Reversible Alkylation of a Methionyl Residue near the Active Site of β-Galactosidase†

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ABSTRACT: Inactivation of β-galactosidase from *Escherichia coli* K-12 by *N*-bromoacetyl-β-D-galactosylamine exhibits saturation kinetics with a K_I of 1.13 mM and a first-order rate constant, $k_3 = 0.063$ min^{-1}, at pH 7.5, 30°. The rate constant of inactivation increases with pH in the pH range 7–10. Inactivation of β-galactosidase with bromo- or iodoacetamide is a bimolecular process with a second-order rate constant $k = 0.25$ min^{-1} M^{-1} at pH 7.5, 30°. Evidence is presented that inactivation of the enzyme by all of these reagents is due to the alkylation of a single methionyl residue near the active site. Enzyme inactivated by alkylation is reactivated by incubation with mercaptoethanol. The half-time for reactivation of enzyme alkylated by *N*-bromoacetyl-β-D-galactosylamine is 11 min and that for enzyme alkylated by iodoacetamide is 155 min at pH 8.9, 0.12 M mercaptoethanol, 30°. Enzyme reactivated with mercaptoethanol is susceptible to inactivation by the above alkylating agents, suggesting that on reactivation an intact methionyl residue is recovered from its alkylsulfonium salt. Replacement of methionine near the active site with norleucine gives enzyme which is not inactivated by *N*-bromoacetyl-β-D-galactosylamine, proving that this methionyl residue does not participate in catalysis.

β-Galactosidase of *Escherichia coli* is composed of four identical subunits each containing one catalytic site (Cohn, 1957). The enzyme contains 19 sulfhydryl groups per subunit but is not inactivated by carboxymethylation in the absence of denaturing agents (Craven *et al.*, 1965). Organic mercurials were found to inactivate the enzyme (Loontiens *et al.*, 1970). β-Galactosidase is activated by sodium and magnesium ions and by mercaptoethanol in relatively high concentrations (Kuby and Lardy, 1953; Reithel and Kim, 1960; Reithel *et al.*, 1966). The mechanism of activation by all of these compounds is still unknown.

We have recently demonstrated that *N*-bromoacetyl-β-D-galactosylamine inactivates β-galactosidase by reacting with a methionyl residue near the active site of the enzyme (Yariv *et al.*, 1971; K. J. Wilson and Z. Bohak, unpublished results). Here we present evidence that the common alkylating reagents, iodoacetamide and bromoacetamide, also inactivate the enzyme by alkylating this strategically located methionyl residue. We also show that this residue is not necessary for catalytic activity since in a biosynthetically modified β-galactosidase, where methionine is replaced with norleucine, catalytic activity is maintained.

In the course of these studies we found that β-galactosidase which was inactivated with the above mentioned haloacetamides can be reactivated with mercaptoethanol. Reactivation is apparently the result of the regeneration of the methionyl residue from its alkylsulfonium salt.

Experimental Section

β-Galactosidase (EC 3.2.1.23) of *E. coli* K-12 strain 3300 was purchased from Worthington Biochemical Corp. (Freehold, N. J.) or prepared locally. It was stored at 4° under 40% saturated ammonium sulfate. Both enzyme preparations were found to hydrolyze *o*-nitrophenyl β-D-galactoside with a rate of approximately 300 μmoles/min per mg, using the assay procedure described below.

N-Bromoacetyl-β-D-galactosylamine (mp 186°, lit. mp 191°) and *N*-bromoacetyl-L-fucosylamine (mp 175°, lit. mp 175°) were prepared as described by Thomas (1970). ^{14}C-Labeled *N*-bromoacetyl-β-D-galactosylamine was prepared using [2-^{14}C]bromoacetic acid (specific activity 55 Ci/mole, Radiochemical Centre, Amersham, Herts., England). To recover the radioactive product, the oil obtained by precipitation in ether was taken up in a small volume of methanol from which the product slowly crystallized. It was then recrystallized from methanol–ether. The final product was homogeneous on thin-layer chromatography (tlc) (silica) in two solvent systems: methanol–acetone (1:10) and isopropyl alcohol–water (7:3).

Iodoacetic acid, bromoacetic acid, and iodoacetamide were purchased from Fluka and recrystallized twice prior to use. Bromoacetamide was prepared according to the procedure of Papendieck (1892). The white crystals obtained after recrystallization from ethanol melted at 90–92°. β-Mercaptoethanol was purchased from Eastman Kodak, *o*-nitrophenyl β-D-galactoside from Koch and Light (Colnbrook, Bucks., England), and isopropyl β-thiogalactoside from Calbiochem (Los Angles, Calif.).

