Resolution of the Individual Steps in the Reaction of Lysozyme with the Trimer and Hexamer of *N*-Acetyl-D-glucosamine at Subzero Temperatures†

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ABSTRACT: The reaction of hen egg white lysozyme with chitotriose and chitohexose has been investigated at temperatures to below −100 °C by using aqueous methanol and dimethyl sulfoxide cryosolvents. Initial investigations involving the effects of increasing cosolvent concentration on the catalytic and structural properties of the enzyme indicated that both methanol and dimethyl sulfoxide cryosolvents, at subzero temperatures, had no adverse effects on lysozyme. Time-dependent changes in the fluorescence emission of the enzyme, under nonturnover conditions, permitted the detection of two intermediates in the reaction with the trimer and three intermediates in the case of the hexamer. The fastest reaction observed for both substrates was complete within minutes at −90 °C and is attributed to initial substrate binding, followed by rapid isomerization to form a "loose" ES complex. The subsequent, slower reactions correspond to successive isomerizations of ES to "tighter" complexes. From dye binding displacement reactions, and comparison of dissociation constants, it is concluded that the predominant mode of binding for the hexamer at subzero temperature is productive. Rate and dissociation constants were determined for the observed reactions as a function of temperature, pH*, and cosolvent concentration. Overall the reaction pathway in methanol cryosolvents at subzero temperatures appears to be very similar to that determined from investigations in aqueous solution at ambient temperatures using a variety of rapid reaction techniques. Conditions necessary to accumulate and stabilize each of the observed intermediates have been determined, thus permitting further studies to delve more deeply into the nature of the intermediates.

A necessary prerequisite to a detailed quantitative explanation of the mechanisms and efficiency of enzyme catalysis is the knowledge of the structure of all enzyme–substrate intermediates on the reaction pathway at atomic resolution. At present the best suited means of obtaining such information involves a combination of high-resolution X-ray crystallography and cryoenzymology (Fink, 1977; Makinen & Fink, 1977; Alber et al., 1976; Fink & Petsko, 1980). Lysozyme is a candidate of particular significance for such a study in view of the proposed major role of substrate strain in its mode of action (Phillips, 1967; Imoto et al., 1972a). In fact, for many years lysozyme catalysis was considered the archetypical example of a mechanism involving a critical ground-state substrate distortion. However, the existence of such strain has recently been questioned on both theoretical (Warshel & Levitt, 1976) and experimental (Schindler et al., 1977) grounds.

The present investigation was undertaken with the aim of supplying necessary information to allow the accumulation and stabilization at low temperatures of enzyme–substrate intermediates in the reaction between lysozyme and a hexasaccharide substrate. With the knowledge of the conditions necessary to trap a particular intermediate, one can then obtain mechanistically useful information regarding intermediate structures by application of a variety of techniques, such as nuclear magnetic resonance, laser Raman spectroscopy, and X-ray diffraction.

Postulated mechanisms of lysozyme catalysis are summarized in reviews by Imoto et al. (1972a) and Hamaguchi & Hayashi (1972). More recent contributions include those of Gorenstein (1977) and Jencks (1975). The generally accepted mechanism at present for chitohexose hydrolysis involves both nonproductive binding to three subsites (A, B, and C) as well as productive binding to all six subsites (A–F). The overall binding process involves at least one subsequent isomerization in the nonproductive mode and at least two isomerizations following the initial enzyme–substrate complexation in the productive mode (Banerjee et al., 1975). In productive binding it has been postulated that distortion of the sugar in the D subsite to some nonchair form (Ford et al., 1974) may occur, although theoretical calculations suggest that electrostatic stabilization alone could account for the rate enhancement (Warshel & Levitt, 1976). It is thought that general acid catalysis by Glu-35 leads to the formation of an oxocarbonium ion which is stabilized by the negative charge of the proximal carboxylate of Asp-52. The potential stabilization to the developing carbonium ion of the antiperiplanar lone pair of the ring oxygen in the boat or sofa conformation lends further support for the need of ground-state distortion (Gorenstein et al., 1977).

The fluorescence emission of lysozyme (hen egg white) originates almost exclusively (>80%) from Trp-62 and Trp-108 (Imoto et al., 1972b; Formoso & Forster, 1975), both residues forming part of the active-site cleft. Several studies have shown that the intrinsic fluorescence of the enzyme is a very useful probe of events at the active site [e.g., Halford (1975), Holler et al. (1975a,b), Teichberg & Shinitzky (1973), Teichberg & Sharon (1970), Lehrer & Fasman (1967), and Rupley & Gates (1967)], and in fact each of the proposed elementary steps can be detected by fluorescence changes (Banerjee et al., 1975).

From a combination of equilibrium and kinetic (including pre-steady-state and steady-state) experiments, Rupley and co-workers (Banerjee et al., 1975) have calculated the individual rate and equilibrium constants, and the corresponding thermodynamic parameters, for each of the steps involved in the catalysis of the hexamer of *N*-acetyl-D-glucosamine. Thus, estimates for the rates of various reactions at subzero temperatures can be made by using the reported enthalpies and rate constants at 25 °C. Halford (1975) has carried out a stopped-flow kinetics investigation of the reaction with the corresponding trimer, using fluorescence to monitor the reaction. His data suggest that binding of the trimer is an excellent model for the nonproductive binding of the hexamer.

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0006-2960/80/0419-0811$01.00/0

In our low-temperature studies we have therefore used the lysozyme–chitotriose reaction as a model for the nonproductive binding of the hexamer.

Even at 25 °C lysozyme is remarkably stable as far as the influence of organic solvents is concerned (Kurono & Hamaguchi, 1964; Hamaguchi, 1964a,b; Hamaguchi & Kurono, 1963; Hamaguchi & Imahori, 1964). Consequently, there should be little difficulty in finding cryosolvents in which the enzyme is stable and unaffected as far as its catalytic and structural properties are concerned. In fact, Douzou et al. (1975) have carried out some preliminary investigations on human leukemic lysozyme which indicate that 40% aqueous methanol (freezing point −35 °C) is a suitable cryosolvent system for preventing turnover with chitohexose at −25 °C. That the turnover reaction can be made negligible at such a relatively high subzero temperature is a reflection of the high energy of activation of glycoside hydrolysis, even when enzyme catalyzed (17–20 kcal mol^{-1}).

In this report we show that methanol and dimethyl sulfoxide (Me_2SO)[1] cryosolvents can be satisfactorily used in the study of lysozyme catalysis at subzero temperatures. Using the enzyme's intrinsic fluorescence and temperatures down to −110 °C, we were able to monitor the discrete steps in the productive binding of the hexamer of *N*-acetylglucosamine and the nonproductive binding of the corresponding trimer.[2] Consequently, the necessary conditions of pH* and temperature to accumulate and stabilize a particular intermediate are now known.

Experimental Procedures

Materials. Twice-recrystallized hen egg white lysozyme was obtained from Worthington Biochemical Corp. Chitotriose and chitohexose were the gift of Dr. J. A. Rupley. The trimer was further purified by gel filtration using Sephadex G-10. Stock solutions of the trimer and hexamer were prepared in the appropriate cryosolvent at concentrations of 1.0 and 0.5 mg/mL, respectively. Cell walls of *Micrococcus lysodeikticus* were from Sigma Chemical Co. Biebrich Scarlet was purchased from Matheson Coleman and Bell and purified by preparative TLC using 9:1 chloroform–methanol. Dimethyl sulfoxide was purified by vacuum distillation, after drying with calcium hydride, at 37 °C. Glass-distilled water and reagent-grade chemicals were used throughout. Cryosolvents were prepared on a volume/volume basis, with an ionic strength of 0.1 M, by using acetate, cacodylate, and morpholine buffers (Fink, 1973, 1976a; Fink & Geeves, 1979).

Methods. In general, the low-temperature experiments were carried out in the manner described previously (Fink, 1973, 1976a; Fink & Angelides, 1976; Fink & Geeves, 1979). A circulating-gas coolant system was used for fluorescence experiments at temperatures below −40 °C; otherwise, the subzero temperatures were obtained by using low-temperature circulating baths with ethanol as coolant.

