Amino Acid Sequence of the Regulatory-Site Glyoxylate Peptide of Biodegradative Threonine Dehydratase of *Escherichia coli*

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Incubation of purified *Escherichia coli* biodegradative threonine dehydratase with glyoxylate resulted in covalent binding of 1 mol of glyoxylate per mol of protein with concomitant loss of enzyme activity. The glyoxylate-binding site was identified as a heptapeptide representing amino acid residues Ser-33-Asn-Tyr-Phe-Ser-Glu-Arg-39 in the protein primary structure. Addition of glyoxylate to a culture of *E. coli* cells led to time-dependent enzyme inactivation. Immunoprecipitation with anti-dehydratase antibody of extract from [¹⁴C]glyoxylate-treated cells revealed labeled dehydratase polypeptide. These results are interpreted to mean that enzyme inactivation by glyoxylate in *E. coli* cells is associated with covalent protein modification.

The biodegradative threonine dehydratase (EC 4.2.1.16) of *Escherichia coli* and *Salmonella typhimurium*, a pyridoxal phosphate-containing enzyme that catalyzes dehydration of L-threonine and L-serine, is subject to several types of regulatory control both at the level of enzyme synthesis and at the level of enzyme function. For example, the purified enzyme is allosterically stimulated by AMP (2, 4, 19), inhibited by α -keto acids (2, 14, 15), and inactivated by certain intermediary metabolites of the glycolytic pathway and the tricarboxylic acid cycle (2, 5, 15, 16). A detailed analysis of the latter phenomenon, designated catabolite inactivation (5), indicated that the mechanism of enzyme inactivation by pyruvate involves covalent attachment of pyruvate to the active tetrameric form of the enzyme followed by dissociation of the protein to yield an inactive dimer (2, 5). The glyoxylate-mediated loss of catalytic activity, on the other hand, is due to covalent binding of glyoxylate to the dehydratase tetramer accompanied by conformational changes in the subunits and resolution of pyridoxal phosphate coenzyme from the enzyme active site (15, 16). Thus, the primary event in catabolite inactivation by both pyruvate and glyoxylate appears to be covalent modification of the enzyme. Recently, we cloned the *E. coli* *tdc* gene, which encodes biodegradative threonine dehydratase (3, 6), and determined the complete amino acid sequence of the protein from the nucleotide sequence of the gene (3). The active-site pyridoxal phosphate-bound lysine residue involved in the dehydration reaction was localized at amino acid residue Lys-58 (3), and a 13-residue tryptic peptide, Thr-230 to Arg-242, implicated in binding of the allosteric modifier AMP on the dehydratase, was mapped on the protein primary structure (17). In this study, we addressed two questions. (i) Where on the protein molecule does glyoxylate bind to potentiate a cascade of events leading to enzyme inactivation, and (ii) does covalent modification of the dehydratase by glyoxylate occur in intact cells? The answers would help to explain the mechanism of catabolite inactivation at the level of protein structure and establish the physiological significance of the regulatory phenomenon in *E. coli*.

MATERIALS AND METHODS

Enzyme purification and assay. *E. coli* K-12 strain KL227 harboring multicopy plasmid pEC61 with the cloned dehydratase gene (6) was used to purify threonine dehydratase as previously described (15). The activity of pure dehydratase was assayed spectrophotometrically as previously described (15); enzyme activity in intact cells was measured colorimetrically with toluene-treated cells (12). The concentration of protein was determined by the method of Lowry et al. (11).

Glyoxylate binding in vitro. AMP-free enzyme prepared by exhaustive dialysis against 100 mM potassium phosphate buffer (pH 8.0) was incubated at 4°C with [¹⁴C]glyoxylate (specific activity, 5.5 Ci/mol; Amersham Corp., Arlington Heights, Ill.) and the amount of [¹⁴C]glyoxylate bound was determined by the dialysis method as previously described (15). The concentrations of various components in the incubation mixture and the time of incubation are described below for each individual experiment.

