Inactivation of β-Galactosidase by Iodination of Tyrosine-253†

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ABSTRACT: β-Galactosidase is rapidly inactivated by iodination catalyzed by lactoperoxidase but is not inactivated in the presence of the substrate analogue, isopropyl β-D-thiogalactoside (IPTG). Enzyme activity is lost upon the incorporation of 1 mol of iodine per mol of monomer, without dissociation of the tetrameric structure. Tryptic digests of β-galactosidase iodinated with ^{125}I in the presence and absence of IPTG were separated by high-performance liquid chromatography and were compared. One fraction was found to be more highly labeled in the digest from the inactivated protein. After isolation of the peptide, amino acid analysis indicated it to be Asp-Tyr-Leu-Arg, residues 252–255. Thus, Tyr-253 is the most reactive tyrosine in β-galactosidase. This suggests that the conformation of this region of the protein may be altered by binding of IPTG to make Tyr-253 less accessible to iodination. Alternatively, Tyr-253 could be an active-site residue.

β-Galactosidase (EC 3.2.1.23) of *Escherichia coli* is a tetrameric enzyme containing four identical polypeptide chains and four active sites per tetramer. The structure of this protein has been under investigation in this laboratory for some time (Zabin, 1980). The amino acid sequence has been determined; each chain has 1021 amino acid residues (Fowler & Zabin, 1978). The topology of the protein has been probed with antibodies prepared from cyanogen bromide and tryptic peptides (Celada et al., 1978) and with iodoacetate (Jörnvall et al., 1978). An intersubunit contact region has been identified by examination of complementing fragments from *lacZ* mutant strains of *E. coli* (Langley et al., 1975; Celada & Zabin, 1979; Welply et al., 1981).

The reaction mechanism has also been a subject of active research (Deschavanne et al., 1978; Withers et al., 1978; Sinnott, 1978). Some evidence has been obtained for a galactosyl–enzyme intermediate in the β-galactosidase-catalyzed reaction (Tenu et al., 1971; Fink & Angelides, 1975; Rosenberg & Kirsch, 1981), but little is known about the amino acid residues involved in substrate binding and in catalysis. Cysteine residues are not involved in the activity of β-galactosidase (Craven et al., 1965; Loontiens et al., 1970; Jörnvall et al., 1978). A number of affinity-labeled studies have been carried out. *N*-(Bromoacetyl)-β-D-galactopyranosylamine inactivates β-galactosidase (Naider et al., 1972), as do diazo and *p*-nitrophenyltriazene derivatives of the β-D-galactopyranosylmethyl group (Brockhaus & Lehmann, 1976; Sinnott & Smith, 1976). The latter reagent reacts with methionine residue 500 of the polypeptide chain (Fowler et al., 1978b; Sinnott & Smith, 1978). It seems likely that methionine-500 is not involved in catalysis but is near the active site.

Kinetic studies at various pH values have led to the suggestion that a tyrosine residue may be involved in the activity of β-galactosidase (Tenu et al., 1971; Sinnott, 1978). The effect on the enzyme of iodination with lactoperoxidase was investigated to test this possibility.

Experimental Procedures

Materials

o-Nitrophenyl β-D-galactoside (ONPG),[1] IPTG, and lactoperoxidase were obtained from Sigma. The $K^{125}I$ was purchased from Amersham/Searle. All other chemicals were reagent grade except for HPLC-grade water and acetonitrile from Mallinckrodt. TFA was obtained from Pierce Chemical Co. Trypsin treated with TPCK was obtained from Worthington.

β-Galactosidase was purified from *E. coli* A324-5 essentially as reported earlier (Fowler, 1972; Brake et al., 1978).

Methods

β-Galactosidase was assayed by the method of Horiuchi et al. (1962). Before iodination, the protein was dialyzed extensively against 0.05 M potassium phosphate buffer (pH 7.0) containing 10^{-4} M 2-mercaptoethanol. This small amount of 2-mercaptoethanol did not interfere with the lactoperoxidase reaction. Lactoperoxidase was dissolved (1 mg/mL) in 0.05 M potassium phosphate buffer (pH 7.0) and frozen in small aliquots (50–200 μL) until used.