Methods

Enzyme Assay. The assay was carried out in a solution containing 0.70 g/l. of *o*-nitrophenyl β-D-galactoside in a 0.1 M sodium phosphate buffer (pH 7.0) which was 10^{-3} M in $MgCl_2$ and 10^{-1} M in β-mercaptoethanol. An aliquot of enzyme was added to 1.6 ml of the above solution and the assay was allowed to proceed at 25° for 1–6 min depending on enzyme concentration. The reaction was stopped by addition of 2 ml of 1 M Na_2CO_3. From the absorbance at 420 nm the *o*-nitrophenol concentration was calculated using a molar extinction coefficient, ε, of 5200. Enzyme concentration was measured spectrophotometrically at 280 nm using an extinction coefficient of 2.09 mg^{-1} cm^2 (Craven *et al.*, 1965). It was found that the release of *o*-nitrophenol was proportional to enzyme con-

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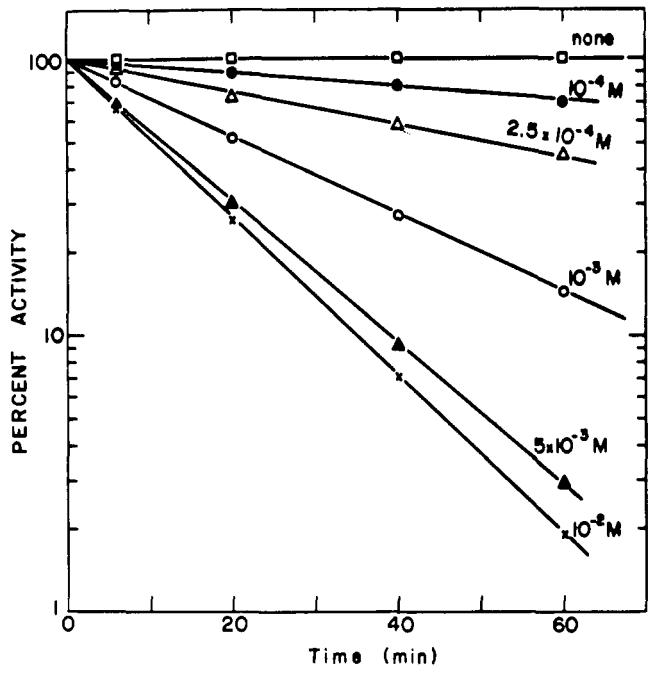

FIGURE 1: The rate of inactivation of β-galactosidase by different concentrations of *N*-bromoacetyl-β-D-galactosylamine in 0.1 M sodium phosphate (pH 7.5), 10^{-3} M in $MgCl_2$ at 30°. Enzyme concentration, 0.02 mg/ml. Inhibitor concentrations are given in the figure.

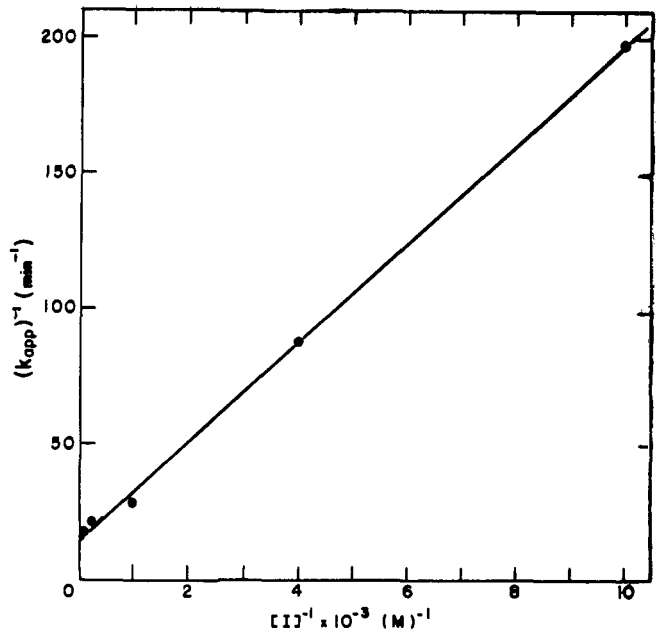

FIGURE 2: The dependence of k_{app} on the concentration of *N*-bromoacetyl-β-D-galactosylamine at pH 7.5 plotted as reciprocals in accordance with eq 5.

centration and, in the time interval used for the assay, the rate of the reaction was zero order in substrate.

Inactivation. The alkylating reagent was dissolved in 0.1 M phosphate buffer (pH 7.5), 10^{-3} M in $MgCl_2$, to a predetermined final concentration. One ml of such solution was placed in a water bath thermostatted at 30°. A small amount of enzyme was added from a stock solution (approximately 0.2 mg/ml in 0.1 M sodium phosphate buffer (pH 7.5), 10^{-3} M in $MgCl_2$) using a microliter pipet. Aliquots were removed at various times and assayed for activity. The determination of the rate of inactivation at different pH values was carried out in a solution of 10^{-2} M inhibitor in 0.1 M phosphate or 0.1 M sodium carbonate buffers 10^{-3} M in $MgCl_2$ and 10^{-3} M in EDTA.

Reactivation. β-Galactosidase was first inactivated as described above and then dialyzed against 0.1 M sodium phosphate buffer (pH 7.5), 10^{-3} M in $MgCl_2$ at 4°, overnight to remove excess inhibitor. An aliquot of inactivated enzyme was added to 1 ml of buffer, 10^{-3} M in $MgCl_2$, containing a specified concentration of β-mercaptoethanol. The regain of activity with time was determined using the assay procedure described above.

