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Oxygen-18 Leaving Group Kinetic Isotope Effects on the Hydrolysis of Nitrophenyl Glycosides. 2. Lysozyme and β-Glucosidase: Acid and Alkaline Hydrolysis†

Steven Rosenberg and Jack F. Kirsch*

ABSTRACT: Oxygen-18 leaving group kinetic isotope effects (KIEs) have been measured for a set of glycosyl transfer reactions with *p*-nitrophenyl β-D-glycosides as substrates. Acid-catalyzed hydrolysis and alkaline hydrolysis exhibit KIEs of k_{16}/k_{18} = 1.0355 ± 0.0015 and 1.0386 ± 0.0032, respectively. Lysozyme and β-glucosidase A show KIEs on V_{max}/K_m (V/K) of $(V/K)_{16}/(V/K)_{18}$ = 1.0467 ± 0.0015 and 1.0377 ± 0.0061, respectively. The large magnitude of these KIEs requires that carbon–oxygen bond scission be far advanced in the transition states for these reactions; therefore in the transition states for the first irreversible steps in these reaction sequences, scission of the glycosidic bond must be essentially complete for the reactions catalyzed by lysozyme and β-glucosidase A, which are thought to proceed via S_N1 and S_N2 mechanisms, respectively. Acid-catalyzed hydrolysis is shown to proceed through a transition state involving at least 80% C–O bond cleavage and only partial proton transfer to the leaving *p*-nitrophenyl oxygen atom.

The mechanisms of enzyme-catalyzed hydrolysis of glycosides and oligosaccharides are among the most studied and best understood in enzymology. Two limiting mechanisms of catalysis have been proposed for these reactions. The first, involving formation of an enzyme-bound oxocarbonium ion with the assistance of general acid catalysis, is typified by the reaction catalyzed by hen egg-white lysozyme (Blake et al., 1967). The second mechanism, that of double displacement first proposed by Koshland (1953), involves nucleophilic attack by the enzyme to form a covalent glycosyl-enzyme intermediate followed by its hydrolysis. More complex schemes involving a combination of these mechanisms, kinetically significant protein conformation changes, and possible intramolecular attack by the C-2 oxygen atom of the substrate have been proposed for β-galactosidase and other enzymes such as β-glucosidase (Sinnott & Souchard, 1973; Brockhaus et al., 1979; Hehre et al., 1977).

Secondary deuterium KIEs[1] have been utilized to study these reactions and to attempt to differentiate between the two mechanisms outlined above (Dahlquist et al., 1969; Sinnott & Souchard, 1973; Sinnott, 1978). However, recent work has led to some question being cast upon the validity of such measurements to differentiate between S_N1 and S_N2 mechanisms (Craze et al., 1978; Knier & Jencks, 1980). The previous paper describes the application of new techniques for the measurement of small kinetic isotope effects to the determination of oxygen-18 leaving group KIEs for reactions catalyzed by *Escherichia coli lacZ* β-galactosidase (Rosenberg & Kirsch, 1981). The measurement of the KIEs due to a second isotope near the reaction center is useful in resolving mechanistic ambiguities, as shown, for example, by Goitein et al. (1978) by using ^{14}C KIEs. This communication extends our studies on β-galactosidase to reactions catalyzed by lysozyme and β-glucosidase and to some relevant nonenzymatic reactions. The results obtained yield unique information on transition-state structure and the nature of the rate-determining step in these reactions.

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[1] Abbreviations used: KIE, kinetic isotope effect; EIE, equilibrium isotope effect; GlcNAc, 2-acetamido-2-deoxy β-D-glucopyranoside; PNPGlu, *p*-nitrophenyl β-D-glucopyranoside; PNPGal, *p*-nitrophenyl β-D-galactopyranoside; GlcNAcGluPNP, *p*-nitrophenyl 4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside.

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Experimental Procedures

Materials

Acetone, dried over anhydrous potassium carbonate, and chloroform were distilled and stored over molecular sieves. Diglyme was stirred over CaH_2 and $LiAlH_4$ overnight, distilled under reduced pressure, and stored under N_2. Sodium methoxide in methanol was prepared by the careful addition of sodium metal to anhydrous methanol. The concentration of the solution was determined by titration with HCl. The synthesis of *p*-nitro[^{18}O]phenol has been described (Rosenberg & Kirsch, 1979b). Unlabeled *p*-nitrophenol was purchased from Aldrich and recrystallized before use. Acetobromoglucose and acetobromogalactose were obtained from Sigma. Chitin, also from Sigma, was ground for 48 h in a ball mill before use and was a gift of Dr. C. Reading. Hen egg-white lysozyme was purchased from Calbiochem as a 3 times recrystallized powder. The purified A isozyme of β-glucosidase from sweet almonds was a gift of J. Weber. Bio-Gel P-2 (100–200 mesh) was obtained from Bio-Rad, as was AG501-X8 mixed-bed ion-exchange resin. All other materials were reagent grade and were used without further purification. Glass-distilled water was used in all isotope effect experiments.

p-Nitrophenyl β-D-Glucoside and p-Nitrophenyl β-D-Galactoside. These compounds were prepared by standard methods from the appropriate protected bromo sugar and either natural abundance or oxygen-18-enriched *p*-nitrophenol with subsequent deacetylation in sodium methoxide in methanol (Conchie & Levy, 1963; Leaback, 1960). Both the natural abundance glycosides and those enriched with oxygen-18 were synthesized from the same batch of acetobromogalactose or acetobromoglucose. The purities of these substrates were determined by melting point (164–165 °C for the glucoside and 178–179 °C for the galactoside), NMR, and UV–visible spectrophotometry. The enriched and natural abundance glycosides were identical by these criteria in all cases. The oxygen-18 contents of enriched glycosides were determined by direct mass spectrometry of the *p*-nitrophenol resulting from complete acid hydrolysis of the glycosides. In all cases, the enrichment was identical with that of the *p*-nitrophenol used in their synthesis.

$GlcNAc_4$. This compound was obtained by partial acid hydrolysis of chitin and purification of the *N*-acetylglucosamine (GlcNAc) oligomers by a combination of the methods employed by Rupley (1964) and Raftery and co-workers (1969). A sample of authentic $GlcNAc_4$ which was used as a standard in gel filtration was the generous gift of C. Reading. Chitin was hydrolyzed in concentrated HCl according to Rupley (1964), neutralized, and loaded onto a 4 × 50 cm charcoal-Celite column. This column was developed with a 5-L gradient from 0% to 60% ethanol and finally washed with 1 L of 60% ethanol. Twenty-milliliter fractions were collected and assayed for reducing sugars by the method of Park & Johnson (1949). This relatively crude purification of the GlcNAc oligomers was supplemented by pooling groups of fractions from the charcoal–Celite column and applying them to a Bio-Gel P-2 column (2.25 × 200 cm) which was developed with 0.1 M NaCl. The desired oligomer, $GlcNAc_4$, to be used in the synthesis of the lysozyme substrate, was subsequently rechromatographed on this column. The identity of this substance was determined by its position of elution from P-2 in relation to standards, by its mobility upon paper chromatography in ethyl acetate/pyridine/water (5:3:2), $R_f = 0.34$, and by its ability to act as a transferase substrate in the presence of lysozyme and the appropriate acceptor, usually *p*-nitrophenyl β-D-glucoside (see below).

