by the linearity of the Scatchard plots (e.g., Figure 4). Furthermore, the values of $\Delta\epsilon_{max}$ for the binding of β-Me-GlcNAc, $(GlcNAc)_3$, and $(GlcNAc)_6$ to the Oxa-62 enzyme are comparable to the values for binding to the native enzyme, indicating that both Oxa-62 lysozyme diastereoisomers bind ligand.

Kinetic Measurements. Least-squares analysis of data for the rate of lysozyme-catalyzed cleavage of $(GlcNAc)_6$ at pH 5, 40 °C, and 0.1 ionic strength (Figure 7) gave values and standard deviations for k_{cat} and K_m of (5.6 ± 0.3) × 10^{-3} s^{-1} and (2.0 ± 0.3) × 10^{-4} M, respectively. K_m is not significantly different from K_s (reciprocal K^{assoc} at 40 °C; Table I), as is also true for native lysozyme.

Table III: Reaction of Oxa-62 Lysozyme and $(GlcNAc)_6$ at 0.1 Ionic Strength, 40 °C, and pH 5[a]

	K^{assoc}			k_{cat}			$K^{assoc}k_{cat}$		
	$\Delta G°$	$\Delta H°$	$\Delta S°$	$\Delta G^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta G^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$
HEW	−7.7	−11.5	−12	19.5	20.9	4.5	11.8	9.4	−7.7
Oxa-62	−5.0	−6.8	−5.7	21.3	20.9	−1	16.3	14.1	−7.0

[a] Values for native lysozyme (HEW) were from Banerjee et al. (1975a). Units of ΔG and ΔH are kcal/mol; units of ΔS are cal/deg mol. K^{assoc} was shown to be equal to $1/K_m$ at 40 °C for Oxa-62 lysozyme and at all temperatures for native lysozyme.

The value of $\Delta H^{\ddagger}$ is 20.9 ± 1.9 kcal/mol, determined from the temperature dependence of k_{cat} (Figure 8). The rates measured for 0.00162 M $(GlcNAc)_6$ at 10 and 25 °C were corrected for competitive inhibition with GlcNAc monomer and for incomplete saturation with $(GlcNAc)_6$ (Banerjee & Rupley, 1975). The value for 40 °C is the extrapolated value from Figure 7.

Values of $\Delta G^{\ddagger}$ and $\Delta S^{\ddagger}$ for the rate-determining step are given in Table III. Table III also contains activation parameters for free enzyme and substrate as reactants, given under the heading $K^{assoc}k_{cat}$, which were calculated by using the activation parameters determined for reaction of the enzyme–substrate complex and the thermodynamic constants for $(GlcNAc)_6$ binding.

Figure 9 gives the fraction of the cleavages of $(GlcNAc)_6$ resulting in transfer as a function of acceptor concentration. The apparent association constant for GlcNAc binding to EP_2', K^*_A (eq 3), is 10^2 M^{-1} for both native and Oxa-62 enzymes. This value for the $(GlcNAc)_6$ reaction is close to estimates for native lysozyme made previously using $(GlcNAc)_3$ as the substrate and GlcNAc as the acceptor (Imoto et al., 1972).

The data closely follow the broken curve of Figure 9, which was drawn assuming limits of 0.1 and 0.9 for the fraction of