Absorption experiments were carried out by using a Cary 118C spectrophotometer. Cell wall hydrolysis was monitored at 711 nm. Fluorescence experiments were performed on a Perkin-Elmer MPF-4 spectrofluorometer; unless otherwise stated excitation was at 280 nm. Excitation and emission slit widths were 2 and 10 nm, respectively. A typical experiment involved the following procedures. An aliquot of the stock enzyme solution was added to cryosolvent at 0 °C, and the solution was added to the precooled cell. After thermal equilibration, the emission spectrum was monitored to ensure stability. Excitation and emission spectra were then recorded. An aliquot of cryosolvent was added, and the emission intensity was monitored to check for the magnitude of the dilution effect and for stability. Then an aliquot of the substrate, dissolved in the cryosolvent, was added and the reaction was monitored. The emission spectrum was scanned at appropriate intervals. Kinetics were usually monitored at 340 nm; dissociation constants were obtained from double-reciprocal, Scatchard, and Hill plots. Excellent agreement among the three techniques was found. Difference spectra in the dye-displacement reactions were obtained by using thermostated cells in both reference and sample beams. Nuclear magnetic resonance spectra were obtained at 100 MHz by using a JEOL PS-100 Fourier transform instrument and at 360 MHz by using the Stanford Magnetic Resonance Facility. The enzyme concentration was 50 mg/mL.

Enzyme assays were routinely performed by using the cell wall hydrolysis procedure.

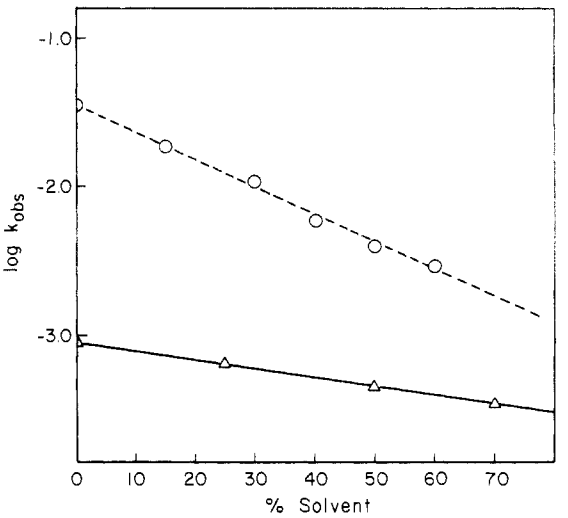

FIGURE 1: Effect of dimethyl sulfoxide (○) and methanol (△) on the rate of lysozyme-catalyzed hydrolysis of cell walls of *M. lysodeikticus*. The Me_2SO experiments were carried out at 25 °C, pH* 7.1, and $[E]_0 = 8.3 \times 10^{-6}$ g/mL. The methanol experiments were performed at 1.1 °C, pH* 7.1, and $[E]_0 = 3.5 \times 10^{-7}$ M.

Results

Effect of Cryosolvents. Hamaguchi and co-workers (Hamaguchi, 1964a,b; Ikeda & Hamaguchi, 1970; Shimaki et al., 1970; Kurono & Hamaguchi, 1964) have previously shown that the observed changes in the circular dichroism and absorbance spectra of hen egg white lysozyme, on varying the concentration of dimethyl sulfoxide or methanol, are consistent with solvent effects on exposed residues and show no evidence of structural perturbations at 25 °C below 60% aqueous dimethyl sulfoxide and 50% aqueous methanol. We have shown previously that increasing concentrations of organic cosolvent lower the midpoint of the thermal denaturation of proteins (Fink & Grey, 1978), and, consequently, at lower temperatures one can expect to use higher cosolvent concentrations without adverse effects.

The effect of increasing dimethyl sulfoxide and methanol concentrations on the rate of hydrolysis of cell walls of *M. lysodeikticus* is shown in Figure 1. The value of k_{cat}/K_m decreases exponentially as the cosolvent concentration increases. This rate decrease presumably results from an in-

[1] Abbreviations used: Me_2SO, dimethyl sulfoxide; ^1H NMR, proton magnetic resonance; pH*, the apparent protonic activity in the aqueous–organic solvent [cf. Hui Bon Hoa & Douzou (1973)].

[2] The terms chitohexose and hexamer are used interchangeably for the hexasaccharide of *N*-acetyl-D-glucosamine. Similarly, chitotriose and trimer refer to the corresponding trisaccharide.

Table I: Rates of Reaction for Lysozyme with Chitotriose and Chitohexose at Subzero Temperatures

substrate	reaction[a]	pH*	% MeOH	T (°C)	$[S]_0$ (M)	k_{obsd} (s^{-1})
trimer	2	7.4	50	−39.5	3.6×10^{-6}	2.9×10^{-3}
	2	7.4	70	−44.0	5.3×10^{-6}	3.7×10^{-3}
	2	4.1	50	−39.5	3.6×10^{-6}	3.1×10^{-3}
	2	7.4	70	−55.0	4.0×10^{-6}	6.2×10^{-4}
	2	7.2	50	−20.1	4.0×10^{-5}	9.0×10^{-3}
	1	7.1	70	−55.6	4.0×10^{-6}	1.3×10^{-1}
	1	7.5	70	−70.8	8.3×10^{-5}	1.5×10^{-2}
	1	7.5	80	−90.4	4.0×10^{-5}	5.3×10^{-3}
	1	7.6	80	−90.8	4.0×10^{-5}	4.3×10^{-3}
hexamer	3	7.4	50	−20.1	2.0×10^{-5}	1.0×10^{-2}
	3	7.4	50	−39.1	4.0×10^{-6}	3.0×10^{-3}
	3	4.1	50	−37.0	4.0×10^{-6}	2.3×10^{-3}
	3	5.8	50	−42.2	2.0×10^{-5}	2.9×10^{-3}
	3	7.1	70	−55.0	2.0×10^{-5}	8.8×10^{-4}
	2	7.1	70	−70.0	1.0×10^{-5}	3.3×10^{-3}
	2	7.1	70	−70.0	4.0×10^{-6}	9.2×10^{-4}
	2	7.5	80	−70.8	2.0×10^{-5}	4.4×10^{-3}
	2	7.5	80	−90.1	2.0×10^{-6}	1.5×10^{-3}
	2	7.5	80	−90.1	4.0×10^{-6}	1.7×10^{-3}
	2	7.5	80	−90.1	6.0×10^{-6}	1.9×10^{-3}
	1	7.1	80	−70.8	1.0×10^{-5}	$\geq 5.8 \times 10^{-2}$
	1	7.5	80	−90.8	1.0×10^{-5}	7.3×10^{-3}

[a] See the text and Figures 2 and 5.

Table II: Dissociation Constants for the Binding of Chitotriose and Chitohexose to Lysozyme at Subzero Temperatures

substrate	pH*	% MeOH	T (°C)	K_d (M)
trimer[a]	7.4	50	−37.2	9.1×10^{-6}
	4.1	50	−39.5	7.6×10^{-6}
	7.4	70	−41.5	7.1×10^{-6}
	7.2	50	−40.0	6.3×10^{-6}
	7.0	50	+0.2	2.4×10^{-5}
	7.1	70	+0.2	4.4×10^{-5}
hexamer[b]	4.1	50	−37.0	1.5×10^{-6}
	7.1	70	−40.5	2.7×10^{-6}
	7.4	50	−39.0	2.2×10^{-6}
	7.4	80	−70.1	4.4×10^{-6} [c]
	7.1	50	+0.2	1.5×10^{-5}
	7.0	70	+0.2	2.9×10^{-5}
	7.0	50	−20.1	8.3×10^{-6}

[a] K_d^{α} for $E + I \rightleftarrows EI_2$; see the text. [b] $K_d^{\beta\gamma}$ for $E + S \rightleftarrows ES_3$, except as noted. [c] K_d^{β} for $E + S \rightleftarrows ES_2$.

crease in K_m, reflecting an increase in the dissociation constant for substrate binding (see Discussion and Table II). The Arrhenius plot for the rate of hydrolysis of the cell wall substrate in 60% aqueous Me_2SO was linear (within experimental error) over the range 25 to −5 °C, with an energy of activation of 23.7 ± 2.3 kcal mol^{-1}. This may be compared with a value of 20 kcal mol^{-1} for the human leukemic lysozyme in 50% aqueous 2-methyl-2,4-pentanediol (Douzou et al., 1975). The reaction was too slow to measure accurately at temperatures much below 0 °C. The effect of pH* on the cell wall hydrolysis in 60% aqueous Me_2SO was investigated. The results yielded a value of $pK^*_1 = 5.9 \pm 0.2$.