p-Nitrophenyl 2-Acetamido-2-deoxy-β-D-glucopyranosyl β-D-[4-^{18}O]Glucopyranoside (GlcNAcGluPNP). This material was prepared from *p*-nitrophenyl β-D-glucoside ~50% enriched with oxygen-18 in the glycosyl oxygen atom and $GlcNAc_4$ by using the transferase activity of lysozyme as described by Rand-Meir et al. (1969). A mixture of 0.38 g of PNPGlu, 50% enriched with oxygen-18, 0.125 g of lysozyme, and 0.125 g of $GlcNAc_4$ was incubated in a solution of 20 mL of 0.1 M sodium citrate (pH 5.2), 3 mL of dry dioxane, and 3 drops of toluene at 40 °C for 18 h. This mixture was then applied to a P-2 column (2.25 × 200 cm) and the column developed in 0.1 M NaCl. Fifteen-milliliter fractions were collected. The presence of *p*-nitrophenyl glycosides was detected by their absorbance at 280 nm. Fractions containing the substrate (GlcNAcGluPNP) and the unreacted oxygen-18-enriched PNPGlu were pooled, desalted by using AG501-X8 mixed-bed ion-exchange resin, and lyophilized. The recovered PNPGlu was recycled to prepare more of the substrate. Typical overall yields of enriched glycosides were in the range 30–40%. Rechromatography of the substrate, GluNAcGluPNP, on P-2 in distilled water yielded a single sharp peak. This material exhibited a melting point of 254–255 °C [lit. mp 256–258 °C (Rand-Meir et al., 1969)].

Methods

Apparatus. All spectrophotometric measurements for isotope effect experiments were made on a Cary Model 118C spectrophotometer interfaced via a microprocessor (Claremont Research) to a teletype and tape punch. Other spectrophotometric measurements were made on a Zeiss Model PMQ spectrophotometer. Mass spectrometric analysis was done on a Du Pont Model 21-491 mass spectrometer equipped with a Columbia Scientific Model 260-722 data system and digital printer. Measurements of pH were carried out on a Radiometer Model pHM4c pH meter equipped with a B-type electrode.

Measurement of Isotope Effects by Mass Spectrometry. The general method used has been described in detail previously (Rosenberg & Kirsch, 1979a). Briefly, the method involves isolating the product of the reaction, in the cases discussed below, *p*-nitrophenol, at ca. 10% and 100% reactions and comparing the isotope ratios at these extents of reaction. This is accomplished by direct mass spectrometric analysis of the *p*-nitrophenol without conversion to a gas, and the necessary precision of ~0.2% is obtained by making 100–1000 measurements of the relevant m/e peaks.

Acid-Catalyzed Hydrolysis. A solution of 90 mL of 2.2 N HCl in a stoppered test tube was temperature equilibrated at 50 °C in a thermostated circulating water bath for 30 min. To this was added 10 mL of a 13 mM solution of PNPGlu 50% enriched with oxygen-18, in glass-distilled water. After mixing was complete, 10 mL of the solution was removed and placed in a separate test tube also at 50 °C. Two 100-μL aliquots were removed from the first solution at 30-min intervals and diluted into 3.00 mL of 0.2 M sodium borate, pH 9.5, and their absorbance at 400 nm was determined. The pseudo-first-order rate constant under these conditions is 1×10^{-5} s^{-1}. When the absorbance measurements indicated that ~10% of the reactant had hydrolyzed, the main reaction mixture was placed on ice to quench it.

The 10-mL aliquot which had been initially removed was then placed at 100 °C for 45 min to force the reaction to completion. Two 100-μL aliquots were removed at the end of this period to determine the end-point absorbance. The *p*-nitrophenol product was isolated from the two solutions by using the same procedures. The cooled solutions were each

extracted twice with ether. The ethereal solutions were combined and extracted twice with 0.1 N sodium carbonate, and the aqueous phases were acidified to pH 2. They were then extracted twice with ether (10 mL), and the ethereal solutions were dried with sodium sulfate and then taken to dryness in a vacuum oven at 40–60 °C. The crystalline residues were dissolved in 200–250 μL of dry chloroform, and three to four samples of both the 10% reaction and 100% reaction points were transferred to mass spectrometry sample tubes. The solvent was removed in a vacuum oven before the mass spectrometric analysis was performed.

β-Glucosidase A Catalyzed Hydrolysis. A solution of 200 mL of 0.1 M sodium phosphate, pH 6.0, containing 0.5 mM PNPGlu 50% enriched with oxygen-18, was equilibrated at 25 °C in a thermostated circulating water bath. The reaction was initiated by the addition of 100 μL of a 22 mg/mL solution of purified β-glucosidase A in 0.1 M sodium acetate, pH 5.5. Subsequent to mixing with an overhead stirrer, ~2 mL of the reaction mixture was transferred to a cuvette, and the time course of the reaction was monitored at 400 nm by using a Cary Model 118C spectrophotometer. The time of the first data point recorded by the teletype interfaced to the spectrophotometer was noted. Approximately 20 mL of the reaction mixture was transferred to a second flask equilibrated in the water bath and allowed to react to completion. The majority of the reaction mixture was quenched at ~10% reaction by the addition of 10 mL of 1.0 N HCl with stirring, and the solution was placed on ice. The *p*-nitrophenol product was isolated from the quenched solution (pH 2.3) by extracting 2 times with ether (150 mL). The ethereal solutions were combined and taken to dryness by rotary evaporation. The residue was dissolved in ~10 mL of 0.1 N sodium carbonate, acidified with HCl to pH 2, and extracted twice with 10 mL of ether. These ethereal solutions were combined, dried with sodium sulfate, and taken to dryness in a vacuum oven. The identical procedure was followed with the aliquot which was allowed to react to completion. Samples were prepared for mass spectrometric analysis as for the acid-catalyzed hydrolysis of this substrate.

Lysozyme-Catalyzed Hydrolysis. Isotope effect experiments were carried out at 40 °C in 0.1 M sodium citrate buffer, pH 5.5, at an enzyme concentration of 3.6 mM and an initial substrate concentration of between 7.4 and 9.1 mM. The substrate GlcNAcGluPNP contained 50% oxygen-18 in the glycosidic oxygen atom initially. Two 25-μL aliquots were removed at ~4-h intervals and diluted into 2.00 mL of 0.05 N NaOH. The solutions were measured at 400 nm to determined the extent of reaction.