Iodination. For reaction in the absence of IPTG, the following components were present in 0.05 M potassium phosphate buffer (pH 6.75): β-galactosidase (3 mg/mL), lactoperoxidase (25 μg/mL), and $K^{125}I$ (4×10^{-4} M). This mixture was incubated at 28 °C for 5–10 min at which time the reaction was started by adding 5 μL of 0.03% H_2O_2 (v/v) per mL. This same volume of 0.03% H_2O_2 was added every 4 min during the period of the experiment. For reaction in the presence of IPTG, the reaction conditions were the same except that 0.06 M IPTG was added. Certain experiments were done in two stages. In the first, iodination with 4×10^{-4} M KI (unlabeled) and 0.06 M IPTG was carried out for 75 min. β-Galactosidase was then separated from the reagents by passage through a Bio-Gel P-2 column (25 × 1 cm) or Sephadex G-25 column (50 × 1 cm) in 0.05 M potassium phosphate, pH 6.75. Iodination of the purified β-galactosidase at 1.5 mg/mL was started again in the absence of IPTG with 1.6×10^{-4} M $K^{125}I$ and fresh lactoperoxidase.

Iodination was stopped by the addition of sodium azide to 0.02% (w/v), and β-galactosidase enzyme activity was measured. The level of iodination was determined by measurement

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[1] Abbreviations: ONPG, *o*-nitrophenyl β-D-galactoside; IPTG, isopropyl β-D-thiogalactoside; TFA, trifluoroacetic acid; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

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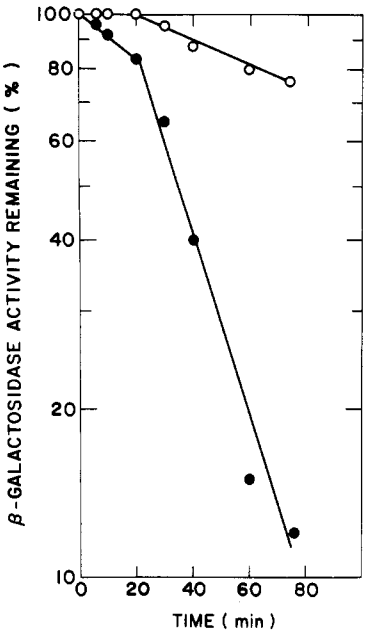

FIGURE 1: Effect of iodination on enzyme activity: (●) no IPTG; (○) 0.06 M IPTG.

of radioactivity after separation of free and protein-bound iodine on a Bio-Gel P-2 column.

Preparation and Separation of Tryptic Peptides. Iodinated β-galactosidase was carboxymethylated (Crestfield et al., 1963) and digested with trypsin (1:100 w/w) in 0.2 M NH_4HCO_3 for 7 h at 37 °C. An equal amount of trypsin was then added, and the digestion was continued overnight at 37 °C. The mixture was acidified with 30% acetic acid and concentrated in vacuo below 40 °C to a small volume. It was then applied in 30% acetic acid to a column of Sephadex G-25 (200 × 2.5 cm), and peptides were eluted with 30% acetic acid. The elution was monitored at A_{280} and by radioactivity. Fractions were purified further by HPLC using an Altex-Beckman Model 332 HPLC instrument with dual detectors. The separations were monitored at 280 and 220 nm, and were on two types of C-18 columns, a Waters preparative Bondapak C-18 column (10 μm, 250 × 10 mm) and in some cases an Altex Ultrasphere C-18 column (5 μm, 250 × 4.6 mm). Gradients of acetonitrile in the presence of 5×10^{-3} M TFA were used to elute the peptides according to the method of Mahoney & Hermodson (1980). All solvents were HPLC grade and were filtered through Millipore filters (0.45- and 0.5-μm pore size) and were degassed before use.

Oligomeric Structure of β-Galactosidase. After iodination, the polymeric structure of the protein was examined by HPLC using two Waters I-250 gel exclusion columns (250 × 5 mm) in series. These columns separated tetramers, dimers, and monomers of β-galactosidase. The elution was carried out with 0.1 M potassium phosphate, pH 7.0, containing 0.2 M NaCl and was monitored at 220 nm.

Results

Effect of Iodination on Activity. The effect of iodination on the enzyme activity of β-galactosidase in the presence and in the absence of the substrate analogue IPTG is shown in Figure 1. In the absence of IPTG, there was a lag period followed by a period of more rapid loss of enzyme activity. After 1 h, less than 20% of the activity remained. No inactivation occurred in the absence of KI, H_2O_2, or lactoperoxidase. These experiments were carried out at pH 6.75. The rate of inactivation increases as the pH is lowered from 8.0 to 6.75. Lower pH values were not tested because the protein tends to precipitate under more acidic conditions.