The effects of varying methanol concentration on the reactions of chitotriose and chitohexose (under nonturnover conditions) with lysozyme at a variety of temperatures were also obtained. Some of the data pertaining to the reaction rates and the dissociation constants are given in Tables I and II. Among the points to note is that for both trimer and hexamer the dissociation constant increases with increasing cosolvent concentration in an exponential fashion. For example, for the trimer the value of K_d is 4.6 times greater at 50%, and 11.5 times greater at 80% methanol, than the reaction in aqueous solution (pH* 7.1; 0 °C). Most of this effect stems from the initial enzyme–substrate complexation, reaction 1 (see Reactions with Chitotriose at Subzero Temperatures).

Two types of experiments were carried out to demonstrate that the enzyme was active toward the hexasaccharide substrate in methanol cryosolvents. The enzyme was incubated with the substrate in 50% methanol at pH* 5.5, 0 °C, and aliquots were removed periodically for chromatographic analysis. Such experiments demonstrated the catalytic conversion of the hexasaccharide into smaller oligosaccharides. Similar experiments were also conducted in which the progress of the reaction was monitored by the intrinsic fluorescence of the enzyme. On addition of substrate substantial quenching of the fluorescence occurred, e.g., 27% for $[E]_0 = [S]_0 = 9.6 \times 10^{-6}$ M. As the reaction progressed the amount of quenching decreased, eventually leveling off at an emission value slightly quenched from the original (due to product binding). The rate of return of fluorescence, although following the anticipated complex rate law, was proportional to the enzyme concentration. At low enzyme concentrations a substantial lag period occurred, as expected.

Reactions with Chitotriose at Subzero Temperatures. The interaction of the *N*-acetylglucosamine trimer with lysozyme was examined by using fluorescence spectrophotometry in methanol cryosolvents ranging from 50 to 90% methanol. Since preliminary experiments indicated that the maximal spectral changes occurred around 340 nm, the time-dependent changes in fluorescence emission were usually followed at fixed wavelengths in this vicinity.

At both 0 and −20 °C a very rapid decrease in fluorescence emission in the 340-nm region was observed. The reaction rate was too fast to measure with the conventional spectrophotometer used. After this initial fluorescence decrease, no further spectral change was detected. At lower temperatures, in the pH* 4–8 range, two reactions could be observed, differing by about 2 orders of magnitude in their rates (Figure 2). The fast reaction, which we will refer to as reaction 1, appeared as a decrease in fluorescence emission in the 340-nm region, and its rate could be measured at temperatures below −55 °C (Figure 2). Reaction 2 also appeared as a decrease in emission in the 340-nm region (Figure 2) and became negligibly slow at temperatures below −70 °C. Representative kinetic data for these two reactions are given in Table I. The rate of reaction 2 was found to be relatively insensitive both to pH* in the 4–8 region and to cosolvent concentration. The observed first-order rate constants for reactions 1 and 2, as well as the magnitude of the spectral changes, were proportional to substrate concentration under conditions of $[S]_0 \gg [E]_0$, up to limiting values.

Reaction 1 appeared monoexponential at temperatures as low as −110 °C (90% methanol). Since the extrapolated bimolecular rate constant for trimer binding from the rate of reaction 1 was slower than expected for a diffusion-controlled reaction, the effect of increasing chitotriose concentration on the first-order rate constant for reaction 1 was measured at −90.0 °C, pH* 7.5. The plot of k_{obsd} vs. $[S]_0$ was hyperbolic over the range $(0.5–15) \times 10^{-6}$ M. Fitting the data to a scheme of the form

$$E + I \underset{}{\overset{K'}{\rightleftharpoons}} EI' \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI_1$$

yielded values of $K' = 4.8 \times 10^{-6}$ M, $k_1 = 4.0 \times 10^{-3}$ s^{-1}, and $k_{-1} = 2.0 \times 10^{-5}$ s^{-1}.

Values for the dissociation constant (K_d) for the product of reaction 2 were obtained from Hill or Scatchard plots (e.g., Figure 3), and representative data are given in Table II. The

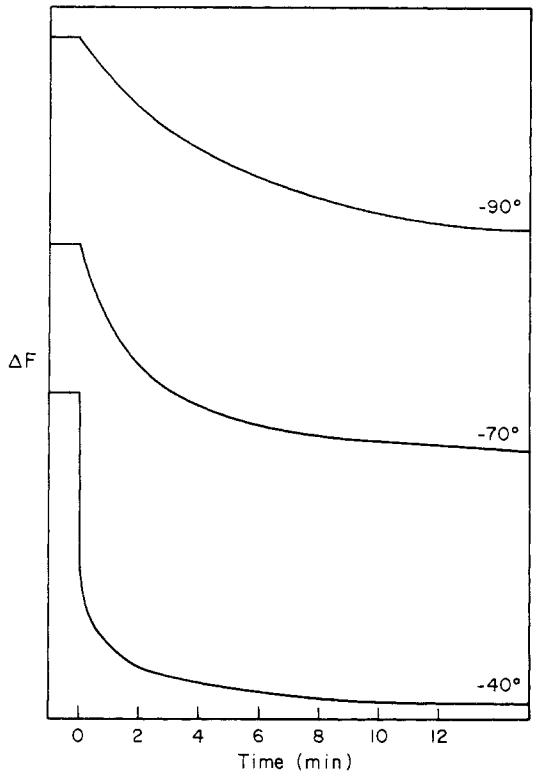

FIGURE 2: Tracings of the time-dependent changes in the fluorescence emission of lysozyme during the reaction with chitotriose. The run at −40 °C was in 50% aqueous methanol; the others were in 80% aqueous methanol, pH* 7.1. Excitation was at 280 nm; emission was monitored at 345 nm. $[E]_0 = 3.6 \times 10^{-7}$ M. Substrate was added at time = 0.

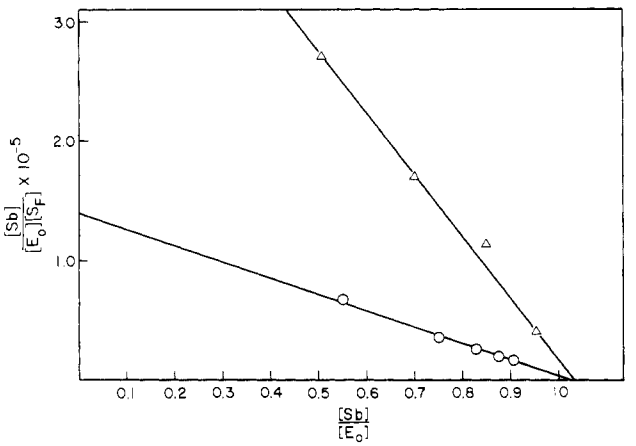

FIGURE 3: Representative Scatchard plots for the binding of chitotriose (O) and chitohexose (Δ) from fluorescence difference spectra. $[E]_0 = 3.6 \times 10^{-7}$ M; pH* 7.1; 50% aqueous methanol; −40.1 °C.

values of the dissociation constants were only slightly affected by changes in pH* or cosolvent concentration.