At a time corresponding to between 15% and 25% of the total reaction, an aliquot amounting to 10% of the total reaction volume was removed. An equivalent volume of 5.65 N HCl was added, and this solution was incubated at 100 °C for 45 min to hydrolyze the substrate to completion. The majority of the reaction mixture was acidified to pH 3 and extracted twice with 10 mL of ether. The high protein concentration used in these reactions, necessitated by the low reactivity of the substrate, caused a suspension to form upon ether addition so that the two phases were separated by centrifugation. The *p*-nitrophenol product was then isolated by the same procedure as for acid-catalyzed hydrolysis described above. After the separation of the phases, the aqueous solution containing the unreacted substrate was loaded onto a P-2 column (2.25 × 200 cm) and eluted with 0.1 M NaCl. The unreacted substrate could then be recovered. This procedure also served as a control on the position of the enzymatic cleavage of the substrate. Other workers have shown that this substrate is uniquely cleaved to yield the disaccharide and *p*-nitrophenol (Rand-Meir et al., 1969).

If cleavage occurred to yield PNPGlu and GlcNAc, the former would have been detected by a peak of 280-nm absorbance of the appropriate mobility on the column. A comparison of the column profiles of the starting material (GlcNAcGluPNP) and the material recovered as described above indicated that little or no production of PNPGlu had occurred. A maximum of 2% cleavage to yield PNPGlu was calculated from the known extinction coefficients of PNPGlu and GlcNAcGluPNP and the column profile, in agreement with the results of Rand-Meir et al. (1969).

Determination of the Extent of Reaction. Since the KIE is determined by the variation of the isotope ratio of the product with the extent of reaction, it is important to measure this quantity precisely. The entire time course of the β-glucosidase A catalyzed hydrolysis of PNPGlu was monitored, and the resulting data were fit to pseudo-first-order kinetics by nonlinear regression analysis. The extent of reaction at which the initial aliquot was quenched was calculated simply from the ratio of the absorbance at the time of quenching to that at the end point of the reaction, determined by computer analysis.

In the cases of acid-catalyzed and lysozyme-catalyzed hydrolysis, the slow rates of reaction and the lack of substantial absorbance changes made it impossible to follow the complete time courses, so two alternative methods were utilized. The method of choice was to compare the absorbance at the time of quenching to that after the reaction had been forced to completion by incubation of the reaction mixture at 100 °C for 45 min in 2–3 N HCl. This method was used for the acid-catalyzed hydrolysis reaction. The high protein concentrations necessary in the lysozyme experiments introduced difficulties with this method, because the *p*-nitrophenol product was trapped to a substantial and variable extent in the denatured protein subsequent to acid hydrolysis. For this reason the theoretical end-point absorbances, calculated from the known initial concentration of the substrate and the extinction coefficient of *p*-nitrophenoxide at 400 nm, were utilized to determine the fraction of reaction when the initial aliquot was quenched. Although this method is certainly less accurate than nonlinear regression analysis of the entire reaction time course, the kinetic isotope effect is relatively insensitive to the fraction of reaction up to 25% reaction if the isotope ratios of the product are measured. This was shown by fitting the observed isotope ratios at ca. 10% and 100% reactions for one of the lysozyme experiments (see Figure 3) to eq 1, assuming different extents of reaction for the aliquot quenched early in the reaction. Variation of the extent of reaction from 5% to 25% altered the calculated KIE only from 1.044 to 1.050 when the isotope ratios of the product were 0.9651 ± 0.0009 early in the reaction and 1.0066 ± 0.0012 at completion.

Mass Spectrometric Analysis. The three or four product samples from ca. 10% and 100% reactions were analyzed alternately. Samples were introduced into the spectrometer via the solid probe at a source temperature of 70–100 °C. The molecular ion peaks of oxygen-18-labeled and unlabeled *p*-nitrophenol (*m/e* 141 and 139) were scanned at ~5-s intervals for the lifetime of the samples in the spectrometer. Thus, the isotope ratio of each sample was measured between 40 and 400 times.

Data Analysis. A complete experiment consisting of three to five samples of the product at 10% and 100% reactions generated 1000–2000 measurements of the isotope ratios. No

allowance was made for the natural abundance of oxygen-18 or other stable isotopes, as this correction is smaller than the experimental error with the relatively high isotopic enrichment used. Thus, only the P and P + 2 peaks were included in the data analysis, corresponding to m/e 139 and 141. These pairs of values were analyzed with the aid of an off-line computer (CDC 6400) in sets of ~20 scans to yield the mean and standard error of the isotope ratio [^{18}O]/[^{16}O]. The weighted mean of the isotope ratio for each sample and its associated standard error were calculated from the isotope ratios for each set by normal statistical methods (Rosenberg & Kirsch, 1979a). This procedure was followed for each product sample at 10% and 100% reactions, and the resulting isotope ratios at these two extents of reaction were used to yield the final isotope ratios and their standard errors by weighted averaging. The kinetic isotope effect was calculated from the isotope ratios at ca. 10% and 100% reactions and from the exact fraction of reaction at the time of quenching. This value is given by eq 1 (Bigeleisen & Wolfsberg, 1958) where f is the fraction

$$\mathrm{KIE} = \frac{\ln\,[1 - [f(R_{100} + 1)/(R + 1)]]}{\ln\,[1 - [fR(R_{100} + 1)/[R_{100}(R + 1)]]]} \quad (1)$$

of reaction (usually about 10%), R is the isotope ratio of the product at f, and R_{100} is the isotope ratio of the product at the completion of the reaction.

Measurement of Isotope Effects by Direct Spectrophotometry—Alkaline Hydrolysis. The method used has been described in detail elsewhere (Rosenberg & Kirsch, 1979b). Briefly, it involves determining directly the difference in rates between natural abundance and highly isotopically enriched reactants by using a high-precision spectrophotometer interfaced via a microprocessor to a teletype and tape punch for direct digital output, coupled with analysis of the resulting data by nonlinear regression analysis.

The KIE on alkaline hydrolysis of PNPGal was measured by this method comparing natural abundance and 74% oxygen-18-enriched galactosides. The experiments were performed at 35 °C in 1.0 N NaOH with an initial concentration of galactoside of 82–87 μM; under these conditions, the pseudo-first-order rate constant is 8×10^{-5} s^{-1}. Reactions were run in sets of 4–5, monitored simultaneously; each set contained two or three enriched and natural abundance galactosides. The kinetics were monitored at 400 nm for 3 or more half-lives.

The resulting data were analyzed by nonlinear regression analysis by fitting the data to a single exponential with the end point and the rate constant as variable parameters. The observed KIEs for each set were used to calculate the final observed KIE by weighted averaging. Lastly, this value was corrected for incomplete labeling of the enriched substrate by using eq 2 (Dahlquist et al., 1969), where f is the fraction of

$$\mathrm{KIE_{cor}} = (\mathrm{KIE_{obsd}} - 1 + f)/f \quad (2)$$

the isotopic enrichment in the labeled substrate, $\mathrm{KIE_{obsd}}$ is the observed KIE for a complete experiment, and $\mathrm{KIE_{cor}}$ is the KIE corrected for incomplete enrichment.