Labeling of dehydratase in *E. coli* cells with glyoxylate. A culture of KL227(pEC61) was grown aerobically for 12 h at 37°C in 5 ml of LB medium (13). The cells were collected by centrifugation, washed twice with 100 mM phosphate buffer (pH 8.0), and suspended in 0.5 ml of the same buffer. One hundred microliters of cell suspension was added to 3 ml of H4 medium (7) consisting of four amino acids (threonine, serine, valine, and isoleucine) and fumarate plus cyclic AMP and incubated anaerobically at 37°C to induce dehydratase synthesis. After 4 h, 5 mM [¹⁴C]glyoxylate (specific activity, 5,280 cpm/nmol) and 100 μ g of chloramphenicol (Sigma Chemical Co., St. Louis, Mo.) per ml were added, and incubation was continued for another 1.5 h. Cells were collected by centrifugation, washed twice with ice-cold phosphate buffer, and suspended in 3 ml of the same buffer. After the cell suspension was kept at 4°C for 15 min, the cells were again centrifuged and suspended in 500 μ l of phosphate buffer. The cells were sonically disrupted at 0°C, and the extract was centrifuged at 12,000 $\times$ g for 15 min to obtain a clear supernatant fluid, which was concentrated to a volume of about 100 μ l with a Diaflo apparatus (Amicon Corp., Danvers, Mass.).

Immunoprecipitation of radiolabeled enzyme. Portions of cell extract (50 μ l) were mixed with 6 μ l of 1.5 M NaCl and either 10 μ l of affinity-purified anti-dehydratase antibody (12) or 10 μ l of preimmune serum and incubated at 4°C for 18 h. Five microliters (25 μ g) of purified soluble protein A (Sigma)

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was then added to each mixture, and the solutions were incubated further at 4°C for 2 h. The precipitates were collected by centrifugation at $12,000 \times g$ for 15 min, dissolved in 20 μ l of sodium dodecyl sulfate (SDS)-sample buffer and heated at 95°C before electrophoresis through an SDS-11% polyacrylamide slab gel as described by Laemmli (9). The gel was stained with Coomassie blue to visualize the molecular weight markers, destained, and dried for autoradiography to locate the labeled protein.

Isolation of glyoxylate-bound peptide. Purified AMP-free enzyme (7 mg; 50 nmol) was incubated for 15 h at 4°C with 20 mM [14 C]glyoxylate (specific activity, 880 cpm/nmol) in 0.7 ml of 100 mM potassium phosphate buffer (pH 8.0), at which time the enzyme was inactivated by 98%. The solution was diluted fivefold with 10 mM phosphate buffer (pH 8.0) and dialyzed against the same buffer to remove free radioactivity. The inactive protein contained 0.7 mol of bound glyoxylate per mol of enzyme. The dialyzed enzyme was lyophilized, dissolved in 100 mM sodium borate buffer (pH 8.8) containing 6 M guanidine hydrochloride, and then reduced and alkylated with sodium iodoacetate as previously described (8). The carboxymethylated protein was digested with L-1-tosylamide-2-phenylmethyl chloromethyl ketone-treated trypsin (Worthington Diagnostics, Freehold, N.J.) (8), and the tryptic peptides were passed through a Bio-Gel P6 column (65 by 1 cm) equilibrated with 10 mM ammonium bicarbonate solution. The fractions eluted early with approximately 75% of the input radioactivity (30,000 cpm) were pooled and concentrated, and the peptides were separated by high-pressure liquid chromatography (HPLC) on a Ultrasphere-ODS C₁₈ column (Beckman Instruments, Inc., Fullerton, Calif.) with a linear gradient of 5 to 40% acetonitrile (J. T. Baker Chemical Co., Phillipsburg, N.J.) in 1 mM sodium-potassium phosphate buffer (pH 6.2) for 35 min at a flow rate of 1 ml/min. Fractions containing radioactivity eluted at 15 min were further purified on a DE-52 ion-exchange column (30 by 1 cm; Bio-Rad Laboratories, Richmond, Calif.) equilibrated in 0.02 M ammonium bicarbonate solution by applying a linear gradient of 0.02 to 0.5 M ammonium bicarbonate solution. A single radioactive peptide, well separated from minor unlabeled peaks, was eluted in fractions 85 to 90 ml at a 0.2 M ammonium bicarbonate concentration. The peptide was pure as judged by rechromatography on HPLC with a linear gradient of 0 to 10% acetonitrile in 0.1% trifluoroacetic acid (Pierce Chemical Co., Rockford, Ill.) for 10 min followed by 10 to 40% acetonitrile in 0.1% trifluoroacetic acid for 50 min at a flow rate of 1 ml/min. The pure peptide was analyzed for amino acid composition, and its amino acid sequence was determined by using the manual Edman degradation procedure of Tarr (20) in the University of Michigan Protein Sequencing Facility.