Difference fluorescence spectra for the products of reactions 1 and 2 compared to enzyme alone under identical conditions are shown in Figure 4. Notable points include the following. The difference spectra for the product of reaction 2 are very similar in overall appearance to those previously reported in aqueous solution, 25 °C [e.g., Halford (1975)], and show maxima around 300 nm and minima around 340 nm at pH* 7. The spectra are very similar in 50, 70, and 80% aqueous methanol, and 60% aqueous dimethyl sulfoxide, for the same pH*. The whole difference spectrum is blue-shifted as the pH* is decreased from 8 to 4, as previously observed in aqueous solution. The shape of the difference spectra is also relatively

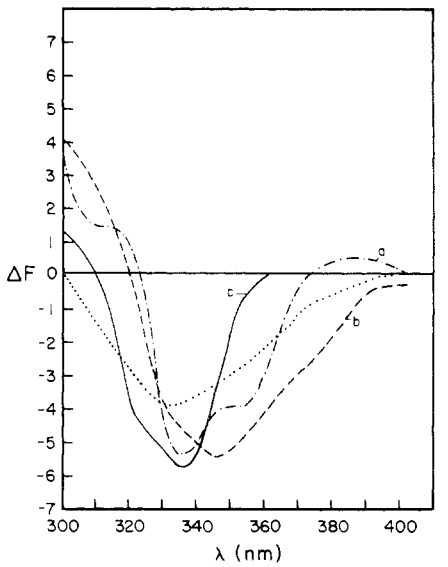

FIGURE 4: Fluorescence difference spectra for the products of the reaction of lysozyme with the trimer of *N*-acetyl-D-glucosamine. Excitation was at 280 nm, $[E]_0 = 3.6 \times 10^{-7}$ M, and $[S]_0 = 1.0 \times 10^{-5}$ M. Product of reaction 1 (−·−) at pH* 7.5, −70 °C, and 80% methanol. Product of reaction 2 at −40 °C in 50% methanol at pH* 7.1 (- - -) and pH* 4.1 (···) and in 60% aqueous Me_2SO at pH* 6.0 (—).

insensitive to temperature. Although excitation was usually done at 290 nm, the emission difference spectra were relatively unaffected by changes in excitation frequency from 280 to 298 nm. The amplitude of the spectral differences (both positive and negative) was maximal with excitation at 280 nm and decreased with increasing excitation wavelength.

The difference spectrum for the product of reaction 1 (Figure 4) was similar at −70 and −90 °C, and in general the outline resembles that of the product of reaction 2 with the exception of pronounced "shoulders" in the vicinity of 315 and 350 nm.

Reactions with Chitohexose at Subzero Temperatures. The reaction of the *N*-acetylglucosamine hexamer with lysozyme was monitored in aqueous methanol cryosolvents by using the intrinsic fluorescence emission of the enzyme. A total of three different reactions (1–3) were discerned over the temperature range 0 to −90 °C (Figure 5). The fastest, reaction 1, was observed as an increase in fluorescence emission at 345 nm and was only detectable at temperatures below −55 °C. Its rate was too fast to measure above −70 °C. Reaction 2 appeared as a decrease in emission at 345 nm, and its rate was readily measured at temperatures in the −60 to −90 °C range. Reaction 3 also appeared as a decrease in emission in the 340-nm region, and its rate was negligible below −60 °C.

Representative kinetic data for reactions 1–3 of the hexamer are included in Table I. Both the observed first-order rate constants and the amplitude of the spectral change are proportional, to a limited value, to the substrate concentration, under conditions of excess substrate. The rate of reaction 3 is essentially unaffected by a change of pH* from 4 to 8 and of cosolvent concentration from 50 to 80% methanol. The rates and apparent energies of activation of reaction 3 for the hexamer and reaction 2 of the trimer are similar (Figure 6). The rate of reaction 1 for the hexamer is close to twice that of reaction 1 for the trimer at −90 °C. In the −70 °C region the relative rates for reactions 1–3 of the hexamer are of the order ≥300:20:1, respectively. A plot of k_{obsd} vs. [chitohexose] at −90 °C, pH* 7.5, for reaction 1 was linear in the (4–10) $\times 10^{-6}$ M range, with an intercept (k_{-1}) of 6×10^{-3} s^{-1} and

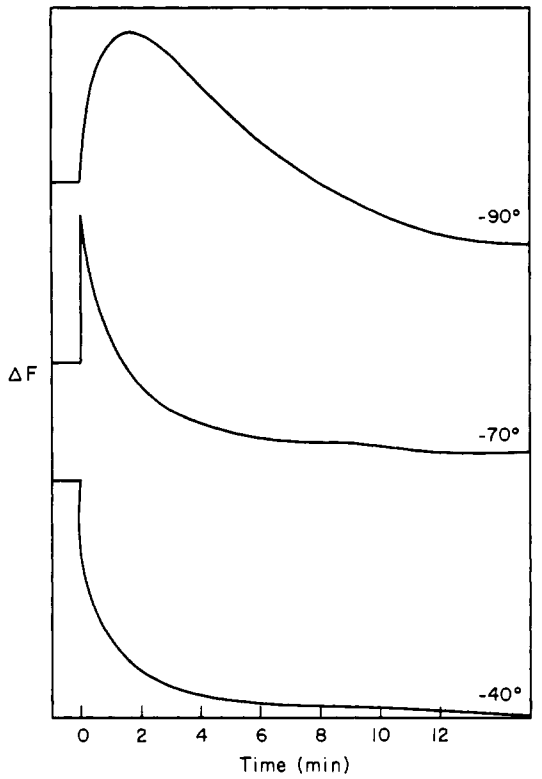

FIGURE 5: Tracings of the time-dependent changes in fluorescence emission in the reaction of lysozyme with the hexamer of *N*-acetyl-D-glucosamine. The runs at −90 and −70 °C were in 80% aqueous methanol; that at −40 °C was in 50% methanol, pH* 7.1; excitation at 280 nm; emission at 345 nm. $[E]_0 = 3.6 \times 10^{-7}$ M. Substrate was added at time = 0.

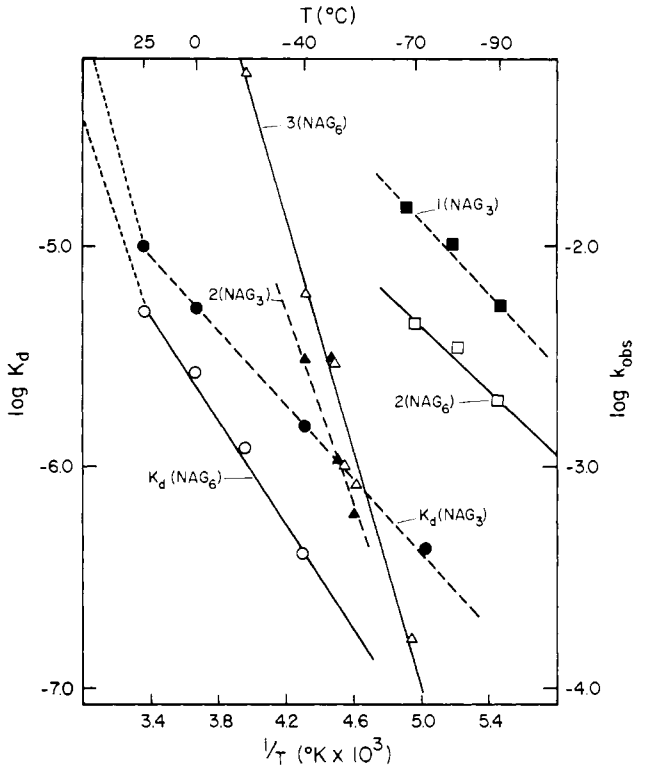

FIGURE 6: Arrhenius plots for the first-order rate constants and dissociation constants for the reaction of lysozyme with the trimer (- - -) and hexamer (—) of *N*-acetyl-D-glucosamine. The numerals 1–3 refer to reactions 1–3 (see the text). The values of K_d were corrected to 0% cosolvent. NAG_3 and NAG_6 refer to the trimer and hexamer, respectively. The broken lines above 25 °C and the points at 25 °C correspond to the data of Banerjee et al. (1975) in aqueous solution.

a slope (k_1) of 1.3×10^{-2} M^{-1} s^{-1}. Unfortunately, technical difficulties prevented measurement of the rates outside this limited concentration range.