Labeling with [^{14}C]N-Bromoacetyl-β-D-galactosylamine. β-Galactosidase was labeled with [^{14}C]*N*-bromoacetyl-β-D-galactosylamine in the presence of 5×10^{-3} M *N*-bromoacetyl-L-fucosylamine as previously described (Yariv *et al.*, 1971). The inactivation at pH 7.5 was allowed to proceed for 60 min; at pH 10 for 20 min. At these times the respective solutions were dialyzed against 0.1 M sodium phosphate (pH 7.5), 10^{-3} M in $MgCl_2$, at 4° until no radioactivity was detectable in the dialysate. The samples were then analyzed for enzymic activity and for radioactivity. Radioactivity was determined by placing aliquots of the samples in Bray's solution and counting in a Packard liquid scintillation spectrometer.

Preparation of Biosynthetically Modified Enzyme. Strain CS-8A of *E. coli* K12 was kindly made available by Professor Y. S. Halpern of the Hebrew University Hadassah Medical School, Jerusalem. This is a β-galactosidase inducible and a met A$^-$ strain. It was grown in a salts medium containing per liter: 13.6 g of KH_2PO_4, 2.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4$, 0.5 mg of $FeSO_4 \cdot 7H_2O$, and 4 g of glycerol. The pH of the medium was adjusted to 7 with 0.1 M KOH. The culture was grown with aeration at 37° with 25 mg of methionine added per l. of culture. The culture (100 ml), harvested in the logarithmic phase, was washed, under sterile conditions, in a Servall centrifuge with the salts medium and suspended in a few milliliters of the salts solution. Salts medium supplemented with 2.5 mg of L-methionine and 500 mg of DL-norleucine per l. was inoculated with a washed suspension of bacteria. The culture grew for two generations and then isopropyl β-thiogalactoside was added to a concentration of 5×10^{-4} M. When the bacterial mass doubled the culture was harvested. This culture will be referred to as "norleucine culture." A control culture was grown with 25 mg of L-methionine/l. as described above and also induced with isopropyl β-thiogalactoside. This culture will be referred to as "methionine culture." Thick suspensions of the two cultures in 0.1 M sodium phosphate buffer (pH 7.5), containing 10^{-3} M magnesium chloride, were sonicated in a Branson sonicator and cleared in a Servall refrigerated centrifuge. The extracts were assayed for β-galactosidase at 28° as described elsewhere (Yariv *et al.*, 1971).

Results

Inactivation of β-Galactosidase by N-Bromoacetyl-β-D-galactosylamine. When β-galactosidase is inactivated using excess *N*-bromoacetyl-β-D-galactosylamine at pH 7.5 and 30° the decrease in activity obeys first-order kinetics with a rate constant that depends on the inhibitor concentration (Figure 1). For an active-site-directed reagent (I) it is expected that the inactive enzyme (E–I) is formed *via* an intermediate complex (E·I) of reagent with enzyme (E). Kitz and Wilson

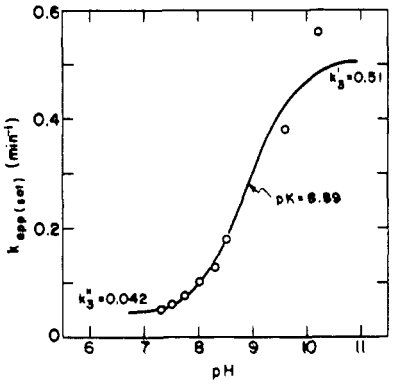

FIGURE 3: The pH dependence of the rate constant of inactivation of β-galactosidase at 30° by *N*-bromoacetyl-β-D-galactosylamine. Experimental points (○) represent the observed pseudo-first-order rate constant for the inactivation of enzyme (0.0175–0.2 mg/ml) by a saturating concentration of the inhibitor (10^{-2} M). Solid line plotted in accordance to eq 9 using the values of the constants indicated in the figure.

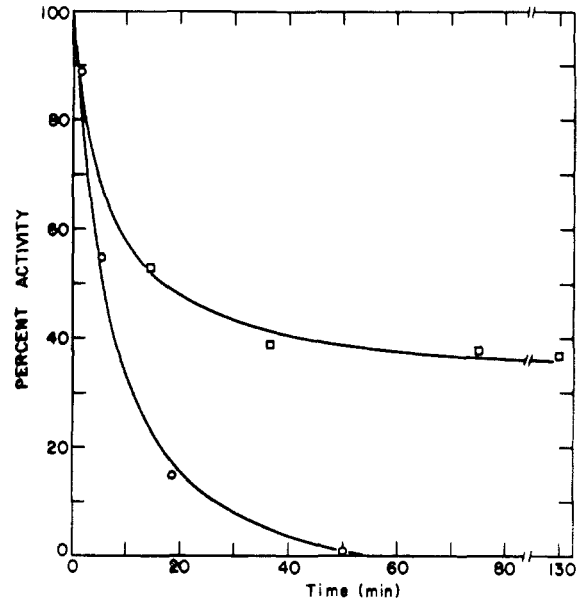

FIGURE 4: The inactivation of β-galactosidase from "norleucine culture" (□) and methionine culture (○) by *N*-bromoacetyl-β-D-galactosylamine (3.3×10^{-3} M) in 0.1 M sodium phosphate (pH 7.5), 10^{-3} M in $MgCl_2$ at 33°.