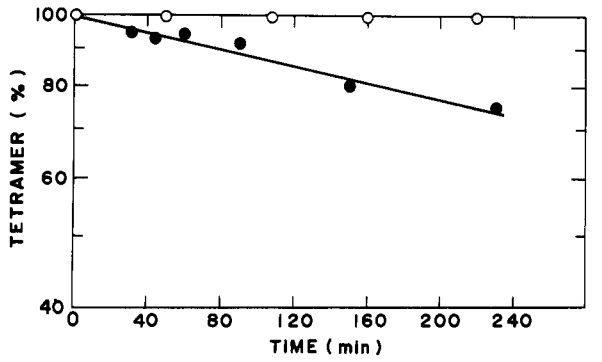

FIGURE 2: Effect of iodination on quaternary structure: (●) no IPTG; (○) 0.06 M IPTG.

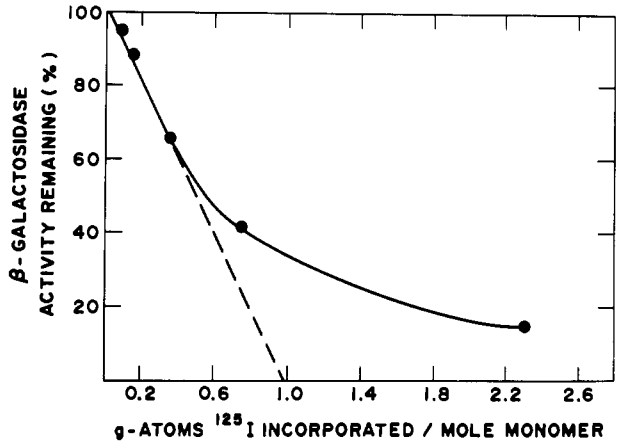

FIGURE 3: Inactivation of β-galactosidase as a function of incorporation of iodine.

By contrast, little activity was lost in the presence of IPTG. After 1 h, about 80% of the activity remained (Figure 1). There was no loss of activity at all for about 20 min, and activity then declined slowly. Substitution for IPTG of glucose or mannitol at 10-fold higher concentrations did not protect the enzyme from inactivation.

Effect of Iodination on Quaternary Structure. As a test of whether iodination had any effect on the tetrameric structure of β-galactosidase, samples of the protein at various time intervals after iodination were examined by HPLC using a gel exclusion column. The results (Figure 2) show that in the absence of IPTG the quaternary structure of β-galactosidase is slowly changed. When only 10% of the activity remained, 90% of the enzyme was still in the tetrameric form. It is clear that loss in activity by iodination is not a consequence of dissociation of the protein. The quaternary structure of IPTG-protected enzyme is not changed at all by iodination.

Stoichiometry of Inactivation by Iodine. The amount of iodine required to inactivate the enzyme was determined by measurement of enzyme activity and of protein-bound ^{125}I at different time intervals. The results of an experiment carried out in the absence of IPTG are shown in Figure 3. Extrapolation to 100% loss of activity indicated that 1 mol of iodine per mol of monomer was sufficient to completely inactivate the enzyme.

Distribution of Label within β-Galactosidase. The stoichiometry of inactivation suggested that a specific residue, presumably a tyrosine, is iodinated when the enzyme is inactivated. However, the biphasic nature of the iodination suggested that the protein might be undergoing a conformational change upon iodination that could expose additional tyrosine residues. Therefore, in the next experiments, samples