Dissociation constants for the product of reaction 3 are given in Table II and were determined from Hill or Scatchard plots (Figure 3). Neither cosolvent concentration nor pH* has *substantial* effects on the dissociation constant. Under similar conditions, at all temperatures below 0 °C, the dissociation constant for the product of reaction 2 of the trimer is greater than that for the product of reaction 3 of the hexamer; i.e., the hexamer exhibits tighter "binding".

From the limited data available we can calculate the entropy associated with some of the processes in lysozyme catalysis at subzero temperatures. For example, values of $T\Delta S^{\ddagger}$ at −40 °C for $ES_2 \rightarrow ES_3$ (hexamer) (Scheme I) and $EI_1 \rightarrow EI_2$ (trimer) are −14.0 and −13.7 kcal, respectively. Similarly, the values for $T\Delta S°$ at −40 °C for the overall processes $E + S \rightleftarrows ES_3$ and $E + I \rightleftarrows EI_2$ are −11.6 and −9.5 kcal, respectively.

In Figure 7 are shown some fluorescence difference spectra for the products of reactions 1–3 of the hexamer. Comparisons of the difference spectra of the product of reaction 3 at pH* 7, 6, and 4 indicate similar minima around 345 nm but the positive maxima around 300 nm at pH* 7 and 6 are absent at the lower pH*. This is in contrast to the effect of pH* on the product of reaction 2 with the trimer (Figure 4) in which both the minima around 340 nm and the maxima around 310 nm are blue-shifted with decreasing pH. The difference spectra at high pH* for the products of reaction 3 with the hexamer and reaction 2 with the trimer are quite similar with the exception of different intensities. The spectra of the product of reaction 2 with the hexamer also show a positive maximum around 300 nm and a minimum at 340 nm. A prominent "shoulder" is visible in the 355–360-nm region, and

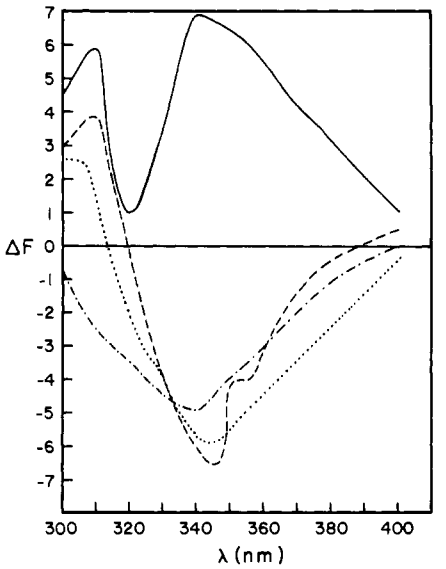

FIGURE 7: Fluorescence difference spectra for the intermediates in the reaction of lysozyme with chitohexose. Excitation was at 280 nm; $[E]_0 = 3.6 \times 10^{-7}$ M; $[S]_0 = 2 \times 10^{-5}$ M. Product of reaction 1 (see the text) at pH* 7.5, −90 °C, and 80% aqueous methanol (—). Product of reaction 2 at pH* 7.5, −70 °C, and 80% aqueous methanol (- - -). Product of reaction 3 at pH* 7.5 (···) and pH* 4.1 (−·−), 50% aqueous methanol, and −40 °C.

"shoulders" are also apparent at 320 and 385 nm. The difference spectra of the product of reaction 1 of the hexamer are quite different. Positive maxima occur at 310 and 340 nm, as well as a large trough with a minimum at 320 nm. The general features of the difference spectra for the product of

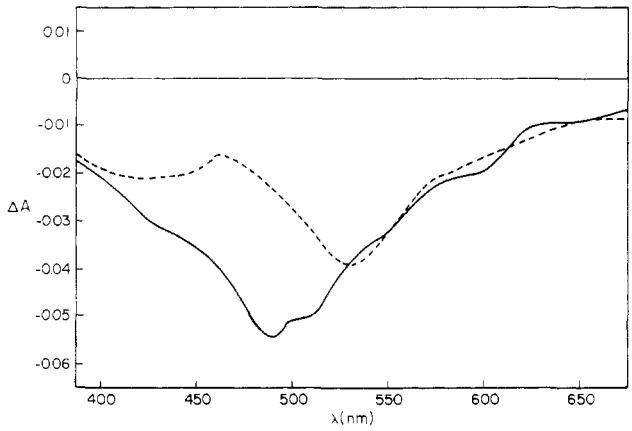

FIGURE 8: Difference spectra for the products of the reaction of lysozyme with chitotriose (---) and chitohexose (—) at −40 °C in the presence of Biebrich Scarlet (7.2×10^{-5} M), $[E]_0 = 3.6 \times 10^{-7}$ M, $[S]_0 = 2 \times 10^{-5}$ M, pH* 7.1, and 50% aqueous methanol. The difference spectra shown are for (enzyme plus dye plus substrate) − (dye plus substrate).

reaction 2 of the hexamer and of reaction 1 of the trimer are similar.

Dye-Displacement Reactions. Holler et al. (1975b) have proposed that the dye Biebrich Scarlet may be used to distinguish between productive and nonproductive binding modes of oligosaccharides to lysozyme. The basis of the method is that the dye binds to the E or F subsite, undergoing perturbation of its visible spectral properties. Subsequent binding of an oligosaccharide, e.g., chitotriose, in a nonproductive manner (to A–C subsites) leads to some spectral perturbation of the bound dye due to the induced conformational change in the active-site cleft. Binding of a substrate in a productive fashion results in displacement of the bound dye, leading to appropriate spectral changes.

Careful examination of the binding of the dye to lysozyme in 50% methanol at −40 °C indicates that 2 mol of Biebrich Scarlet binds per mol of enzyme, the dye-binding sites having different affinities (Hui Bon Hoa and Fink, unpublished observations). Further investigation suggests that the tighter binding site is not directly in the E–F subsite region of the cleft but is perturbed on the binding of hexasaccharide (Compton and Fink, unpublished observations). The dye difference spectra in the presence of tri- and hexasaccharide at subzero temperatures vary as a function of dye concentration, pH*, and temperature. On the basis of the available data, the relationship between the observed dye difference spectra and substrate in the E–F subsite region must be considered unresolved. However, if trisaccharide and hexasaccharide bind identically, e.g., to the A–C subsites, then identical difference spectra are to be expected.

The difference spectra for the bound dye in the presence of the trimer and hexamer of *N*-acetylglucosamine (at −40 °C, under conditions where the products of reactions 2 and 3, respectively, predominate) are shown in Figure 8. The difference spectra between (enzyme plus dye plus substrate) − (dye plus substrate) are quite different for the two substrates, indicating that the products of reaction 2 with the trimer and reaction 3 with the hexamer were different. Difference spectra of (enzyme plus dye plus substrate) − (enzyme plus dye) are also different for the trimer and hexamer.

Reaction with N-Acetylglucosamine. The interaction of lysozyme with *N*-acetylglucosamine was investigated over the −80 to 0 °C range. The only reaction detected was an initial rapid decrease in the fluorescence emission at 340 nm. At the high concentrations of sugar necessary to result in a detectable signal (>1 mM), the reaction was complete within a few seconds, even at −80 °C. Binding studies at −40 °C indicated an apparent dissociation constant of 1.25×10^{-3} M and that 1.5 mol of monomer bound per mol of enzyme.

The fluorescence difference spectra of the resulting lysozyme–*N*-acetylglucosamine complex were similar to those of the product of reaction 1 with the trimer, with a minimum at 330 nm and a pronounced shoulder at 350 nm. However, at pH* 7.0, in contrast to the trimer, the difference spectrum for the monomer remained negative in the 320–300-nm region.