Results

Acid and Alkaline Hydrolysis. Some typical results of the measurement of the oxygen-18 leaving group KIE for the acid-catalyzed hydrolysis of PNPGlu (50.1% enriched with oxygen-18) are shown in Figure 1. The magnitude of the KIE observed, $k_{16}/k_{18} = 1.0358 \pm 0.0023$, requires that a large degree of carbon–oxygen bond scission occurs in the transition state for this reaction. A similar result ($k_{16}/k_{18} = 1.03$) was

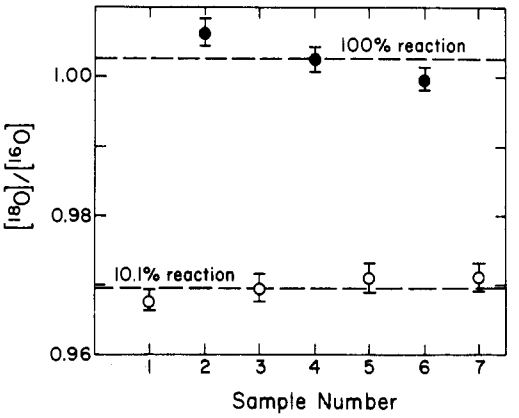


FIGURE 1: Typical data for the determination of the oxygen-18 kinetic isotope effect on acid-catalyzed hydrolysis of p-nitrophenyl β-D-glucoside. The observed isotope ratios of the product, p-nitrophenol, are shown after hydrolysis of the reactant at 50 °C in 2.0 N HCl at 10.1% (○) and 100% reactions (●). The isotope ratios were determined by direct mass spectrometric analysis of the product. Each point represents between 140 and 360 individual measurements of the isotope ratios, and the error bars show one standard error. The weighted means of the isotope ratios, given by the dashed lines, are 0.9697 ± 0.0009 and 1.0026 ± 0.0019 at 10.1% and 100% reactions, respectively. The isotope effect calculated from these data is $k_{16}/k_{18} = 1.0358 \pm 0.0023$.

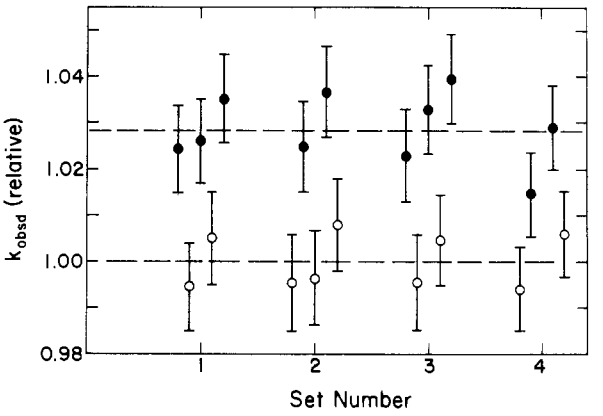


FIGURE 2: Measurement of the kinetic isotope effect on alkaline hydrolysis of p-nitrophenyl β-D-galactoside. The relative values of the pseudo-first-order rate constants for the alkaline hydrolysis of natural abundance PNPGal (●) and 74% oxygen-18-enriched PNPGal (○) at 35 °C in 1.0 N NaOH are shown. The average value of k_{obsd} for the enriched substrate for each set was arbitrarily set at 1.000. The error bars represent the standard errors in k_{obsd} for each individual kinetic run as calculated by nonlinear regression analysis. The upper dashed line indicates the observed isotope effect, $k_{16}/k_{18} = 1.0286 \pm 0.0024$, which is the weighted mean of all the isotope effect determinations in this experiment (nine enriched and ten unenriched) calculated as described in the text.

obtained by earlier workers for the acid-catalyzed hydrolysis of methyl α-D-glucopyranoside (Banks et al., 1961).

The alkaline hydrolysis of phenyl glycosides is thought to proceed via intramolecular attack of the C-2′ hydroxyl in its ionized form on the anomeric carbon atom (Ballou, 1954). In contrast to acid-catalyzed hydrolysis, this reaction represents nucleophilic attack at (presumably) C-1, rather than an S_N1-like process. The results of a series of measurements of the rates of hydrolysis of natural abundance PNPGal and that substituted with oxygen-18 (74% enrichment) in the glycosidic oxygen atom are shown in Figure 2. It is clear that ^{18}O substitution substantially reduces the rate of hydrolysis; the *observed* KIE is $k_{16}/k_{18} = 1.0286 \pm 0.0024$. Thus, the rate-determining step in this reaction must be characterized by a transition state with substantial carbon–oxygen bond cleavage.

Table I: Oxygen-18 Leaving Group Kinetic Isotope Effect Experiments on Glycosyl Transfer Reactions of *p*-Nitrophenyl Glycosides

catalyst	conditions	expt[a]	method[a]	KIE	final KIE[c]	% bond cleavage[d]
H_3O^+	2.0 N HCl, 50 °C	1	competitive	1.0358 (0.0023)	1.0355 (0.0015)	see Table II
H_3O^+	2.0 N HCl, 50 °C	2	competitive	1.0353 (0.0020)		
^-OH	1.0 N NaOH, 35 °C	1	direct	1.0386[b] (0.0032)		*e*
lysozyme	pH 5.5, 40 °C	1	competitive	1.0468 (0.0017)	1.0467 (0.0015)	110 ± 4%
lysozyme	pH 5.5, 40 °C	2	competitive	1.0465 (0.0031)		
β-glucosidase A	pH 6.0, 25 °C	1	competitive	1.0391 (0.0068)	1.0377 (0.0061)	89 ± 14%
β-glucosidase A	pH 6.0, 25 °C	2	competitive	1.032 (0.014)		

[a] Each experiment is a completely independent measurement of the isotope effect determined either by the competitive technique using mass spectrometric analysis of the product as a function of the extent of the reaction or by the direct technique of spectrophotometric measurement of the difference in rate due to isotopic substitution (Rosenberg & Kirsch, 1979a,b). Values in parentheses are the propagated standard errors of the isotope effects. [b] The alkaline hydrolysis reaction was not measured with 100% enriched substrate, so the observed KIE (1.0286 ± 0.0024) was corrected for the fraction of enrichment (0.74) by using eq 2, yielding the value shown above. [c] The final KIEs are the weighted means of the two independent experiments for the reactions shown. [d] The fraction of bond cleavage is calculated from the theoretical maximum KIE for complete bond scission (1.0425), and the observed KIE, assuming that bond cleavage is completely rate determining and a linear relationship between the KIE and remaining bond order (Bilkadi et al., 1975; Saunders, 1975; Hogg et al., 1980). [e] The fraction of bond cleavage for alkaline hydrolysis is unknown because of the complication of two concurrent pathways for hydrolysis (see text).