Identification of free [14 C]glyoxylate. The fractions containing a small amount of radioactivity eluted at around 50 ml during Bio-Gel P6 fractionation were found to contain free [14 C]glyoxylate. The fractions were pooled, lyophilized, and then dissolved in 50 μ l of deionized water. Unlabeled glyoxylate was added to this solution, and the mixture was separated by paper chromatography by using the butanol-pyridine-water (3:2:1.5, vol/vol) solvent system (10). The paper was dried and sprayed with a 0.05% solution of bromophenol blue to visualize glyoxylate (10). The spot representing authentic glyoxylate (R_f , 0.39) was cut out and upon scintillation counting was found to contain >80% of the applied radioactivity.

RESULTS AND DISCUSSION

Covalent binding of glyoxylate. Previous reports (15, 16) indicated that incubation of 600 μ g of pure threonine dehydratase per ml with glyoxylate resulted in rapid binding within 1 to 5 min of about 1 mol of glyoxylate per mol of enzyme, followed by slow binding of several additional moles of glyoxylate over a period of several hours. Assay for dehydratase activity during incubation with glyoxylate revealed loss of less than 10% enzyme activity with 1 mol of bound glyoxylate; by the time the enzyme was completely inactivated, a little over 3 mol of glyoxylate was bound per mol of enzyme. This complex pattern of incorporation of glyoxylate associated with enzyme inactivation posed a technical problem for identification of the glyoxylate-binding site involved in the loss of catalytic activity. Therefore, we reexamined the various parameters required for enzyme inactivation by glyoxylate. Incubation of 10 mg of AMP-free dehydratase per ml with 20 mM [14 C]glyoxylate in 100 mM potassium phosphate buffer, pH 8.0, at 4°C resulted in time-dependent enzyme inactivation with a gradual increase in incorporation of glyoxylate into protein (Fig. 1). After 10 h of incubation, complete enzyme inactivation was achieved, with approximately 0.8 mol of glyoxylate bound per mol of protein. With continued incubation of the enzyme, more glyoxylate was incorporated, and 60 h after the enzyme was completely inactivated, a total of approximately 3 mol of glyoxylate was bound per mol of the enzyme.

The results obtained with a high dehydratase concentration showed no rapid incorporation of glyoxylate during the first few minutes, as seen earlier with a low protein concentration (15, 16); however, slow binding of glyoxylate molecules continued to occur after enzyme inactivation, as observed previously (15, 16). The reason for the lack of rapid binding of glyoxylate at a high enzyme concentration remains unclear. Because the *E. coli* dehydratase exhibits an association-dissociation equilibrium in solution influenced by protein concentration (15), it is conceivable that changes in protein conformation at a high dehydratase level can affect glyoxylate incorporation. In any case, the binding data presented above indicate that covalent attachment of about 1 mol of glyoxylate to active dehydratase was sufficient to cause complete loss of catalytic activity; inactivation of threonine dehydratase with pyruvate (5) and 8-azido-AMP (17) also involved covalent attachment of 1 mol of ligand per mol of protein.