NMR Spectra of Lysozyme in Cryosolvent. The possibility of using NMR as a technique to obtain structural information of accumulated enzyme–substrate intermediates, stabilized at subzero temperatures, was investigated as follows. Lysozyme solutions in aqueous 65% Me_2SO cryosolvent were prepared in which deuterated enzyme and solvent were used. These were examined at 100 and 360 MHz. The demonstrated stability of the enzyme in this cryosolvent at 25 °C (Hamaguchi, 1964a,b; this study) meant that spectra could be obtained of the native enzyme at 25 °C as well as subzero temperatures. The ^1H NMR spectra in the aromatic region in the cryosolvent at 360 MHz were similar to the enzyme in aqueous solution under similar conditions at 25 °C. On the solution being cooled, the peaks gradually broadened and at −40 °C were almost completely lost due to line width broadening.

Discussion

Our strategy of using cryoenzymology to provide detailed "time-lapse" pictures of the step-by-step transformation of substrate to product in an enzyme-catalyzed reaction has been outlined in detail in recent reviews [e.g., Fink (1977), Makinen & Fink (1977), and Fink & Petsko (1980)]. The key points are (1) to demonstrate that the cryosolvent and subzero temperatures have no adverse effect on the catalytic and structural properties of the enzyme, (2) to use suitable probes to monitor the interaction of dissolved enzyme with substrate at subzero temperatures and thereby determine conditions necessary to accumulate and stabilize intermediates on the productive catalytic pathway (i.e., kinetic and thermodynamic characterization of the intermediates), and (3) to use high-resolution techniques to obtain the detailed atomic structure of the intermediates, currently through the use of X-ray diffraction on the crystalline intermediate (Fink & Ahmed, 1976; Alber et al., 1976). The present investigation substantially fulfills the first two of these goals.

Cryosolvent Effects. Previous studies by Hamaguchi and co-workers have shown that, at 25 °C, concentrations up to about 60% methanol or dimethyl sulfoxide cause no detectable structural perturbations in lysozyme as monitored by UV absorbance, circular dichroism, and viscosity (Hamaguchi, 1964a,b; Hamaguchi & Kurono, 1963; Ikeda & Hamaguchi, 1970). Expectations that higher methanol concentrations would also have no effects at lower temperatures (Fink & Grey, 1978) are borne out in the data on the rates and dissociation constants for the reactions with chitotriose and chitohexose, which indicate that no adverse effects, either structural or catalytic, occur at subzero temperatures in cryosolvents up to at least 80% methanol, as well as in related studies by Douzou (Douzou et al., 1975; Maurel & Douzou, 1976).

That the presence of organic solvents causes an increase in K_m is a fairly general phenomenon (Fink, 1976b), which can be attributed to an increase in the dissociation constant for substrate binding (Fink, 1974; Fink and Tsai, unpublished results). The underlying cause is less favorable partitioning

of the substrate to the active site, compared to the bulk solvent, and probably involves the greater hydrophobicity of the cryosolvent relative to water (Maurel, 1978). We attribute the observed exponential decreases in the pseudo-first-order rate constant for cell wall hydrolysis (Figure 1), and the exponential increase observed in the dissociation constants for chitotriose and chitohexose (Table II) with increasing cosolvent, to such an effect. The data indicate that substrate binding is about 4 times poorer in 50% methanol and 60% dimethyl sulfoxide and 12 times poorer in 80% methanol. Maurel & Douzou (1976) have observed the expected decrease in k_{cat} for cell wall hydrolysis as the water concentration decreases in going from aqueous to 50% methanol solvent.

The observed exponential increase in substrate dissociation constant with increasing cosolvent concentration allows one to extrapolate K_d values measured at a particular cosolvent concentration to other values. We have taken advantage of this fact to compare our values of K_d measured in cryosolvent with those previously measured in aqueous solution by extrapolation to 0% cosolvent (Figure 6). For example, at 0 °C, the values of K_d for trimer and hexamer (under nonturnover conditions) extrapolated to 0% methanol are 5.3×10^{-6} M and 2.7×10^{-6} M, respectively. Since the kinetic expressions for the reaction rates include a term in the denominator involving the substrate dissociation constant (Fink, 1976b), all rates will be slowed down correspondingly, at substrate concentrations below saturation.

The pH dependence of cell wall, but not chitohexose, hydrolysis is shifted substantially from an optimum of 5.6 in water to pH* 7.1 in 60% aqueous Me_2SO. This type of phenomenon has been attributed to an electrostatic effect by Maurel & Douzou (1976) due to the highly charged surface of the cell walls. A similar effect is observed in aqueous methanol (Douzou et al., 1975).

The similar ^1H NMR spectra of lysozyme in water and cryosolvent are excellent evidence that the enzyme structure is not significantly perturbed by the cryosolvent. The disappearance of the signal with decreasing temperature is due to aggregation of the enzyme. This is a well-known phenomenon of lysozyme [e.g., Studebaker et al. (1971)] and effectively precludes the use of ^1H NMR in most subzero-temperature investigations of dissolved lysozyme. The fluorescence spectra (emission and excitation) exhibited anomalous shape when aggregation occurred and provided a simple means of monitoring the process. We found that aggregation in the cryosolvent was strongly temperature and cosolvent dependent and that the maximum enzyme concentration without aggregation was 3.5×10^{-7} M at −90 °C in 80% methanol.

On the basis of results of the present investigation, in conjunction with previously reported studies, we conclude that dimethyl sulfoxide and methanol cryosolvents have no adverse effects on the catalytic mechanism of hen egg white lysozyme at *subzero temperatures*.

Reactions of Lysozyme with Oligosaccharides of Chitose at Subzero Temperatures. The reactions observed under the nonturnover, subzero-temperature conditions may be compared with those obtained in other studies in aqueous solution, by using rapid reaction techniques, as a means of identifying them in terms of known events in lysozyme catalysis.

In an extensive and detailed study of the kinetics and thermodynamics of the reaction of lysozyme with *N*-acetylglucosamine oligosaccharides, Rupley and co-workers (Banerjee & Rupley, 1973a,b; Holler et al., 1975a,b; Banerjee et al., 1975) have pieced together the following picture for the reaction with the hexamer. The substrate may bind in a

Scheme I

$$E + S \overset{\alpha_1}{\rightleftharpoons} ES_1^{NP} \overset{\alpha_2}{\rightleftharpoons} ES_2^{NP}$$

$$E + S \overset{\beta_1}{\rightleftharpoons} ES_1 \overset{\beta_2}{\rightleftharpoons} ES_2 \overset{\gamma}{\rightleftharpoons} ES_3 \rightleftharpoons ES^* \rightarrow E + P$$

Scheme II

$$E + I \overset{i}{\rightleftharpoons} EI' \overset{1}{\rightleftharpoons} EI_1 \overset{2}{\rightleftharpoons} EI_2$$

nonproductive mode to the A, B, and C sites, in which the reducing end of the oligosaccharide binds to the C subsite of the enzyme. This is a two-step process, called the α process by Rupley. The substrate also binds *productively*, again a two-step process, the β process, followed by a third step, the γ process. In the productive complex the hexasaccharide is "lined up" with the A–F subsites. The first stage in the α and β processes corresponds to initial substrate binding or complexation; the subsequent steps, and the γ process, correspond to isomerizations (Banerjee et al., 1975). The reaction pathway may be envisioned as shown in Scheme I, where the superscript NP denotes nonproductive and ES* represents the putative oxocarbonium intermediate. From a large body of experimental data Rupley and co-workers were able to measure, or estimate, the rate and equilibrium constants, and the enthalpies and entropies, for the reaction with chitohexose (Banerjee et al., 1975). Points of particular interest are that at 25 °C, aqueous solution, the ratio of productive to nonproductive binding is approximately 1:1, and the α process is about 10 times faster than the β process, which is about 100 times faster than the γ process; the cleavage reaction is 10-fold slower than the γ process and has nearly twice the energy of activation; the enthalpies for the overall binding processes are such that binding should increase with decreasing temperature, but the lower temperatures will favor the nonproductive binding mode.