(1962) have shown that such reactions will obey kinetic eq 1–5:

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E\cdot I \xrightarrow{k_3} E\text{–}I \qquad (1)$$

$$K_I = \frac{[E][I]}{[E\cdot I]} \qquad (2)$$

$$[E_0] = [E] + [E\cdot I] + [E\text{–}I] = \epsilon + [E\text{–}I] \qquad (3)$$

where $[E_0]$ is equal to total enzyme concentration and ϵ is the concentration of active enzyme. The decrease of activity with time is then given by

$$\ln \frac{\epsilon}{[E_0]} = -\frac{k_3 t}{1 + K_I/[I]} = -k_{app} t \qquad (4)$$

and the dependence of k_{app} on inhibitor concentration is given by

$$\frac{1}{k_{app}} = \frac{1}{k_3} + \frac{K_I}{k_2}\frac{1}{[I]} \qquad (5)$$

A plot of the calculated first-order rate constants (k_{app}) for the inactivation of β-galactosidase by *N*-bromoacetyl-β-D-galactosylamine according to eq 5 describes a straight line (Figure 2). From the slope and intercept of this line we were able to compute the value of K_I = 1.13 mM and k_3 = 0.063 min^{-1} at pH 7.5 and 30°.

Examination of the inactivation reaction at different hydrogen ion concentrations shows that the reaction rate is pH dependent (Figure 3). The change in the inactivation rate of β-galactosidase with pH suggests that the titration of some group in the protein changes the activity of the enzyme toward *N*-bromoacetyl-β-D-galactosylamine. This effect may be represented by the following kinetic scheme:

$$E + I \rightleftharpoons E\cdot I \xrightarrow{k_3'} E\text{–}I \qquad (6)$$

$$EH^+ \overset{K}{\rightleftharpoons} E + H^+ \qquad (7)$$

$$EH^+ + I \rightleftharpoons EH^+\cdot I \xrightarrow{k_3''} EH^+\text{–}I \qquad (8)$$

At pH values near the pK of reaction 7 the dependence of the rate of inactivation on inhibitor concentration differs from that given by eq 4 and 5 since the two species of the enzyme E and EH^+ react simultaneously with inhibitor. Cleland (1970) has shown that when substrate reacts with two forms of an enzyme, reciprocal plots of rate *vs.* substrate concentration may be concave down. Curves concave down have in fact been observed when the experimental data obtained at pH 8.5 and 9.6 were plotted according to eq 5. To determine the constants k_3' and k_3'' inhibition experiments had to be carried out using saturating concentrations of inhibitor. At conditions approaching saturation the observed rate of inactivation will obey eq 9, where α is the fraction of the enzyme

$$k_{app(sat)} = k_3'\alpha + k_3''(1 - \alpha) = k_3'\frac{K}{K + [H^+]} + k_3''\frac{[H^+]}{K + [H^+]} \qquad (9)$$

in the unprotonated form and K is the dissociation constant of the protonated group. Numerical analyses of the experimental results according to eq 9 yield the values for k_3' = 0.51 min^{-1}, k_3'' = 0.042 min^{-1}, and $K = 1.29 \times 10^{-9}$ M (pK = 8.89). The theoretical curve calculated using these values is compared to the observed rate constants in Figure 3.

Inactivation of β-Galactosidase Synthesized in the Presence of Norleucine. Extracts obtained from norleucine culture and from methionine culture had similar activity of around 20 μmoles/min per ml of extract, which was invariant for 3 hr, the duration of the experiment. Both extracts were incubated with 3.3×10^{-3} M *N*-bromoacetyl-β-D-galactosylamine at 33° and 20-μl aliquots were removed for assay of β-galactosidase activity. The time course of the inactivation of the two extracts, expressed in per cent activity, is given in Figure 4. It can be seen that enzyme induced in the presence of an optimal methionine concentration is completely inactivated by the reagent, similarly to enzyme of the constitutive strain. Enzyme obtained in suboptimal concentration of L-methionine and a

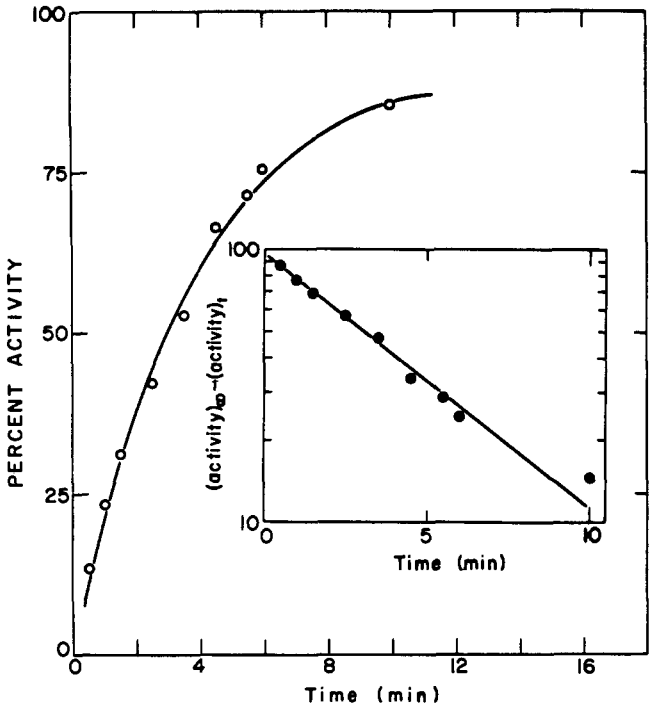

FIGURE 5: Reactivation of β-galactosidase alkylated with *N*-bromoacetyl-β-D-galactosylamine on incubation with 0.12 M mercaptoethanol at pH 9.9 and 30°. Insert: results plotted as a first-order reaction.