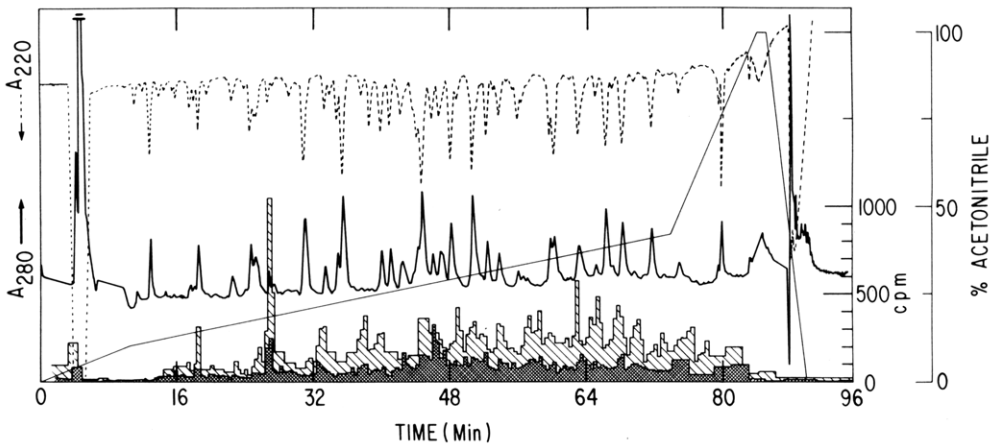

FIGURE 4: Elution profiles and ^{125}I counts from HPLC of tryptic digests of 2.0 nmol of iodinated β-galactosidase. A μBondapak C-18 column (10 μm, 250 × 10 mm) was used. The adsorption profiles of samples iodinated in the absence of IPTG were identical and are superimposed. (Solid spectrum) A_{280}; (broken spectrum) A_{220}; (hatched bars) ^{125}I counts in the absence of IPTG; (cross-hatched bars) ^{125}I counts in the presence of 0.06 M IPTG; (—) percent acetonitrile in a 5×10^{-3} M TFA/acetonitrile gradient. The flow rate was 3 mL/min. The recovery of ^{125}I was greater than 90%.

of β-galactosidase were iodinated only for 30 min with ^{125}I in the presence and in the absence of IPTG. The reaction was stopped, the proteins were carboxymethylated and hydrolyzed overnight with trypsin, and each tryptic digest was chromatographed by reverse-phase HPLC. Adsorption at A_{280} and A_{220} was used to monitor elution (Figure 4). The profiles of the two samples were identical. However, the ^{125}I levels were different. For enzyme iodinated in the presence of IPTG, radioactivity was spread throughout the peptides. A few components were more highly labeled, but not to a marked extent. For enzyme iodinated in the absence of the substrate analogue, radioactivity was also distributed among the peptides. However, one component eluting at 18.5% acetonitrile was more highly labeled than any other and contained about 10% of the total radioactivity.

Considerably more iodine was incorporated into the whole protein in the absence of IPTG; 0.35 mol of ^{125}I per mol of monomer was found compared to 0.1 mol of ^{125}I per mol of monomer when IPTG was present.

Identification of the Specific Tyrosine. For isolation of the highly labeled peptide, a single, larger sample of β-galactosidase (430 nmol) was iodinated in two steps. Reaction with nonradioactive KI in the presence of IPTG was carried out for 75 min, the reagents and IPTG were separated by gel filtration, and iodination of the protein with $K^{125}I$ in the absence of IPTG was allowed to proceed. Under these conditions, the rate of inactivation in the absence of substrate analogue becomes much more rapid; the half-time of inactivation is reduced by a factor of about 4. After 13 min of reaction with labeled iodine, 45% of the activity remained, and about 2 mol of ^{125}I per mol of monomer had been incorporated. The reaction was carried out in this manner to ensure that a sufficient quantity of labeled tryptic peptide could be isolated.

The protein was carboxymethylated and treated with trypsin, and a small portion was chromatographed by HPLC as before. The elution profile was similar to that shown in Figure 4 although the background level was higher (data not shown). The remainder of the tryptic digest was passed through a Sephadex G-25 column, and pooled fractions were examined by HPLC in the same manner to locate the highly labeled peak. Only one Sephadex fraction was found to contain this material. Its HPLC elution profile is shown in Figure 5A. The highly labeled fraction was collected and found by TLC and by amino acid analysis to contain two peptides. These were separated on a reverse-phase (5 μm) column of higher resolving power to give a labeled and an unlabeled peptide (Figure 5B). From the amino acid composition (Table I), the labeled peptide was found to be T19, Asp-Tyr-Leu-Arg, residues 252–255, and the unlabeled peptide T42, Trp-Ser-Ile-Lys, residues 516–519 of β-galactosidase (Fowler et al., 1978a). The specifically labeled amino acid residue is thus Tyr-253.