The loss of enzyme activity by covalent protein modification by all three ligands (pyruvate, 8-azido-AMP, and glyoxylate) at less-than-stoichiometric amounts has been previously correlated with changes in the subunit organization of the native enzyme (5, 15–17). For example, binding of 1 mol of pyruvate altered the association-dissociation equilibrium of the enzyme in favor of the dissociated form to yield an inactive dimer (5), and inactivation of threonine dehydratase by the photoaffinity analog 8-azido-AMP was most likely due to formation of an inactive dimeric species with covalently attached 8-azido-AMP (17). Furthermore, several lines of evidence, especially perturbation of spectral properties of the enzyme due to glyoxylate binding (15, 16), strongly suggested that a change in the conformation of the enzyme subunits was accompanied by resolution of the pyridoxal phosphate coenzyme. These findings suggest that changes in protein conformation associated with ligand binding result in actual loss of catalytic activity.

As reported previously (16), glyoxylate inactivation of threonine dehydratase at a low protein concentration re-

FIG. 1. Kinetics of enzyme inactivation and incorporation of glyoxylate into protein. A sample of AMP-free enzyme at 10 mg of protein per ml was incubated at 4°C with 20 mM [^{14}C]glyoxylate (specific activity, 1,760 cpm/nmol) in 100 mM potassium phosphate buffer, pH 8.0, for various time periods, and samples were removed to measure residual enzyme activity, protein concentration, and incorporation of radioactivity into protein after dialysis against phosphate buffer (15). The enzyme activity was determined spectrophotometrically (16) and is expressed as a percentage of that found in the control not treated with glyoxylate. The amount of glyoxylate incorporated into protein was calculated from protein concentration and radioactivity measurements.

leased 2 mol of pyridoxal phosphate coenzyme from the enzyme active site; aggregation of the protein and further binding of glyoxylate followed the enzyme inactivation process. Before attempting to isolate the peptide containing glyoxylate, it was thought important to obtain evidence that

incubation of dehydratase at a high protein concentration with glyoxylate exhibits similar characteristics. The results of these experiments are summarized below. (i) A solution of threonine dehydratase (10 mg of protein per ml) was inactivated to 98% with 20 mM [^{14}C]glyoxylate, and the incuba-

FIG. 2. Purification of glyoxylate-bound peptide. (A) Fractionation of tryptic peptides on a Bio-Gel P6 column. One-milliliter fractions were collected and monitored for A_{235} (○) and radioactivity (●). (B) Pooled material in fractions 20 to 28 ml eluted from the Bio-Gel P6 column was concentrated, and the peptides were separated on HPLC by using a phosphate-acetonitrile gradient. The material eluted at 15 min (solid bar) contained 85% of the radioactivity applied to the column. (C) DE-52 ion-exchange chromatography of radioactive material obtained during HPLC separation. The hatched peak represents the radioactive peptide. (D) Rechromatography of the labeled peptide from the DE-52 step on HPLC by using a trifluoroacetic acid-acetonitrile gradient. For details, see Materials and Methods.

tion mixture was passed through a Bio-Gel P6 column. The fractions containing the 388-nm absorbing material, representing free pyridoxal phosphate (18), were pooled and concentrated. Direct fluorometric measurement (1) of this material showed that 2 mol of pyridoxal phosphate was released per mol of protein. The inactive enzyme had 0.8 mol of bound glyoxylate per mol of tetramer. (ii) The ^{14}C -labeled enzyme isolated in the above-described experiment was passed through a Bio-Gel P200 column. The inactive enzyme was eluted as an aggregated species with an M_r of 600,000. (iii) An enzyme solution was incubated at 4°C with 20 mM [^{14}C]glyoxylate for 96 h. After exhaustive dialysis to remove free [^{14}C]glyoxylate, the protein contained approximately 3.5 mol of bound glyoxylate per dehydratase tetramer. These results, taken together, suggest that enzyme inactivation by glyoxylate at high protein concentrations influences the structure and chemistry of the protein in a manner similar to that seen at low protein concentrations (15, 16). It may be recalled that the monoglyoxylated form of the enzyme generated after rapid initial binding of 1 mol of glyoxylate per mol of enzyme at a low protein concentration was structurally and catalytically indistinguishable from the native enzyme (16).