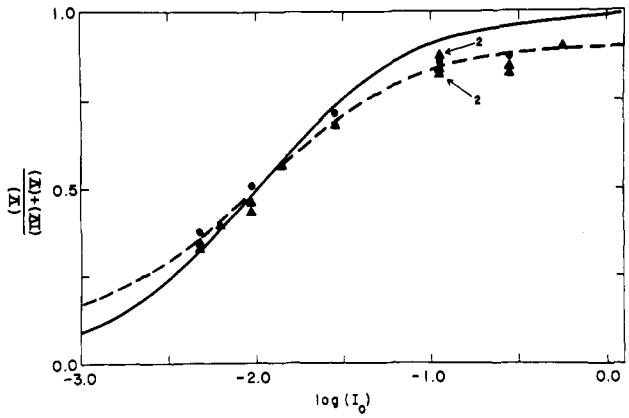

FIGURE 9: Oxa-62 lysozyme catalyzed glycosyl transfer from $(GlcNAc)_6$ to GlcNAc at pH 5, 40 °C, and 0.1 ionic strength. The ordinate gives the fraction of cleavages that results in transfer. (I_0) is the initial GlcNAc concentration. The solid curve is calculated for 1:1 complex formation with asymptotic limits of 0.0 and 1.0. The dashed curve represents 1:1 binding with asymptotic limits of 0.1 and 0.9.

cleavages resulting in transfer. This observation is consistent with there being a small proportion (~10%) of cleavage of $(GlcNAc)_6$ at the fifth or third bonds from the nonreducing end. Also, the rate of cleavage of $(GlcNAc)_5$, produced in the transfer process, is about one-tenth the rate of cleavage of $(GlcNAc)_6$ and should be reflected in the value of the product ratio (0.9) at high acceptor concentrations.

Imoto et al. (1974), in a study using Oxa-62 lysozyme purified by an affinity procedure, found for this derivative a value of V_{max} that was 8% of the native value, measured by using glycol chitin. The results obtained by using glycol chitin are similar to the results obtained in this work by using $(GlcNAc)_6$ (k_{cat} for Oxa-62 lysozyme is 3.7% of the value with native enzyme). Imoto also compared the transglycosylation reactions of native and Oxa-62 lysozyme, using glycol chitin as the substrate. He observed a fivefold lower rate of reaction of acceptor with the Oxa-62 enzyme. This finding is different from the present results obtained from using $(GlcNAc)_6$ and may be due to the more complex structure or the low rate of hydrolysis of glycol chitin.

Discussion

A variety of thermodynamic studies of binding ligands to lysozyme have given results that are interpretable in terms of particular enzyme–ligand interactions, specifically those within regions ABC[3] of the acitve site. For example, the enthalpy change for reaction of $(GlcNAc)_3$ with native lysozyme is ~4 kcal less negative at pH 2 than at pH 5, and this difference is understood to represent principally the effect of protonation of Asp-101, which has a pK near 4.2 in the unliganded protein. This conclusion was confirmed by the comparison of hen and turkey lysozymes (Banerjee & Rupley, 1975). Also, both the free energy and enthalpy of saccharide binding to the native enzyme become more negative with increased saccharide size, comparing monomer, dimer, and trimer, and the $\Delta G°$ values are substantially similar for binding of trimer and higher oligosaccharides (Imoto et al., 1972). The point to be emphasized is that the thermodynamics of oligosaccharide binding to native lysozyme generally appear to reflect additivity of the individual interactions.

The values given in Table II and Figure 6 that compare the thermodynamics of saccharide binding to native and Oxa-62 lysozymes show that the interactions at regions ABC of the active site are affected greatly by oxidation of Trp-62. In some of the complexes of saccharides with Oxa-62 lysozyme, regions A and B of the active site apparently are not filled as they are in complexes formed with native lysozyme. In this regard, change in pH from 5 to 2 does not weaken the binding of $(GlcNAc)_3$ and $(GlcNAc)_6$ to Oxa-62 lysozyme, and the values of $\Delta G°$ and $\Delta H°$ at pH 5.3 for binding $(GlcNAc)_3$ are less negative than those for binding $(GlcNAc)_2$. This behavior of the Oxa-62 lysozyme is opposite to that found in the native enzyme, for which interactions of Asp-101 at regions AB of the active site produce a chain-length and pH dependence of $\Delta G°$. The irregularity of the pattern of changes produced by oxidation of residue 62, as shown in Figure 6 and discussed under Results, suggests that the placement of the oligosaccharide ligands (the mode of binding) within the ABC region is different for the Oxa-62 and native enzymes.