Reactions with the N-Acetylglucosamine Trimer. Under the nonturnover conditions used in this study, the reaction of the trimer with lysozyme involves it binding to the A, B, and C subsites. Substantial kinetic and thermodynamic data support the assumption that the binding of the trimer serves as a model for the nonproductive binding of the hexamer (Halford, 1975; Banerjee et al., 1975). We interpret the two reactions observed at subzero temperatures with the trisaccharide to correspond to the formation of an initial "loose" complex, followed by an isomerization involving at least changes in the conformation of the active-site cleft to form a "tighter" complex (Halford, 1975) (Scheme II).

Saturation kinetics observed for reaction 1 imply that this reaction actually corresponds to a two-step process, the initial enzyme–substrate complexation, followed by a subsequent isomerization. Reaction 2 corresponds to a further isomerization, also involving at least changes in the vicinity of Trp-62 and Trp-108. Thus, the minimum pathway for formation of the stable lysozyme–trimer complex at subzero temperatures is represented by Scheme II. As will become apparent in the following discussion, we believe reaction 1 corresponds to the α_1 and reaction 2 to the α_2 process of Rupley and co-workers (Banerjee et al., 1975). From comparison of the fluorescence difference spectra of EI_1 and the stable complex with *N*-acetylglucosamine (see later discussion), we believe that the major conformational change involving narrowing of the active-site cleft and movement of Trp-62 (Imoto et al., 1972a) has already occurred in EI_1.

The possibility that reaction 2 with the trimer involves an additional molecule binding to the E and F subsites can be

eliminated by the Scatchard plots and the results of the dye binding experiments, which show that reaction 2 of the trimer does not result in displacement of the dye.

The fluorescence difference spectra for the products of reaction 2 (Figure 4) have very similar shapes and pH dependencies to those observed by Halford (1975) and Lehrer & Fasman (1967) at 25 °C, aqueous solution, except that the whole subzero difference spectrum is blue-shifted ~30 nm. This shift is probably not due to dielectric constant effects since the difference spectra are essentially identical in 50 and 70% methanol at −40 °C, where the dielectric constants are 84 and 67, respectively (Douzou et al., 1976). Rather, the spectral shift probably reflects a combination of the more hydrophobic solvent and perturbations of the ionizations of Asp-101 and, particularly, Glu-35 in the cryosolvent, both of which would lead to blue shifts in the difference spectrum.

Halford (1975) has articulated the evidence to support the minimum in the lysozyme–trimer fluorescence difference spectrum being due to perturbations of the environment of Trp-62, whereas the maximum in the fluorescence difference spectrum at lower wavelength is due to changes associated with Trp-108. Thus, the minimum in the vicinity of 340 nm in the fluorescence difference spectra (Figure 4) is attributed to perturbations of Trp-62, the maximum near 300 nm being associated with perturbations of Trp-108.

A van't Hoff plot of K_d for the trimer (the dissociation constant for $E + I \rightleftharpoons EI_2$), adjusted to 0% cosolvent, shows a linear relation for the range 25 to −40 °C, pH* 7.2 (Figure 6), with an estimated ΔH of 3.8 kcal mol^{-1}. This is in contrast to a reported value of 13.7 kcal mol^{-1} in aqueous solution for the temperature range 8–60 °C at pH 5.3 (Banerjee & Rupley, 1973a,b). Thus, a major change in the enthalpy occurs for the reaction, probably near 25 °C at pH 7 in the cryosolvent. This change in the enthalpy of the reaction occurs in the temperature region where lysozyme has been reported to undergo a structural transition which also affects the catalytic parameters, especially at pH >5 (Jollès et al., 1975; Saint-Blanchard et al., 1977). Given our current understanding of protein dynamics, it is not at all surprising that large temperature changes (or other environmental perturbations) may induce different conformations in either the free enzyme or the enzyme–substrate complex, resulting in altered catalytic properties.

The value of K_d for chitotriose binding at subzero temperatures, extrapolated to 25 °C, aqueous solution, gives a value (1×10^{-5} M) in excellent agreement with that previously measured by Halford (1975) of 1×10^{-5} M. Since the expressions for the observed rates for reactions 1 and 2 are complex and contain several rate and dissociation constants, each of which has its own temperature dependence, and because of the need for corrections to take into account the effect of the cosolvent and viscosity effects, it is difficult to extrapolate the rate data shown in Figure 6 to estimate accurate values at 25 °C, aqueous solution.

For reaction 2, at a trimer concentration of 4.0×10^{-6} M, the rate expression will reduce to $k_{obsd} \simeq k_{-2}$, which is reported as 10 s^{-1} for pH 5.9, 25 °C (Halford, 1975). The value estimated from Figure 6 with appropriate corrections is 2 s^{-1}. For reaction 1 the value of k_{obsd} at −55 °C indicates a break in the Arrhenius plot around −70 °C. If the −70 and −55 °C data are extrapolated to 25 °C, 0% methanol, one obtains an estimated k_{obsd} of 10^3 s^{-1}. Since aqueous studies of trimer binding showed no evidence of saturation kinetics with ligand concentrations as high as millimolar for the fast reaction (Halford, 1975), comparison of the extrapolated data for reaction 1 with observed data in aqueous solution at 25 °C cannot be made.

Scheme III

$$E + S \underset{\beta_1}{\overset{1}{\rightleftharpoons}} ES_1 \underset{\beta_2}{\overset{2}{\rightleftharpoons}} ES_2 \overset{3}{\rightleftharpoons} ES_3$$

It is clear that the enthalpy of activation of reaction 1 must increase at temperatures above −70 °C, or this step would become rate limiting. At least part of this apparent change in the energy of activation is attributable to the viscosity of the cryosolvents, which will result in substantial rate reductions of the initial binding (and subsequent steps under nonsaturating conditions) at temperatures below −70 °C.

The following similarities between the reactions observed for trimer binding at subzero temperatures and those in aqueous solution, 25 °C, suggest that the reaction mechanism is basically the same in both cases: the shape of the fluorescence difference spectra; the pH dependencies of the fluorescence difference spectra; the magnitude of the overall binding constant; the accord observed in the rate of reaction 2; the observed biphasicity.

Our data indicate that in solution EI_2 is effectively indefinitely stable at −40 °C and that EI_1 can be stabilized for periods of hours at −70 °C or below. In addition, kinetic analysis (Fink, 1976b) indicates that both species can be accumulated in stoichiometric concentrations equivalent to the limiting reagent.

Reaction of the N-Acetylglucosamine Hexamer. There are two most likely interpretations of the results with the hexamer. One is that the three reactions observed by fluorescence changes at subzero temperatures correspond to the β_1, β_2, and γ processes, respectively, i.e., to productive binding and subsequent isomerizations. Alternatively, in view of the similarities in the properties of reactions 1 and 2 of the trimer and reactions 2 and 3 of the hexamer, it is possible that reactions 2 and 3 of the hexamer correspond to nonproductive binding, i.e., the α process, and that reaction 1 may be connected with the initial binding in the productive mode, i.e., the β_1 process. Since the results of the fluorescence experiments did not allow an entirely unambiguous resolution of this issue, we examined the interaction of the dye Biebrich Scarlet, which has previously been shown to bind to the E or F subsite (Holler et al., 1975a,b), with the products of reaction 2 of the trimer and reaction 3 of the hexamer. If the latter two reactions observed with the hexasaccharide did involve nonproductive binding, then the difference spectra (with the dye) should be essentially identical with that for the trimer (under substrate saturation conditions).

As shown in Figure 8, the results of dye-binding studies clearly indicate that the product of reaction 3 with the hexamer is different from that of the product of reaction 2 of the trimer. Another significant difference between reaction 2 of the trimer and reaction 3 of the hexamer is the fact that the difference spectra of the products are quite different at low pH*. Reaction 2 of the hexamer and reaction 1 of the trimer also differ significantly in their rates. The results will therefore be considered in the light of the interpretations that the three reactions observed with the hexamer correspond to the β_1, β_2, and γ processes of Rupley and co-workers, i.e., Scheme III. The fluorescence difference spectra for the products of each of the three reactions with the hexamer are clearly different from each other, indicating significant differences in the environment about Trp-108 and -62 in the different intermediates.