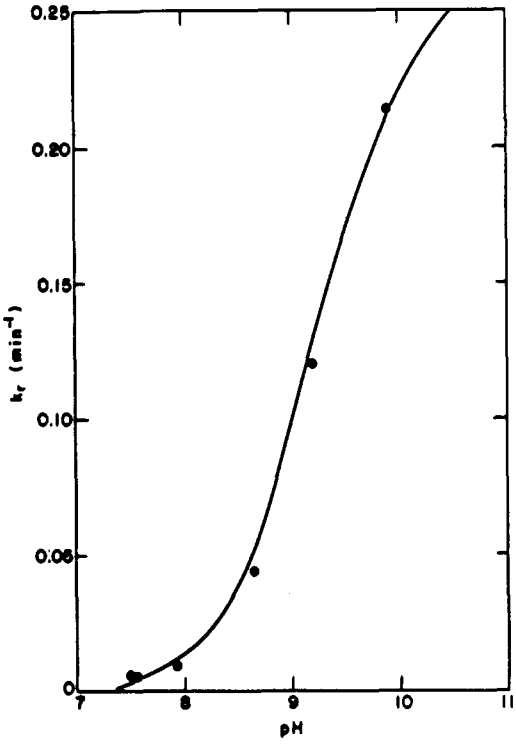

FIGURE 6: pH dependence of the rate constant of reactivation using mercaptoethanol (0.12 M). Experimental points (O) represent the observed pseudo-first-order rate constant. Theoretical curve calculated using an intrinsic rate constant k_i = 0.267 min^{-1} and pK = 9.3 for the reacting species.

100-fold excess of L-norleucine is inactivated to 38% of the original activity. Inactivation in both cases proceeds at the same rate, as can be verified when the logarithm of per cent activity *vs.* time is plotted.

Reactivation of Alkylated β-Galactosidase. When β-galactosidase inactivated with *N*-bromoacetyl-β-D-galactosylamine is kept at pH 8.65 in the presence of 0.12 M mercaptoethanol, 94% of the original activity is regenerated in 90 min. The regeneration of enzymatic activity is observed to obey first-order kinetics (Figure 5). When the inactivated enzyme was incubated with varying concentrations of mercaptoethanol, at pH 7.5, the rate of reactivation was proportional to the mercaptoethanol concentration up to 0.60 M. In the absence of mercaptoethanol no measurable gain in activity is observed at pH 7.5 and 30° for 4–5 hr and at 4° for 2 weeks. The rate of reactivation in the presence of 0.12 M mercaptoethanol is found to increase as the pH was raised (Figure 6). Such an increase indicates that mercaptide ion is involved in the reaction. The change in the reactivation rate constant (k_r) with pH could be correlated with the ionization constant (K_a) of a single species in solution and an intrinsic reactivation rate constant (k_i) by eq 10. Graphical analysis yields k_i = 0.267

$$k_r = \frac{k_i K_a}{[H^+] + K_a} \qquad (10)$$

min^{-1} (at 0.12 M mercaptoethanol) and the pK_a = 9.3 in good agreement with reported pK_a values for mercaptoethanol (9.32–9.6) (Danehy and Noel, 1960; Jencks and Salvesen, 1971). In Figure 6 the theoretical curve, constructed using the above constants, is compared to the observed reactivation rates at different pH values.

To determine whether the reaction of mercaptoethanol with inactivated β-galactosidase also results in the regeneration of the starting methionyl residue, we utilized enzyme alkylated with ^{14}C-labeled *N*-bromoacetyl-β-D-galactosylamine. Inactivation by *N*-bromoacetyl-β-D-galactosylamine both at pH 7.5 and 10 results in the incorporation of approximately 1 mole of label/mole of subunit. Reactivation of alkylated enzyme is accompanied by loss of the radioactivity from the protein (Table I), indicating the removal of the acetamidogalactosylamine residue. The reactivated enzyme is kinetically in-

TABLE I: Alkylation of β-Galactosidase with [^{14}C]*N*-Bromoacetyl-β-D-galactosylamine.[a]

Enzyme	% Act.	Mole of Label/Moles of Subunit	Rate of Inactivation × 10^2 (min^{-1})	Rate of Reactivation × 10^2 (min^{-1})
Native	100	0	33	
After first inactivation	3	0.87		39[b]
After first reactivation	88	0.21	32	
After second inactivation	4	0.99		33[c]

[a] The enzyme (8 mg/ml) was inactivated with the radioactive reagent at pH 7.5 and 30° as described in Methods. Reactivation was carried out in 0.12 M mercaptoethanol at 30° at the pH values indicated. [b] pH 8.7. [c] pH 8.6.