It is noteworthy that a specific and apparently minor chemical modification produces such substantial effects on protein properties. The results described above were not foreseen. The side chain of Trp-62 is on the surface of the molecule and does not participate in intramolecular interactions. Thus, it was not anticipated that oxidation of Trp-62 would alter the mode of binding of saccharide. The unexpected chemistry of binding saccharides to the Oxa-62 derivative emphasizes the difficulty of drawing detailed conclusions from comparisons of a native protein with a derivative that by physical measurements appears identical with the native protein. Estimates of the contribution of Trp-62 to the free energy of binding substrate at the ABC region of the active site that have been based on comparison of the native and Oxa-62 proteins (Imoto et al., 1972) are thus incorrect and likely overestimate the contribution.

The absorbance changes produced through reaction of Oxa-62 lysozyme with saccharides have been interpreted (Imoto et al., 1975) as indicating that in these complexes region C of the active site is filled as it is in the complex of the native protein with monosaccharide. The different chemistry of binding of saccharides to the native and Oxa-62 proteins requires that interactions at the A and B regions in the Oxa-62 lysozyme complexes be substantially different from those found for the native protein. A rationalization of these observations is suggested by the crystallographic results (Blake et al., 1967), which demonstrated that GlcNAc binds at site C of lysozyme in two modes: one "normal" [i.e., bound as is the reducing end unit of $(GlcNAc)_3$, which fills the sites ABC] and the other "anomalous", in that the ligand lies along the wing of the cleft.

Native and Oxa-62 lysozymes show the same dependence of the transglycosylation reaction on GlcNAc concentration. This indicates that the difference in free energy between the transition states for hydrolysis and transglycosylation is the same for native and Oxa-62 proteins. Several significant conclusions follow from this observation. (a) The glycosyl enzyme, presumed to be a carbonium ion intermediate that is formed through cleavage of the DE glycosidic bond and that reacts with water or with acceptor to complete the hydrolysis or transfer reaction, must have the same geometry in the DE region for both Oxa-62 and native lysozymes, in order for the competition between acceptor and water to be the same. (b) Oxidation of Trp-62 does not change the rate-determining step of the reaction. Banerjee et al. (1975a) have shown that the transition state energy for an isomerization of the enzyme–substrate complex (the γ process) is only 1.5 kcal/mol lower than the transition state energy for the rate-determining step, i.e., the cleavage of the glycosidic bond. If the γ process or another isomerization were to become rate determining for formation of the glycosyl enzyme, owing to Trp-62 oxidation,

the parallel effect on the hydrolysis reaction could be explained only as a remarkable coincidence. (c) The data of Figure 9 also show that the cleavage pattern for $(GlcNAc)_6$ is the same for Oxa-62 and native lysozymes. This suggests that the substrate has the same interactions with the A region of the active sites of both Oxa-62 and native lysozymes. In this regard, protonation of Asp-101, which weakens interaction at site A (Banerjee & Rupley, 1975), changes the cleavage pattern from the predominantly DE reaction observed at pH 5 to a pattern with a higher proportion of CD reaction. A measurement of the cleavage pattern of Oxa-62 lysozyme at pH 2 showed it to be similar to that for the native enzyme at pH 2 and different from the pH 5 pattern. This conclusion, that the A region interactions within the *productive* complex are similar for Oxa-62 and native lysozymes, stands in strong contrast to the difference in AB region interactions inferred above from the equilibrium data. In explanation, it is possible that nonproductive binding of $(GlcNAc)_6$ is important for the Oxa-62 enzyme. This suggestion is in accord with the complex pattern of thermodynamic values for binding to the Oxa-62 protein (Figure 6). (d) The concept that in productive complexes substrate is bound much differently than in the nonproductive complexes, reflected in K_m and the thermodynamic parameters, explains the effect of Trp-62 oxidation being the same for transglycosylation and hydrolysis of the glycosyl enzyme. The productive complexes found with Oxa-62 lysozyme would be all at higher free energy levels (ΔG = 4.5 kcal/mol; Table III) than the corresponding complexes of the native enzyme. Thus, the last preequilibrium complex, the glycosyl enzyme, and the rate-determining transition states for its hydrolysis and transglycosylation are all elevated equally in free energy, owing to the distortion of the Oxa-62 enzyme that is required for binding of substrate in sites A and B of the active site.