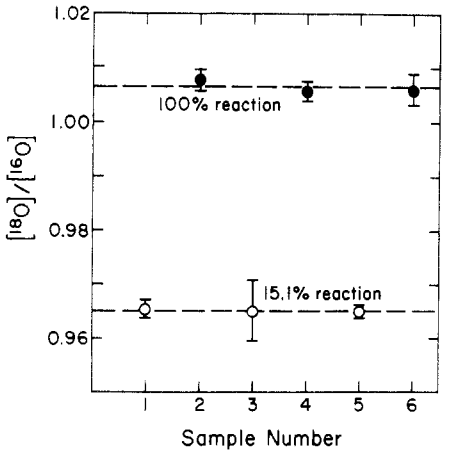


FIGURE 3: Typical data determining the oxygen-18 kinetic isotope effect on V/K for lysozyme-catalyzed hydrolysis of GlcNAcGluPNP. The observed isotope ratios of the *p*-nitrophenol product determined by mass spectrometric analysis after reaction at 40 °C, pH 5.5, are shown. The isotope ratios at 15.1% (○) and 100% (●) are shown; each point represents between 30 and 370 measurements of the isotope ratio for a given sample. The weighted means of the isotope ratios at the two extents of reaction, given by the dashed lines, are 0.9651 ± 0.0009 and 1.0066 ± 0.0012 at 15.1% and 100% reactions, respectively. The isotope effect calculated from these data is $(V/K)_{16}/(V/K)_{18}$ = 1.0468 ± 0.0017.

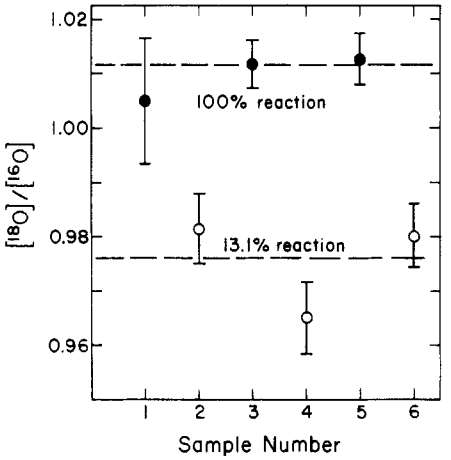
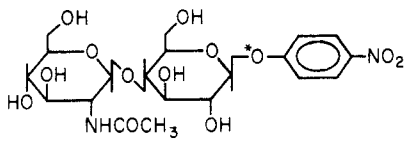


FIGURE 4: Typical data determining the oxygen-18 kinetic isotope effect on V/K for β-glucosidase A catalyzed hydrolysis of PNPGlu. The observed isotope ratios of the *p*-nitrophenol product, resulting from hydrolysis of PNPGlu (initially 50% enriched with oxygen-18) at 25 °C, pH 6.0, are shown. The isotope ratios at 13.1% (○) and 100% reactions (●) were measured by direct mass spectrometric analysis of the *p*-nitrophenol product. Each point represents between 80 and 280 measurements of the isotope ratio for a given sample. The weighted means of the isotope ratios, given by the dashed lines, are 0.9761 ± 0.0051 and 1.0116 ± 0.0031 at 13.1% and 100% reactions, respectively. These data yield an isotope effect of $(V/K)_{16}/(V/K)_{18}$ = 1.0391 ± 0.0068.

Enzymatic Studies. The oxygen-18 leaving group KIE for the lysozyme-catalyzed hydrolysis of GlcNAcGluPNP (I) was



I

determined by mass spectrometric analysis of the product as a function of the extent of reaction at 40 °C, pH 5.5. Some typical results are shown in Figure 3. The observed KIE, $(V/K)_{16}/(V/K)_{18}$ = 1.0468 ± 0.0017, is the largest observed in this study and requires that C–O bond scission be essentially complete in the transition state for the first irreversible step in the reaction sequence (see Discussion).

In contrast to the mechanism of lysozyme-catalyzed hydrolysis, which is believed to have a largely carbonium ion like transition state, it has been proposed that β-glucosidase hydrolyzes glycosides via a nucleophilic mechanism since a burst of aglycon has been observed with reactive substrates (Fink & Good, 1974; Weber & Fink, 1980). It has been postulated that a protein conformation change may be rate determining for some reactions catalyzed by this enzyme (Sinnott & Withers, 1974). Some typical results from the measurement of the oxygen-18 leaving group KIE on V/K for the β-glucosidase A catalyzed hydrolysis of PNPGlu are shown in Figure 4. The value of V/K for β-glucosidase A is $\sim 5 \times 10^3$ M^{-1} s^{-1}, assuming a molecular weight of 67000 for the active form of the enzyme (J. Weber and A. L. Fink, personal communication). The KIE is $(V/K)_{16}/(V/K)_{18}$ = 1.039 ± 0.007, indicating that as for lysozyme-catalyzed hydrolysis, C–O bond scission is quite far advanced in the transition state for the first irreversible step. All of the isotope effect data obtained in this study are summarized in Table I.

Discussion

These oxygen-18 leaving group kinetic effects result from differences in force constants to the isotopically labeled glycosidic oxygen atom between the ground states and transition states for the reactions studied. A KIE of substantially greater than 1.00 indicates that the force constants to the labeled atom have decreased in the transition state as compared to the

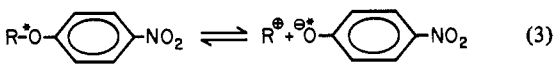
Table II: Relationship of Fraction Carbon–Oxygen Bond Breaking with Oxygen–Proton Bond Formation in the Transition State for Acid-Catalyzed Hydrolysis of *p*-Nitrophenyl Glycosides[a]

	model A[c]		model B[c]	
X_{C-O}[b]	X_{O-H}[b]	charge on leaving oxygen atom[d]	X_{O-H}[b]	charge on leaving oxygen atom[d]
1.0	0.5	−0.5	0.84	−0.16
0.95	0.38	−0.57	0.64	−0.31
0.90	0.26	−0.64	0.43	−0.47
0.80	0.01	−0.79	0.02	−0.78

[a] For transition-state structure V. Calculations are from eq 5 with $KIE_{obsd} = 1.0355$ and $EIE_{C-O} = 1.0446$. [b] X_{C-O} = fraction C–O bond *broken* and X_{O-H} = fraction new O–H bond *made* in the transition state. [c] Model A is based on $EIE_{O-H} = 1.0181$ and model B on $EIE_{O-H} = 1.0108$. See text. [d] $X_{O-H} - X_{C-O}$.

ground state and, for the reactions studied here, that cleavage of the glycosidic bond is advanced in the transition state. The KIEs measured for the reactions catalyzed by lysozyme and β-glucosidase A are the KIEs on V/K, the second-order rate constant extrapolated to zero substrate concentration, since they were measured by a competitive technique (O'Leary, 1978). Thus, these KIEs represent the effect of isotopic substitution up to the first irreversible step in these reaction sequences.