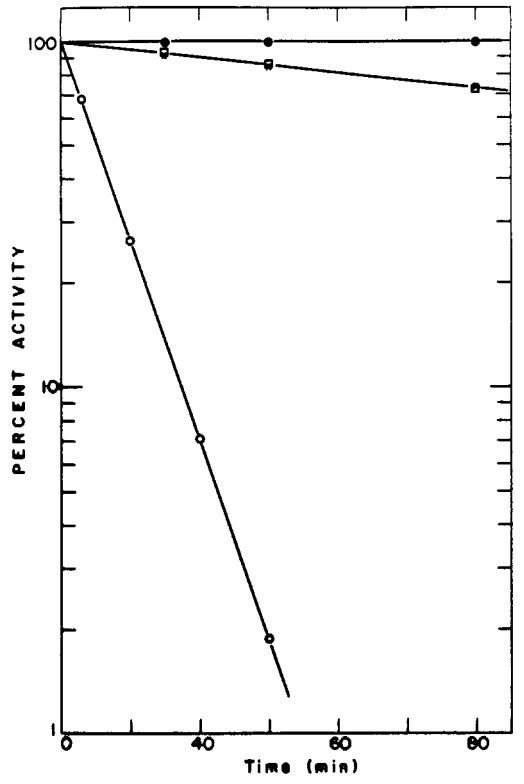

FIGURE 7: Inactivation of β-galactosidase by different alkylating reagents at 30°. The enzyme (0.02 mg/ml) in 0.1 M sodium phosphate (pH 7.5), 10^{-3} M in $MgCl_2$ was incubated with 10^{-2} M *N*-bromoacetyl-β-D-galactosylamine (○), bromoacetamide (□), iodoacetamide (×), and bromoacetic acid or iodoacetic acid (○).

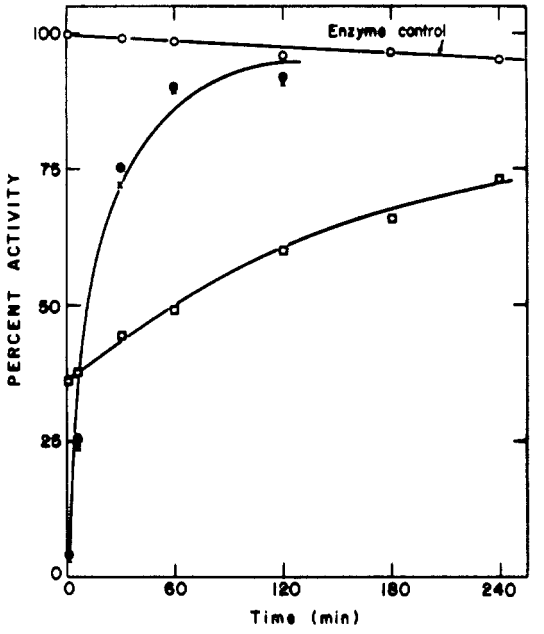

FIGURE 8: Reactivation by mercaptoethanol of β-galactosidase inhibited with: *N*-bromoacetyl-β-D-galactosylamine (○), iodoacetamide (□), and *N*-bromoacetyl-β-D-galactosylamine followed by iodoacetamide (×). Reactivation was carried out at 30° in 0.1 M sodium phosphate (pH 8.9), 10^{-3} M $MgCl_2$, 0.12 M in mercaptoethanol. Enzyme concentration, 0.027–0.033 mg/ml.

distinguishable from native enzyme in its inactivation by *N*-bromoacetyl-β-D-galactosylamine and reactivation by mercaptoethanol (Table I).

Inactivation by Iodo- and Bromoacetamides. At pH 7.5 and 30° both iodoacetamide and bromoacetamide inactivate β-galactosidase whereas the corresponding acids have no effect on activity (Figure 7 and Table II). Under conditions where high concentrations of iodo- and bromoacetamides were used, the decrease in enzymic activity with time obeys first-order kinetics. The rate constant for the inactivation by iodoacetamide is proportional to the concentration of this alkylating agent, indicating a second-order reaction.

β-Galactosidase inactivated with iodoacetamide is reactivated with mercaptoethanol similarly to enzyme alkylated with *N*-bromoacetyl-β-D-galactosylamine (Figure 8). The rate of reactivation, however, is approximately 12 times slower than for enzyme inactivated using the affinity label (Table III). In order to determine the identity of the residue alkylated by iodoacetamide the following experiments were performed. β-Galactosidase was treated with iodoacetamide, *N*-bromo-

TABLE II: Rate Constants for the Inactivation of β-Galactosidase by Different Alkylating Reagents at pH 7.5.[a]

Inhibitor[b]	k_{app} (min^{-1})[c]	Intrinsic Rate Constant
N-Bromoacetyl-β-D-galactosylamine	0.06	0.063 min^{-1} [d]
Bromoacetamide	0.0025	
Iodoacetamide	0.0025	0.25 min^{-1} M^{-1} [e]

[a] For details of experimental procedure and computation of the rate constants, see text and Figures 1 and 7. [b] Inhibitor concentration was 10^{-2} M. [c] The pseudo-first-order rate constant computed from the decay of activity with time. [d] First-order rate constant k_3 (eq 1). [e] Rate constant computed assuming a simple bimolecular reaction.

TABLE III: Reactivation Rates of Alkylated β-Galactosidase.