The values given in Table III under the heading $K^{assoc}k_{cat}$, which is equivalent to k_{cat}/K_m, are estimates of the activation parameters for formation of the transition state of the rate-determining step from free enzyme and substrate. Considering the varied modes of binding of ligands to the Oxa-62 enzyme, $K^{assoc}k_{cat}$ is the most appropriate measure of the effect of Trp-62 oxidation.[4] The difference between the values for native and Oxa-62 lysozymes gives the total effect of oxidation of Trp-62 on binding and bond rearrangement. The effect of the modification on catalysis is even more profound than on oligosaccharide binding. In the native enzyme Trp-62 forms one hydrogen bond with the saccharide moiety bound at site C and a hydrophobic interaction involving one side of the indole ring with the saccharide moiety bound at site B (Blake et al., 1967). The changes of 4.5 kcal/mol in $\Delta G^{\ddagger}$ and 4.7 kcal/mol in $\Delta H^{\ddagger}$ (Table III) are greater than could be attributed plausibly to loss of these interactions. In this regard, the hydrogen bonding of Trp-62 to the hydroxymethyl group of the GlcNAc moiety at site C apparently does not make a significant contribution to the free energy of substrate binding, as inferred from the similar binding of GlcNAc oligosaccharides and corresponding *N*-acetylxylosamine (van Eikeren & Chipman, 1972) and 6-deoxy-*N*-acetylglucosamine (Kuramitsu et al., 1973) derivatives.

The conclusions to be drawn from this discussion of the chemistry of Oxa-62 lysozyme are as follows. (a) The geometry of the transition state of the rate-determining step is the same for Oxa-62 and native lysozyme. (b) Oxidation of Trp-62 strongly affects substrate binding, particularly interactions at the A site, judging from the modification's altering of $(GlcNAc)_3$ more than $(GlcNAc)_2$ binding and from its changing of the pH dependence of the thermodynamics of binding (Figure 6). (c) In view of the above two points, the 2000-fold decrease in catalytic effectiveness produced by Trp-62 oxidation reflects the difficulty of positioning the substrate properly within the active site. (d) In the productive complex of Oxa-62 lysozyme with $(GlcNAc)_6$ that is the reactant in the rate-determining step, interactions have been established at site A between enzyme and substrate. Thus, the observed values of K_m and k_{cat}, like the thermodynamic parameters for $(GlcNAc)_6$ binding, reflect nonproductive binding. This explains why k_{cat} is affected by the oxidation. The free energy difference between the transition states for native and Oxa-62 lysozymes presumably is the cost of rearranging the active site in the region near Oxa-62, in order to accommodate productive binding of substrate. (e) Conformational changes in the wing of the cleft containing Asp-52 have been observed in the crystal to be associated with saccharide binding (Blake et al., 1967). In view of the chemistry of Oxa-62 lysozyme described here, it is not likely that interactions between Trp-62 and substrate are important for proper positioning of substrate and catalytic groups. An observation that supports this conclusion is that the value of k_{cat} for human lysozyme, measured by using $(GlcNAc)_6$, is identical with the value for hen lysozyme (J. A. Rupley and P. Adams, unpublished results). In human lysozyme, residue 62 is tyrosine, which has different interactions than Trp-62 with substrate.