The equilibrium oxygen-18 isotope effect for the reaction shown in eq 3 (where R = Gal and $k_{16}/k_{18} = 1.0425$) has been



(3)

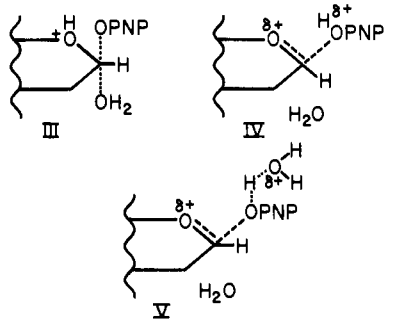
II

determined from force-field calculations based on infrared frequency measurements of isotopically substituted molecules (G. Burton, M. Liskin, and J. F. Kirsch, unpublished results; Rosenberg, 1978). This value coupled with the experimentally determined equilibrium oxygen-18 isotope effect on the ionization of *p*-nitrophenol ($K_{16}/K_{18} = 1.0181 \pm 0.0019$; Rosenberg, 1978) gives $K_{16}/K_{18} = 1.0240 \pm 0.0019$ for the formation of R^+ and *p*-nitrophenol from II. Protonation of the leaving group decreases the KIE because it increases the force constants to the labeled atom in the product, as compared with the anionic species (see below).

An observed KIE close to the maximum value can be unambiguously interpreted; i.e., scission of the bond to the isotopically labeled atom must be nearly complete in the transition state for the first irreversible step for the enzyme-catalyzed reactions. Three explanations can be offered for the observation of smaller isotope effects: (1) the step involving cleavage of the bond adjacent to the isotopically substituted atom is only partially rate determining (Northrop, 1975), (2) the structure of the transition state is such that the bond in question is only partially cleaved, and/or (3) there is a parrtially compensating inverse isotope effect due to bond-forming processes (e.g., see Acid-catalyzed Hydrolysis). Fortunately, all of the KIEs observed in this study are close to the maximum expected, and the interpretation is not beclouded by the above.

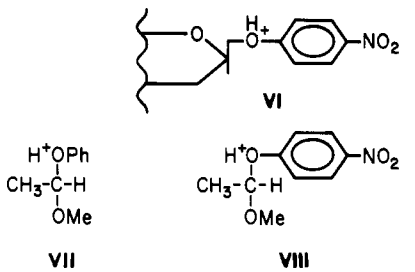
Estimates of the fraction of C–O bond cleavage in the transition states for these reactions are included in Table I. A large KIE is observed ($k_{16}/k_{18} \geq 1.035$) for all of the reactions, and the clear interpretation of these data is that C–O bond scission is nearly complete in the corresponding transition states. It has been suggested that most reactions of glycosides proceed through "exploded" transition states with large amounts of C–O bond cleavage and little nucleophilic participation (Young & Jencks, 1977; Sinnott & Jencks, 1980). Structure–reactivity correlation investigations on several of the reactions studied here are consistent with this hypothesis (Nath & Rydon, 1954; Lowe et al., 1967). The observation of large leaving group KIEs requires that the transition states for these reactions be characterized by a large degree of C–O bond scission.

Acid-Catalyzed Hydrolysis. Possible transition states for the acid-catalyzed hydrolysis reaction of PNP glycosides include



Water, the accepting nucleophile, is present, at least as a nonbonding spectator, in all three cases in view of the recent theoretical (Young & Jencks, 1977) and experimental (Sinnott & Jencks, 1980) demonstrations that a fully solvent-equilibrated glycosidic oxocarbonium ion has too short a lifetime to exist. Species III represents a classical S_N2 transition state with a protonated *cyclic* oxygen atom. This mechanism requires 100% inversion of anomeric configuration if an alcohol rather than water is used as the nucleophile. This has not been observed (see Sinnott & Jencks, 1980), and III can be ruled out as the major contributing transition state.

Species IV and V both represent sp^2-like transition states but differ in the extent of protonation of the aryl oxygen atom. Transition-state IV is reached after preequilibrium protonation of the original glycoside (Capon, 1969). The pK_a of the fully protonated PNP glycoside (VI) can be roughly estimated from



the pK_a's of $CH_2(OMe)_2H^+$ (−4.6), $(CH_3)_2C(OMe)_2H^+$ (−5.2), and $CH_2(OPh)_2H^+$ (−6.5) (Cordes & Bull, 1974). Substitution of one of the dimethoxymethane hydrogens for a CH_3 ($\Delta pK_a = -0.3$) and one phenoxy residue for a methoxy ligand [$\Delta pK_a = (-1.9/2)$] gives an estimated pK_a of −5.9 for the species VII. Assuming a similar difference in pK_a between VII and VIII as that which exists between *p*-nitroanilinium and anilinium ions or *p*-nitrophenol and phenol, one obtains $pK_a \simeq -9$ for VIII. While this estimate fails by two pK units to be too low to exclude transition-state IV from consideration on the basis of the observed rate constants and diffusion-controlled rates of proton transfer, recent investigations of acetal hydrolysis argue that V is the more likely candidate. Jensen et al. (1979) report that the hydrolysis of substituted benzaldehyde acetals is general acid catalyzed and the value of the Brønsted coefficient, α, decreases with increasing electron withdrawal in the alkyl moiety, demonstrating that the transition state is reached with less than complete proton transfer to weakly basic leaving groups. Similar findings were

obtained by Capon & Nimmo (1975) with benzaldehyde aryl methyl acetals. In addition, Fife and co-workers (1972) find that the hydrolysis of 2-(*p*-nitrophenoxy)- but not 2-ethoxytetrahydropyrans is subject to general acid catalysis. The demonstration of Cocker & Sinnott (1975) of a significant pH-independent hydrolysis reaction for 2,4-dinitrophenyl glycosides between pH 1.6 and 8 where no proton transfer to the leaving 2,4-dinitrophenoxide ion occurs further suggests that *complete* proton transfer to *p*-nitrophenoxide is unnecessary for displacement. Some proton transfer to the departing *p*-nitrophenoxide is, however, required by the slightly negative Hammett ρ value obtained for the acid-catalyzed hydrolysis of *p*NP β-glucosides (Nath & Rydon, 1954). These results all support transition-state V with or without some chemical bonding to the incoming water molecule as being the most likely transition state for the acid-catalyzed hydrolysis of *p*NP glycosides. This corresponds to general acid catalysis by the solvated proton and predicts that under certain conditions (see below) buffer-catalyzed solvolysis should be detectable.