As mentioned in the discussion of the trimer, van't Hoff plots

for both trimer and hexamer show that changes in the enthalpy for overall binding of both substrates occur around 25 °C. For the hexamer the energy of activation at lower temperatures is of the order of 5.3 kcal mol^{-1}, somewhat larger than that for the trimer. Under comparable conditions the dissociation constant for the trimer may be used to obtain an estimate of the dissociation constant for nonproductive binding of the hexamer and, hence, a measure of the ratio of productive to nonproductive binding in the hexamer. In contrast to the case at 25 °C where an approximately 1:1 ratio of productive to nonproductive binding occurs (Banerjee et al., 1975), at −40 °C the hexamer binds 4 times more tightly, indicating much more favorable productive binding at the lower temperature. This is in marked contrast to the extrapolation of the data of Banerjee et al. (1975) above 25 °C, which predicts essentially only nonproductive binding at such low temperatures. Thus, the change in enthalpy accompanying the temperature-induced structural transition leads to more favorable binding in the productive mode.

The observed tighter binding of the hexamer relative to the trimer as the temperature is decreased (Figure 6) is in accord with expectations based on the crystallographic data. Examination of the contributions of hydrogen bonding and nonpolar (hydrophobic) interactions, in terms of contacts between the enzyme and oligosaccharide, indicates that the relative contribution of hydrogen binding is greater for the hexasaccharide than for the trimer (Imoto et al., 1972a). Since hydrogen-bond strength will increase and hydrophobic interaction strength will decrease, as the temperature decreases, one would predict relatively tighter binding of the hexasaccharide to the low-temperature form of lysozyme.

The value of the observed K_d for the hexamer at −70 °C cannot be compared with those at higher temperatures because reaction 3 is negligible at these temperatures. The measured value of K_d at −70 °C thus corresponds to that of K_{13}^{β} of Banerjee et al. (1975). Extrapolation of the van't Hoff plot for the K_d values at higher temperature (i.e., $K_d^{\beta\gamma}$) allows an estimate of K_d^{γ} (i.e., k_{-3}/k_3) to be made. The value calculated is 0.1 at −70 °C, pH* 7.2. This may be contrasted with a value of 0.3 at 25 °C (Banerjee et al., 1975).

Although the data for reaction 1 indicate a linear relationship between k_{obsd} and $[S]_0$, it was not technically feasible to carry these experiments out over a sufficiently broad range of substrate concentration to determine whether or not saturation occurs. In fact it seems likely, based on the estimated[3] value of k_1 (3×10^6 M^{-1} s^{-1} at 25 °C; 0% methanol), that reaction 1 is not the initial diffusion-controlled complexation but, analogous to the case with the trimer, includes a subsequent isomerization. Productive binding of the hexasaccharide is also favored kinetically as indicated by the faster rate for reaction 1 of the hexamer compared to that for the trimer. Since reaction 2 for chitohexose remains faster than reaction 3 at higher temperatures, it is apparent that E_a for reaction 2 must increase in the −70 to −40 °C range.

The data in Table I reveal that the ES_3 complex may readily be trapped at −40 °C in cryosolvent solution. At −90 °C the ES_2 complex can be accumulated and is stable for many hours whereas the ES_1 complex has a rather short lifetime ($t_{1/2}$ = 90 s).

Reaction with N-Acetylglucosamine. Previous studies have shown that the monomer binds to the C subsite, due to the strong interactions involving the acetamido group (Imoto et al., 1972a). It is also known that a sugar in the C subsite has strong interactions with Trp-62 and weaker ones with Trp-108 (Imoto et al., 1972a; Ford et al., 1974). The fluorescence difference spectrum for the lysozyme–monomer complex indicates very similar perturbations of Trp-62 to those in the EI_1 complex with the trimer, whereas the spectral change attributable to Trp-108 is quite different. We thus conclude that the environment about Trp-62 is very similar in EI_1 and that the narrowing of the active-site cleft has already occurred in EI_1. Differences in the environment of Trp-62 and -108 between the monomer and trimer complexes have also been observed by chemical shift changes in NMR experiments (Blake et al., 1978).

The 1000-fold poorer binding of the monomer compared to the trimer and hexamer, at subzero temperatures, is also consistent with results under normal conditions and reflects the lesser number of enzyme–ligand contacts. The high concentration of monomer required to achieve a detectable signal precluded experiments to determine whether isomerization steps also occurred in monomer binding. Since Scatchard plots indicated 1.5 mol of *N*-acetylglucosamine per mol of lysozyme, it appears that under the low-temperature conditions, in contrast to results at 25 °C, a second molecule of monomer binds, somewhat more weakly. Since this latter molecule perturbs the Trp-62 fluorescence, it is probably occupying subsite B, as Trp-62 is known to have contacts with sugar residues in both sites B and C (Imoto et al., 1972a).

Crystalline Enzyme–Substrate Intermediates. We have previously shown that crystalline enzyme–substrate intermediates can be formed at subzero temperatures by adding crystalline enzyme to the cryosolvent and then allowing the substrate to diffuse in at the appropriate temperature (Fink & Ahmed, 1976; Alber et al., 1976). One advantage of this procedure is that the reduced rate of diffusion through the interstices of the crystal results in substantial additional rate reduction (typically 1–2 orders of magnitude). However, in the case of lysozyme this approach does not seem feasible at present because the known forms of the crystalline enzyme (with the possible exception of that from tortoise) have structures such that large substrates (e.g., chitohexose) are unable to diffuse into the active site due to occlusion by neighboring enzyme molecules. If a suitable crystalline form can be found, the anticipated rate reductions due to the reduced diffusion rates would probably allow the stabilization of all the observed intermediates. This would then allow the detailed structures of these species to be determined at atomic resolution and would allow unambiguous resolution of the issue of the role of substrate distortion. In the meantime we are attempting to crystallize, at subzero temperatures, the trapped ES_3 complex of the hexamer, since this intermediate should have the residue of the D subsite in the distorted form, if distortion does occur. Initial experiments have shown that crystals can indeed be obtained in this manner.

Conclusion

The results of the present investigation indicate that methanol cryosolvents may be satisfactorily used in the study of lysozyme reactions at subzero temperatures. On the basis of changes in the fluorescence emission and dye-displacement experiments, we conclude that the reaction of lysozyme with chitohexose at subzero temperatures involves predominantly productive binding to the A–F subsites. This reaction, under the nonturnover conditions, occurs via at least a three-step process. The latter two stages of the process correspond to isomerizations of the enzyme–substrate complex in which it is presumed that binding energy is used to achieve optimal alignment of the substrate and enzyme catalytic groups. With

[3] Assuming an energy of activation of 3 kcal mol^{-1} and correcting for viscosity and cosolvent effects.

the trimer, a two-step process is observed, corresponding to initial complexation and subsequent isomerizations.

Comparison of the data at subzero temperatures with those at higher temperatures indicates that the enthalpy of substrate binding changes significantly in the temperature range (25 °C) where the enzyme undergoes a structural reorganization. The low-temperature form of the enzyme binds hexasaccharide substrate predominantly in the productive mode because of kinetic as well as thermodynamic control. The fluorescence emission spectra serve as probes for the A–C subsite region of the enzyme. Thus, the observed fluorescence changes reflect the enzyme environment in this region. The similarities in many of the characteristics of the two isomerizations in the reaction of the hexamer to the binding and isomerization of the trimer suggest underlying structural similarities, which, in turn, are consistent with the hypothesis of substrate distortion in the D subsite.

Acknowledgments

The 360-MHz ^{1}H NMR spectra were obtained at the Stanford Magnetic Resonance Laboratory which is supported by National Institutes of Health Grant No. RR00771 and National Science Foundation Grant No. GR23633. We are also indebted to Professor J. Rupley for the gift of the chitose oligosaccharides.

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