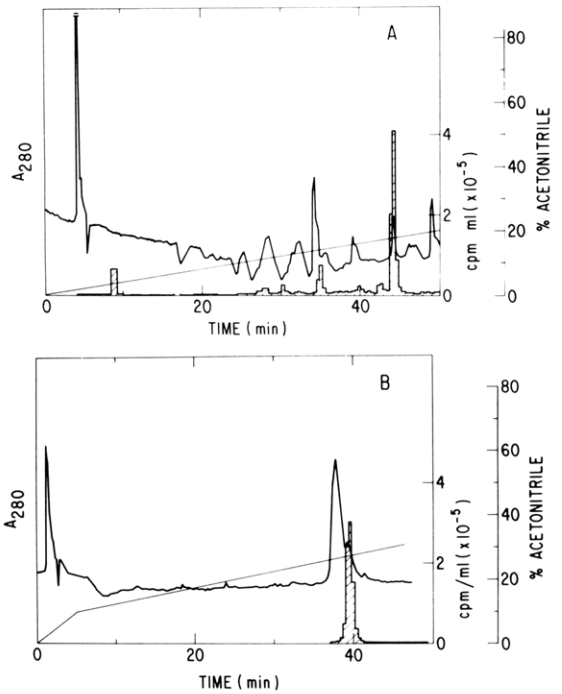

FIGURE 5: (A) Elution profile of the highly labeled peptide fraction. The fraction was obtained from a tryptic digest of 430 nmol of iodinated β-galactosidase after preliminary separation on Sephadex G-25. The same HPLC conditions were used as in Figure 4. (B) Elution profile of the highly labeled fraction of Figure 5A. HPLC separation was on an Altex Ultrasphere C-18 column (5 μm, 250 × 4.6 mm). The flow rate was 1.5 mL/min with the same solvent system. Symbols are the same as in Figure 4.

Discussion

The results presented here show that β-galactosidase is inactivated by iodination of a specific tyrosine residue at position 253 in the polypeptide chain. The enzyme is protected from inactivation by the substrate analogue, IPTG. Data obtained by extrapolation of initial rates indicate that inac-

Table I: Amino Acid Composition of β-Galactosidase Tryptic Peptides T19 and T42 from the Protein Inactivated by Iodination

	T19[a]	T42
lysine	0.1	1.1 (1)
arginine	0.9 (1)[b]	0.2
tryptophan		1.0 (1)
aspartic acid	1.0 (1)	0.3
threonine	0.1	0.1
serine	0.3	0.9 (1)
glutamic acid	0.2	0.3
proline		0.2
glycine	0.4	0.3
alanine	0.1	0.2
isoleucine	0.1	1.0 (1)
leucine	1.1 (1)	
tyrosine	0.9 (1)	0.3
phenylalanine		0.4

[a] Specifically iodinated peptide. [b] Integral numbers are based on the sequence (Fowler & Zabin, 1978).

tivation occurs when one iodine atom is incorporated per monomer of enzyme. At the earliest times of reaction, much of the iodine was incorporated into Tyr-253. Continued reaction results in iodine labeling throughout the molecule. There are 31 tyrosine residues in the β-galactosidase monomer, and some of these as well as other residues must also be iodinated. It is interesting that the background level of labeling is considerably higher in the absence as compared to the presence of a substrate analogue. This suggests that a more compact or "tightened" structure results when IPTG is added. Results of studies concerned with the action of effectors on enzyme kinetics suggested that a conformational change occurs on addition of IPTG to β-galactosidase. A direct effect was seen by difference spectra between free enzyme and IPTG enzyme (Deschavanne et al., 1978). This may account for differences in iodination in the two states.

Loss of activity on iodination in both the presence and absence of ligand occurred with a lag period, although the loss was very much less in the presence of IPTG. It is possible that an initial iodination loosens or opens the tetramer somewhat so that it becomes more susceptible to further iodination. This could account for the two rates. However, actual dissociation of the whole protein to subunits occurs very slowly when IPTG is absent and not at all in its presence. Other changes causing inactivation of the protein, such as oxidation of certain residues (i.e., tryptophan and methionine), have not been ruled out.

In the experiments reported here, iodination was carried out with lactoperoxidase, which is a surface-oriented probe for proteins (York & Blombäck, 1979). Evidently in the absence of IPTG, Tyr-253 must be close to the surface rather than buried within the protein.

The high reactivity of Tyr-253 may be explained by assuming that this residue is the most accessible of all tyrosine side chains in the free enzyme to iodination. On addition of substrate analogue, however, Tyr-253 is made inaccessible by a conformational change. A second possible explanation is that Tyr-253 is in the substrate binding region and might be the acid catalytic group involved in the catalytic mechanism of β-galactosidase. In an earlier study with an active site specific reagent for β-galactosidase, a galactopyranosylmethyl group was bound to Met-500 (Fowler et al., 1978b). This result suggested that Met-500 was at or near the active site. The present results suggest that if indeed Tyr-253 is an active-site residue, the polypeptide chain is folded so as to bring Tyr-253 and Met-500 close to each other. This would mean that for β-galactosidase, as for enzymes in general, residues far apart in the primary structure are brought together to form the active site.

These experiments also illustrate the great resolving power of reverse-phase HPLC columns for peptides. In a tryptic digest of β-galactosidase, about 60 separate peaks of absorption at 220 nm were obtained. This is more than three-fourths of the theoretical number (Fowler et al., 1978a). These separation procedures can be useful for additional active-site studies of β-galactosidase.

Acknowledgments

We thank David Dupont and Paulette Osborne for excellent technical assistance.

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