Identification of the glyoxylate-bound peptide. The data presented thus far established that incubation of threonine dehydratase (10 mg of protein per ml) with 20 mM [^{14}C]glyoxylate for about 10 h resulted in complete loss of catalytic activity with about 1 mol of bound glyoxylate per mol of tetramer, thereby facilitating identification of the ligand-binding site involved in enzyme inactivation. To isolate the glyoxylate-bound peptide, the inactive protein was reduced and alkylated and then subjected to trypsin digestion. In a preliminary experiment, when the tryptic peptides were separated by HPLC with a trifluoroacetic acid-acetonitrile gradient, all radioactivity emerged at an elution time of 2 min (data not shown); the radioactive material was identified as free [^{14}C]glyoxylate as described in Materials and Methods. The data indicate that although the protein-glyoxylate linkage was stable during the alkylation-reduction reaction, release of free glyoxylate occurred at the low, acidic pH of the solvent system. Attempts to stabilize the protein-glyoxylate linkage by various chemical modifications, including acetylation reaction and sodium borohydride reduction (P. Datta, unpublished data), proved unsuccessful. Therefore, the tryptic peptides were separated first by fractionation on a Bio-Gel P6 column followed by HPLC with a neutral phosphate-acetonitrile gradient and then on a DE-52 ion-exchange column (Fig. 2), yielding a pure radioactive peptide. It should be mentioned that a small amount (<15%) of enzyme-bound [^{14}C]glyoxylate was lost during Bio-Gel fractionation and the HPLC and ion-exchange steps.

The radioactive peptide recovered from the DE-52 column was desalted by HPLC by using a trifluoroacetic acid-acetonitrile gradient before amino acid analysis and sequence determination. Although this treatment released all radioactivity as free [^{14}C]glyoxylate (see above), the loss of radioactivity was judged to be inconsequential because recovery of the labeled amino acid-[^{14}C]glyoxylate adduct was not expected during composition and sequence analyses because of the acid-labile nature of the protein-glyoxylate linkage.

The amino acid composition of the glyoxylate-binding peptide revealed the following residues (moles per mole of peptide): Asx (0.5), Arg (1.3), Glx (0.9), Gly (0.8), Phe (0.9), Ser (2.4), and Tyr (0.8). The amino acid sequence of the

FIG. 3. Glyoxylate-mediated loss of dehydratase activity in *E. coli* cells. Washed cells from a culture of strain KL227(pEC61) previously grown in LB medium were incubated anaerobically at 37°C in 3 ml of H4 medium (7) to induce threonine dehydratase. At 4 h (arrow), one culture was supplemented with 5 mM glyoxylate and 100 μg of chloramphenicol per ml (Δ), and the second received only chloramphenicol ($\circ$). Incubation was continued, and samples of cultures were withdrawn at the times indicated to measure enzyme activity in washed, toluene-treated cells as previously described (12).

purified peptide determined by the manual Edman degradation procedure was found to be Ser-Asn-Tyr-Phe-Ser-Glu-Arg. On the basis of the known amino acid sequence of the *E. coli* biodegradative threonine dehydratase (3), the heptapeptide corresponds to Ser-33 to Arg-39 in the protein primary structure. The Gly residue detected during amino acid analysis but not found during sequence determination appears to be a contaminant encountered in composition analysis.