Alkylating Reagent	Residual Act. (%)	$t_{1/2}$ Reactivation (min)
N-Bromoacetyl-β-D-galactosylamine, 5×10^{-3} M[a]	<1	11
Iodoacetamide, 10^{-2} M[a]	36	155
N-Bromoacetyl-β-D-galactosylamine, 5×10^{-3} M, followed by iodoacetamide, 10^{-2} M[b]	<1	11
Iodoacetic acid, 2×10^{-1} M, followed by *N*-bromoacetyl-β-D-galactosylamine, 5×10^{-3} M[b]	<1	12

[a] β-Galactosidase (2.5–3 mg/ml) was incubated with various alkylating reagents for a total of 5.5 hr at 30°. [b] The second reagent was added after incubating the enzyme with the first reagent for 30 min. [c] Reactivation was carried out at pH 8.9 in the presence of 0.12 M mercaptoethanol.

acetyl-β-D-galactosylamine, or with both these reagents, and reactivated with mercaptoethanol. It was expected that if iodoacetamide inactivates the enzyme by attacking a residue other than the methionine near the active site it should alter the reactivation rate of β-galactosidase alkylated by the affinity label. The results summarized in Table III demonstrate that enzyme inactivated by *N*-bromoacetyl-β-D-galactosylamine and enzyme inactivated by the galactosyl reagent followed by iodoacetamide are reactivated by mercaptoethanol at the same rate.

Discussion

The results presented above show that various alkylating reagents inactivate β-galactosidase by alkylating a single methionyl residue near the active site of the enzyme. This inactivation is reversible upon the nucleophilic decomposition of the alkylsulfonium salts with mercaptoethanol.

The alkylating reagents examined constitute three distinct classes in their behavior toward β-galactosidase. *N*-Bromoacetyl-β-D-galactosylamine exhibits saturation kinetics during the inactivation of the enzyme, indicating the formation of a reversible complex prior to covalent bond formation. The inactivation of β-galactosidase by iodoacetamide and bromoacetamide obeys a second-order rate law, suggesting that these reagents alkylate the enzyme in a simple bimolecular reaction. The results in Table III indicate that inhibition by these reagents is due to the reaction with the same methionyl residue alkylated by the active-site-directed reagent. The third class of reagents tested, bromoacetic acid and iodoacetic acid, does not inactivate β-galactosidase under conditions identical with those employed for the corresponding amides (see also Craven *et al.*, 1965). Furthermore enzyme modified by treatment with iodoacetic acid can subsequently be inactivated with *N*-bromoacetyl-β-D-galactosylamine, showing that the halo acids do not attack the methionyl residue near the active site (Table III). The failure of the α-halo acids to react with this residue may serve as an indication of the presence of a negatively charged group in its vicinity. Tenu *et al.* (1971) have presented kinetic evidence suggesting that a group with a pK below six influences the activity of β-galactosidase.

As can be seen in Table II, the first-order rate constant (k_3) for the alkylation of β-galactosidase by *N*-bromoacetyl-β-D-galactosylamine is similar in magnitude to the second-order rate constant (k) for the alkylation of methionine by iodo- and bromoacetamides. These rate constants are also similar in magnitude to those found for the alkylation of a model peptide, glycylmethionylglycine, by bromoacetamide (F. Naider, unpublished results). The rapid inactivation of the enzyme by *N*-bromoacetyl-β-D-galactosylamine is therefore a consequence of binding and not of an enhanced chemical reactivity of this particular methionyl residue. The alkylation of methionine is known to be independent of pH and is not subject to acid–base catalysis (Gundlach *et al.*, 1959; Jencks, 1969). It can be seen, however, from Figure 3 that the rate of inactivation of β-galactosidase with *N*-bromoacetyl-β-D-galactosylamine increases with pH. Inactivation at all pH values was a result of the alkylation of a methionyl residue as judged from labeling and reactivation experiments. The reason for the observed pH dependence could be existence of the enzyme in two interconvertible forms which are alkylated at different rates. Indeed the experimental data fit equations derived from a model that includes two forms of the enzyme. The equilibrium between the two forms of the enzyme depends on the deprotonation of a group with pK = 8.9. Interestingly, Tenu *et al.* (1971) have shown that the catalytic activity decreases on deprotonation of a group with pK = 8.4.

As can be seen in Figure 4, β-galactosidase synthesized in the presence of norleucine is composed of two kinds of enzyme, one which is sensitive to alkylation and the other which is not. Since norleucine replaces methionine uniformly at the various methionine loci of *E. coli* proteins, as was shown by Cowie *et al.* (1959) (see also Bruton and Hartley, 1968), we conclude that the β-galactosidase which is not sensitive to alkylation by *N*-bromoacetyl-β-D-galactosylamine is one in which norleucine replaces the methionyl residue normally located at the active site. Therefore, this methionine does not participate in the catalytic mechanism of β-galactosidase, since norleucine can replace it without loss of catalytic activity.