Recently, an elegant combination of X-ray crystallographic and NMR approaches (Blake et al., 1978; Cassels, 1979; C. M. Dobson, personal communication)[5] has given a detailed picture of the effects of Trp-62 oxidation on the structure of lysozyme and its complex with $(GlcNAc)_3$ and other oligosaccharides. The following observations that were made in this structural work give an explanation of the chemistry described above. Oxidation of Trp-62 prevents interactions at the A and B sites found in the complexes of $(GlcNAc)_3$ with native lysozyme. Thus, the preferred mode of oligosaccharide binding is related to the "anomalous" mode of binding observed for the α anomers of GlcNAc and $(GlcNAc)_2$ (Blake et al., 1967), in which the sugar residues that would be in sites A and B of native lysozyme lie out of the cleft of Oxa-62 lysozyme. One of these residues interacts with Asn-59 and Arg-61; the nonreducing end residue of $(GlcNAc)_3$ makes little contact with the Oxa-62 enzyme. These observations rationalize the greater effect of oxidation on $(GlcNAc)_3$ than on $(GlcNAc)_2$ or β-Me-GlcNAc binding (Table II and Figure 6). The 4.5 kcal/mol increase in the free energy level of productive complexes and transition states that follows oxidation of Trp-62 becomes understandable as being the cost principally of moving the Oxa-62 side chain out of the cleft and less significantly of breaking a weak hydrogen bond and several nonpolar contacts made between Trp-62 and substrate. Both diastereoisomeric conformations of Oxa-62 block the A and B sites, which explains the apparently homogeneous behavior of Oxa-62 lysozyme in binding oligosaccharides. The thermodynamic and catalytic properties of Oxa-62 lysozyme are seen to be in close accord with the structural information.

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[4] For mechanisms in which nonproductive binding is important, the steady-state kinetic parameter k_{cat}/K_m reflects the catalytic reaction, i.e., the properties of productive complexes.

[5] A reviewer was very helpful in apprising us of Dr. Cassels' work.

Cassels' thesis and other unpublished information on the NMR and X-ray crystallographic studies.

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Sites of D-Domain Interaction in Fibrin-Derived D Dimer†

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ABSTRACT: We have examined the plasmic digestion products of fibrin formed in the presence of dansylcadaverine, the fluorescent D dimer, to determine whether they are held together not only by the cross-link region on the γ chain but also by other interactions on the D domain. Antibodies to the D dimer reacted 8× more strongly with sites on the D dimer (purified or in the presence of E) than with sites on fibrinogen or plasmin-digested fibrinogen. The reactivity of this surface site was lost when the γ chain was cleaved by plasmin after the molecule had been destabilized by the removal of calcium ions, thus breaking the covalent linkage of the homodimer. The noncovalent D dimer retained its dimeric structure by the criteria of molar volume, measured by fluorescence polarization, and molecular sieving. The noncovalently attached, cross-link-containing peptide bound tightly to the parent molecules at higher temperatures but rotated more freely below 15 °C, and could be lost from the parent molecules without destroying the dimeric structure. We therefore propose that the forces maintaining the dimeric structure of the noncovalently joined molecule are not solely located at the γ-chain cross-link region. These other sites on the D domain are therefore candidates for the initial fibrinogen polymerization site and may also have a role in fibrinogen half-molecule assembly.

The early stages of the fibrinogen to fibrin conversion can be divided into three separate steps (Doolittle, 1973). The first, catalyzed by the proteolytic enzyme thrombin, involves cleavage of the A and B fibrinopeptides from the α and β chains of the fibrinogen molecule to yield a fibrin monomer. Cleavage of only the A fibrinopeptide is required for the second step, polymerization of fibrin monomers to form a urea-dissociable gel, to proceed (Blomback et al., 1978). The third step, stabilization of this gel, is carried out by calcium-requiring transglutaminase (fibrin-stabilizing factor XIIIa), which covalently links the γ chains on adjacent molecules by an isopeptide bond (Lorand, 1972). The later stages of fibrin formation involve lateral association of the initially formed linear polymers and covalent cross-linking of the α chains, through an acceptor site in the highly mobile COOH-terminal end of

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