The interpretation of the observed KIE = 1.0355 in terms of structure V is not possible in a quantitative sense because the C–*O bond breaking and *O–H bond forming processes affect the KIE in different directions. Nonetheless, rather restrictive limits can be set, taking advantage of the fact that Pauling bond orders appear to be linearly related to observed KIEs for nucleophiles in carbonyl addition reactions and, therefore, by microscopic reversibility, for leaving groups in S_N1-like decompositions (Saunders, 1975; Bilkadi et al., 1975; Hogg et al., 1980). By assuming that the C–*O and the H–*O KIEs are relatively independent of each other, i.e., that they are orthogonal, one can write

$$KIE_{obsd} - 1 = (KIE_{C-*O} \times KIE_{O*-H}) - 1 \quad (4)$$

or in terms of fractional bond orders in the transition state

$$KIE_{obsd} - 1 = X_{C-*O}(EIE_{C-*O} - 1) - X_{O*-H}(EIE_{O*-H} - 1) \quad (5)$$

where X_i and EIE_i represent the fraction of bond cleavage or formation in the transition state and the observed or calculated equilibrium isotope effects for the processes in question.[2]

The value of EIE_{C-O} is taken as 1.0446, intermediate between the largest experimentally determined KIE in this study (1.0467 for lysozyme) and the calculated EIE (1.0425; G. W. Burton et al., unpublished results). No such clear value is available for EIE_{O-H}. The calculations in Table II are based on two models; the first (model A) assumes the same value for the protonated *p*NP glycoside as was obtained for *p*-nitrophenol (1.0181; Rosenberg, 1978), and the second employs an estimate of $EIE_{O-H} = 1.0108$, which is 60% of the first, and is based on the same ratio as that calculated by Thornton (1962) for the ^{18}O EIE for the ionization of H_2O vs. H_3^+O (model B). The quantities X_{C-O} and X_{O-H} are related through eq 5; if their sum is >1, there will be some positive charge on the leaving oxygen atom, while a sum of <1 results in some negative charge on this atom in the transition state. A choice of one of these values based on independent observations does serve to fix the other, as shown in Table II.

A number of useful conclusions and predictions emanate from Table II: (1) Only transition states having >80% C–O bond cleavage are consistent with the experimental results. Earlier transition states on the C–O coordinate would not be subject to acid catalysis; i.e., X_{O-H} becomes negative. This is consistent with the conclusions of Sinnott & Jencks (1980) reached by completely independent criteria. (2) The value of X_{O-H} should be roughly equivalent to the Brønsted coefficient; therefore, general acid catalysis of the hydrolysis of these compounds may be detectable. (3) A substantial negative charge resides on the *p*-nitrophenyl oxygen atom, and thus the leaving group possesses substantial *p*-nitrophenoxide character at an acidity which is 7 pH units lower than the pK_a of its conjugate acid. This helps to understand the appearance of a substantial pH-independent hydrolysis rate of 2,4-dinitrophenyl glucoside where the leaving group is 3 pK_a units less basic (Cocker & Sinnott, 1975).

Alkaline Hydrolysis. The alkaline hydrolysis of phenyl β-D-glucosides is thought to proceed via intramolecular attack of the C-2′ oxygen atom on C-1 (Ballou, 1954). This hypothesis is supported by the observation that the 2′-*O*-methyl derivatives react much more slowly than the unblocked glycosides (Gasman & Johnson, 1966). Furthermore, the α-secondary ^2H KIE for a closely related reaction, the methoxide-catalyzed methanolysis of phenyl β-D-glucoside, is $k_H/k_D = 1.03$, consistent with a mechanism involving substantial nucleophilic participation and little carbonium ion character in the transition state (Dahlquist et al., 1969). The observed oxygen-18 KIE, $k_{16}/k_{18} = 1.0386 \pm 0.0032$, requires a transition state with a large degree of C–O bond scission.

The interpretation of this result is complicated by the observation that a significant depletion of oxygen-18 occurs in the enriched product as compared with the starting material during the course of the reaction. *p*-Nitrophenyl β-D-galactoside, shown to be 74% enriched with oxygen-18 in the glycosidic oxygen atom by mass spectrometric analysis of the *p*-nitrophenol after acid- or β-galactosidase-catalyzed hydrolysis, yields *p*-nitrophenol only 46% enriched after alkaline hydrolysis (data not shown). A possible mechanism for this reduction of enrichment is suggested by the results of Horton & Luetzow (1971) who have shown that *p*-nitrophenyl α-D-glucopyranoside is hydrolyzed in alkali via a mechanism involving stepwise migration of the nitrophenyl group. In addition, they reported that alkaline hydrolysis of the β anomer also proceeds via this pathway, although less rapidly. Thus, if the attack of the C-2′ oxyanion occurs at both the anomeric carbon atom and the C-1 position on the aromatic ring at comparable rates, the depletion of label observed in this study can be explained. However, the migration pathway involving

[2] The assumption of orthogonality, although not yet tested experimentally, says, for example, that the magnitude of the ^{18}O KIE for >C–*OPNP cleavage is uninfluenced by the extent of protonation of the leaving group in the transition state. While this is probably quite reasonable in the present instance where the extent of such bonding to the proton is small, it is unlikely to be true generally as suggested by Thornton's (1962) calculations of the ^{18}O EIEs for the dissociation of $H_3{}^*O^+$ and $H_2{}^*O$ whose values are substantially different. Equation 5 follows from eq 4 by writing $KIE_{C-*O} = X_{C-O}(EIE_{C-*O} - 1) + 1$ and $KIE_{O*-H} = X_{O*-H}(EIE_{O*-H} - 1) + 1$. Multiplication of the two expressions leads directly to eq 5 after elimination of the cross term, $X_{C-*O}X_{O*-H}(EIE_{O*-H} - 1)(EIE_{C-*O} - 1)$, which is negligible compared to the remaining quantities. The bond order approach ignores the imaginary frequency contribution ($\nu_{L(1)}^{\ddagger}/\nu_{L(2)}^{\ddagger}$), which is always a positive multiplier of the other factors in the expression for the KIE, both in bond making and in bond breaking processes. This factor has the value

$$\left[\left(\frac{1}{m_1'} + \frac{1}{m_1''}\right)\Big/\left(\frac{1}{m_2'} + \frac{1}{m_2''}\right)\right]^{1/2} \geq \frac{\nu_{L(1)}^{\ddagger}}{\nu_{L(2)}^{\ddagger}} \geq \left[\left(\frac{1}{M_1'} + \frac{1}{M_1''}\right)\Big/\left(\frac{1}{M_2'} + \frac{1}{M_2''}\right)\right]^{1/2}$$

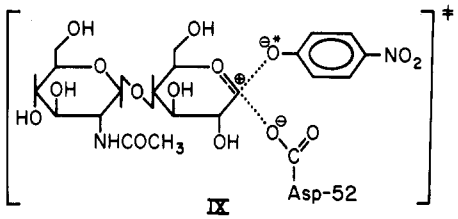
where the quantity on the left side of the inequality represents the masses of the *atoms* being joined or broken and that on the right hand side those of the *molecular fragments* (Bigeleisen & Wolfsberg, 1958; Melander, 1960). The large masses of the isotopically substituted fragments employed in this study would be expected to reduce this quantity to a factor that is negligible compared with the experimental error.

aromatic nucleophilic substitution is not the only pathway for hydrolysis since isotopic label is retained in the majority of the product.

The fact of the occurrence of two parallel reactions in alkaline medium precludes quantitative analysis of transition-state structure in this instance because the maximum KIE for nucleophilic aromatic substitution is unknown and the relative proportions of the two pathways are uncertain. It is still possible to conclude that release of *p*-nitrophenol by the dominant pathway is characterized by a large oxygen-18 leaving group KIE; thus, the transition state is late with respect to C–O bond cleavage. Structure–reactivity correlations indicate that the charge density at the glycosidic oxygen atom in the transition state for alkaline hydrolysis of a series of phenyl glycosides closely resembles that for the free phenoxides (β_{lg} = −1.1; Nath & Rydon, 1954), in qualitative agreement with the magnitude of the KIE observed.