The reactivation of alkylated β-galactosidase is due to the nucleophilic attack of a mercaptide ion on the methionylsulfonium salt. Reactivation is apparently the result of the regeneration of the methionyl residue. Thus, when β-galactosidase inactivated with radioactive *N*-bromoacetyl-β-D-galactosylamine is treated with mercaptoethanol the regeneration in enzymatic activity is accompanied by a loss of radioactivity from the modified protein (Table I). Furthermore, the active enzyme obtained after reactivation could subsequently be inactivated and reactivated, exhibiting kinetic constants almost identical with that of the native enzyme. This conclusion is in agreement with results obtained with model alkylsulfonium salts of methionine as reported in the accompanying communication (Naider and Bohak, 1972).

Acknowledgments

We thank Professor E. Katchalski and Dr. S. Blumberg for their help and interest. We are grateful to Mr. T. Amarant for his expert technical assistance, to Mr. N. Kaldor of the Weizmann Institute Fermentation Unit for his help with growing the bacteria, and to Dr. S. Bauer of the Hebrew University, Jerusalem, for his help in preparing the enzyme.

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Regeneration of Methionyl Residues from Their Sulfonium Salts in Peptides and Proteins†

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ABSTRACT: The reaction of methionylsulfonium derivatives of the model peptide Gly-Met-Gly with several nucleophiles was found to result in the regeneration of an intact methionyl residue. Sulfonium salts derived from this peptide were especially susceptible to attack by sulfur nucleophiles. The rate of regeneration of the methionyl side chain depends on the nature of both the sulfonium salt and the attacking nucleophile. Reaction with mercaptoethanol was found to reactivate chymotrypsin alkylated at Met-192 with phenacyl bromide. Regeneration by thiols may be used to distinguish the alkylation of methionyl residues from that of other amino acid residues in biologically active proteins.

Modification of proteins is often accomplished using various alkylating agents. Such reagents have been found to attack methionyl residues and yield alkylsulfonium derivatives (Stark, 1970). Methionylsulfonium salts are generally stable to performic acid oxidation (Neumann *et al.*, 1962) and cyanogen bromide cleavage (Spande *et al.*, 1970) but decompose on acid hydrolysis to give a variety of products (Gundlach *et al.*, 1959; Goren *et al.*, 1968). Sulfonium salts prepared from acylated methionine were found by Toennies and Kolb (1945a,b) to yield several materials when incubated with different nucleophiles at high temperature for long periods of time.

During a recent investigation it was found that β-galactosidase can be inactivated by the alkylation of a methionyl residue near its active site (Naider *et al.*, 1972; Yariv *et al.*, 1971). On incubation with mercaptoethanol the protein regained more than 90% of its enzymatic activity, suggesting the recovery of the methionyl residue. In view of the findings of Toennies and Kolb (1945b) the decomposition of the methionylsulfonium salt was not expected to result in one product. It is therefore of particular importance to determine whether "thiolysis" is a general method for the regeneration of the methionyl side chain from methionylsulfonium salts. In this report we present evidence that reaction with sulfur nucleophiles leads to the quantitative regeneration of methionyl residues from sulfonium salts in peptides and proteins.

Experimental Section

Materials. Glycyl-L-methionylglycine was purchased from Mann Laboratories (lot R1956). It was homogeneous in paper electrophoresis at pH 3.5. *Anal.* Calcd for $C_9H_{17}N_3O_4S$: N, 15.98; S, 12.12. Found: N, 15.91; S, 13.22. Amino acid analysis after performic oxidation indicated 1.07 methionyl residues/2 glycyl residues.

Chymotrypsin was purchased from Worthington (three-times crystallized, lot CDI SLK). All other materials employed in this investigation were reagent grade chemicals.

Methods

Electrophoresis. Electrophoresis was carried out with a Model LT-36 electrophoresis tank, EC-123 coolant, and an HV-5000 power supply (Savant Instruments). Pyridine–acetate buffer (pH 3.5) was prepared from glacial acetic acid–pyridine–water (10:1:89, v/v). Samples were applied to Whatman No. 3MM paper and run at a gradient of 50 V/cm for 45–60 min. After electrophoresis, the paper was dried, dipped in a solution of ninhydrin (0.5% w/v) in 95% aqueous acetone, and developed by heating at 80–90°.

Amino Acid Analysis after Performic Acid Oxidation. Samples of 10–25 μl were mixed with 0.5 ml of performic acid (Moore, 1963) and kept at 0° for 4–6 hr. The samples were then dried under reduced pressure using a rotary evaporator at 40°. The last traces of performic acid were removed by adding water (0.5 ml) and again evaporating to dryness. Removal of excess performic acid by the addition of HBr (Moore, 1963) was found to decompose some of the sulfonium salts investigated to yield methionine sulfone. The oxidized samples were hydrolyzed with 6 N HCl for 22 hr in evacuated sealed tubes at 110°. Analyses were carried out using a Beckman 121 automatic amino acid analyzer.

Assay of Chymotrypsin. Chymotryptic activity was measured in a pH-stat (Radiometer, Copenhagen) with acetyl-L-tyrosine ethyl ester as substrate. Assays were performed in solutions 2×10^{-3} M in substrate, 0.2 M in KCl and 0.1 M in $CaCl_2$, at pH 8.0 and 24°.

Preparation of Sulfonium Salts. A solution of Gly-Met-Gly (0.05 M) in 0.05 M sodium acetate buffer (pH 4) was made 0.1 M in alkylating agent. The reaction was allowed to proceed at

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