The glyoxylate-binding heptapeptide Ser-Asn-Tyr-Phe-Ser-Glu-Arg revealed no unusual property. In analogy to other proteins that are subjected to covalent modification by various reagents, several amino acid residues in this peptide, notably Ser and Tyr, may be potential sites for glyoxylate binding in a hemiacetal linkage. Because of its extreme acid lability, new techniques have to be developed for positive identification of the amino acid-glyoxylate adduct. It may be mentioned in this context that the peptide(s) containing bound glyoxylate during long-term incubation of the inactive enzyme (15, 16; Fig. 1) has not been identified. However, previous experiments with the *S. typhimurium* threonine dehydratase (8) showed that the enzyme inactivated to 80% contained one tryptic peptide with bound glyoxylate, whereas the enzyme modified with 3 mol of glyoxylate contained an additional peptide with covalently attached glyoxylate. These data suggest that a distinct enzyme site is involved in the nonspecific binding of glyoxylate following enzyme inactivation.

Glyoxylate modification of threonine dehydratase in *E. coli* cells. Since the initial report (5) of catabolite inactivation of

FIG. 4. SDS-polyacrylamide slab gel electrophoresis and autoradiography of immunoprecipitated material from extract of [^{14}C]glyoxylate-labeled cells. Lanes: 1, 5 μg of pure threonine dehydratase labeled with [^{14}C]glyoxylate in vitro; 2, precipitate obtained after incubation of affinity-purified antibody with extract from cells labeled with [^{14}C]glyoxylate; 3, extract from [^{14}C]glyoxylate-labeled cells treated with preimmune serum. For details, see Materials and Methods. The numbers on the left indicate molecular weights in thousands.

threonine dehydratase by intermediary metabolites, much information about the physical and chemical bases of enzyme inactivation by covalent protein modification has been gathered (2, 5, 8, 15, 16). However, in physiological terms, there is no experimental evidence to decide whether covalent modification of the dehydratase by metabolites implicated in catabolite inactivation occurs in intact cells. To test this experimentally, 5 mM glyoxylate was added to a culture of *E. coli* KL227(pEC61) at the mid-point of the enzyme induction curve, and loss of enzyme activity was monitored (Fig. 3). To prevent new enzyme synthesis, chloramphenicol was also added at a concentration of 100 $\mu\text{g}/\text{ml}$ of culture along with glyoxylate. In 90 min, about 85% of the enzyme activity was lost compared with that of a control culture without glyoxylate.

In an identical experimental protocol described above, 5 mM [^{14}C]glyoxylate plus chloramphenicol was added to label the enzyme in intact cells. After 90 min of incubation, a cell extract was prepared and reacted with affinity-purified anti-dehydratase antibody, and the immunoprecipitate was analyzed by SDS-polyacrylamide slab gel electrophoresis (Fig. 4). A labeled 35-kilodalton polypeptide was precipitated by the dehydratase antibody from the extract of [^{14}C]glyoxylate-treated cells (lane 2), whereas no radioactive polypeptide was found when the labeled cell extract was reacted with preimmune serum (lane 3). Lane 1 shows the position of dehydratase subunits when a sample of pure enzyme labeled with [^{14}C]glyoxylate was subjected to SDS-gel electrophoresis. These results indicate that glyoxylate inactivation of threonine dehydratase in intact cells is associated with covalent protein modification.

Physiological significance. The physiological significance of catabolite inactivation of biodegradative threonine dehydratase by intermediary metabolites may be rationalized in the context of the proposed role of this inducible enzyme in anaerobic ATP production from threonine (21). As men-

tioned earlier (5), addition of glucose to the culture medium would lead to increased production of ATP (and certain metabolites derived from glucose, such as pyruvate and glyoxylate) and spare the need for anaerobic threonine degradation. For cellular economy, pyruvate and glyoxylate would then shut off threonine catabolism by inactivating threonine dehydratase. The experiments summarized here indicate that addition of glyoxylate to *E. coli* cells resulted in enzyme inactivation, as well as incorporation of glyoxylate into dehydratase protein. These data, in conjunction with those reported with the purified enzyme correlating glyoxylate-mediated loss of enzyme activity with covalent protein modification (15, 16), support the notion that catabolite inactivation functions as an important physiological regulatory device in *E. coli* metabolism.

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