Enzymatic Studies. Lysozyme. The isotope effect observed for this reaction is the KIE on V/K and measures the isotopic sensitivity of steps up to the first irreversible step in the reaction sequence. It has been shown by both equilibrium and rapid kinetic measurements that the lysozyme-catalyzed hydrolysis of *N*-acetylglucosamine oligomers is a complex multistep process (Holler et al., 1975a,b; Bannerjee et al., 1975). The isotope effect measured in this study, $(V/K)_{16}/(V/K)_{18}$ = 1.0467 ± 0.0015, requires that C–O bond scission be virtually complete in the transition state for the first irreversible step. Thus, the chemical cleavage is the first irreversible step in the reaction catalyzed by lysozyme. There is almost certainly little proton transfer to the leaving group with this substrate since the KIE is so large. As discussed above (see acid-catalyzed hydrolysis), proton transfer to the leaving group would reduce the KIE.

The oxygen-18 leaving group KIE observed here coupled with the demonstration of a substantial α-secondary ^2H KIE of $(V/K)_H/(V/K)_D$ = 1.11 for a closely related substrate (Dahlquist et al., 1969) makes the likely transition-state structure for lysozyme-catalyzed hydrolysis that shown as IX.

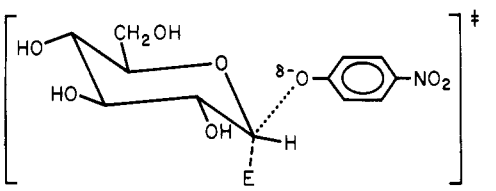


IX

The available isotope effect data are certainly consistent with the formation of an oxocarbonium ion in the transition state. However, uncertainties as to the interpretation of the magnitude of the α-secondary ^2H KIE make it possible that some nucleophilic stabilization by Asp-52 may occur, as this residue is located close to the C-1 position of the substrate (Blake et al., 1967). Thus, Asp-52 would play a role analogous to that of the water molecule in the transition state for acid-catalyzed hydrolysis, shown above as V. The demonstration by Raftery and co-workers (Smith et al., 1973) that the α-secondary ^3H KIE for lysozyme-catalyzed hydrolysis of $GlcNAc_3$ yields a result similar to that for the substrate with a phenyl leaving group suggests strongly that the transition-state structure IX is not a special case and that all lysozyme-catalyzed hydrolyses will proceed through such an "exploded" transition state, with essentially complete bond cleavage to the leaving group and little bond formation to Asp-52 (Sinnott & Jencks, 1980).

β-Glucosidase A. The isotope effect observed for this enzyme catalyzed reaction is $(V/K)_{16}/(V/K)_{18}$ = 1.038 ± 0.006, corresponding to 89 ± 14% C–O bond cleavage in the transition state. There is a considerable body of evidence which suggests that this enzyme reacts via nucleophilic (S_N2) as opposed to a carbonium (S_N1) mechanism. The α-secondary ^2H KIE observed with a commerical preparation of β-glucosidase with phenyl β-D-glucoside as substrate is small [$(V/K)_H/(V/K)_D$ = 1.015; Dahlquist et al., 1969]. This result is consistent either with a S_N2 mechanism of hydrolysis or with a mechanism in which the first irreversible step involves no changes in hybridization at the anomeric carbon atom (e.g., a protein conformational change). A burst of *p*-nitrophenol has been observed at −15 °C with PNPGlu as substrate (Fink & Good, 1974). These results were confirmed by a rapid kinetic study at 20 °C (Takahashi, 1975). More recent investigations have demonstrated a temperature-dependent change in rate-determining step from the breakdown of an intermediate at less than 25 °C to its formation at higher temperatures (Weber & Fink, 1980). This explains the observed absence of a burst for some substrates at 35 °C (Legler, 1975).

The oxygen-18 leaving group KIE observed in this study coupled with the above data suggests a transition-state structure with little change in hybridization at the anomeric carbon atom and almost complete C–O bond scission. Thus, a covalent intermediate, resulting from nucleophilic participation of the enzyme, is likely since trivalent carbon would otherwise result. The transition-state structure is schematically shown as X. This transition state differs from that for lysozyme-catalyzed hydrolysis (IX) in that nucleophilic participation by the enzyme must be considerably further advanced in the β-glucosidase A reaction. It has been suggested that a conformational change might be the rate-determining step in β-glucosidase hydrolysis as has been proposed for β-galactosidase (Sinnott & Souchard, 1973; Sinnott & Withers, 1974). The large oxygen-18 leaving group KIE observed in this study eliminates the possibility that such a rate-limiting conformation change precedes C–O bond scission since the former would not be isotopically sensitive.



X

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Selective *N*-Bromosuccinimide Oxidation of the Nonfluorescent Tryptophan-31 in the Active Center of Thioredoxin from *Escherichia coli*†

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ABSTRACT: The two tryptophan residues (Trp-28 and Trp-31) of thioredoxin-S_2 from *Escherichia coli* were selectively tritiated with trifluoroacetic [^{3}H]acid. The ^{3}H label was introduced to permit quantitative amino acid sequence analyses of the result of *N*-bromosuccinimide oxidation of tryptophan to oxindolylalanine. Addition of 3-fold molar excess of *N*-bromosuccinimide at pH 4 modifies a tryptophan in thioredoxin-S_2 that is nonessential for enzyme activity with thioredoxin reductase and has a strongly quenched fluorescence in both oxidized and reduced thioredoxin. This residue was shown to be Trp-31 by amino acid sequence analyses of ^{3}H-labeled chymotryptic peptides from the modified protein. The results demonstrate that the second tryptophan residue, Trp-28, signals a conformational change on reduction of the active-center disulfide to a dithiol by increasing its fluorescence quantum yield about 6-fold at pH 7. The differential reactivity of the tryptophan residues agrees with the known three-dimensional structure of thioredoxin-S_2.

A 3-fold increase in the quantum yield of tryptophan fluorescence at pH 7 accompanies the reduction of *Escherichia coli* thioredoxin-S_2[1] to thioredoxin-$(SH)_2$ (Stryer et al., 1967; Holmgren, 1972a). This useful change is evidence for a localized protein conformational change involved in the reaction mechanism of thioredoxin-$(SH)_2$ as a disulfide reductase (Holmgren, 1979a,b). Both tryptophan residues of thioredoxin (Trp-28 and Trp-31) are located close to the functional di-

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[1] Abbreviations used: dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; thioredoxin-S_2 and thioredoxin-$(SH)_2$, the oxidized and reduced forms of thioredoxin, respectively; 1-NBS-thioredoxin, thioredoxin with one tryptophan residue modified to oxindole with *N*-bromosuccinimide.

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