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Inactivation of *Escherichia coli* 2-Amino-3-ketobutyrate CoA Ligase by Phenylglyoxal and Identification of an Active-Site Arginine Peptide[1]

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Treatment of homogeneous preparations of 2-amino-3-ketobutyrate CoA ligase from *Escherichia coli*, a pyridoxal 5′-phosphate-dependent enzyme, with phenylglyoxal, 4-(oxyacetyl)phenoxyacetic acid, 2,3-butanedione, or 1,2-cyclohexanedione results in a time- and concentration-dependent loss of enzymatic activity. Phenylglyoxal in 50 mM phosphate buffer (pH 7.0) is the most effective modifier, causing >95% inactivation within 20 min at 25°C. Controls establish that this inactivation is not due to modifier-induced dissociation or photoinduced nonspecific alteration of the ligase. The substrate, acetyl CoA, or the coenzyme, pyridoxal 5′-phosphate, gives >50% protection against inactivation. Enzyme partially inactivated by phenylglyoxal has the same K_m value for glycine but the V_{max} decreases in proportion to the observed level of inactivation. Whereas the native apoligase shows good recovery of activity with time in parallel with an increase in 428-nm absorptivity when incubated with pyridoxal 5′-phosphate, no such effects are seen with the phenylglyoxal-modified apoligase. Reaction of the enzyme with [^{14}C]phenylglyoxal allowed for the isolation of a peptide which, by amino acid composition and sequencing data, was found to correspond to residues 349–378 in the intact enzyme. These results indicate that arginine residue-366 and/or residue-368 in the primary structure of *E. coli* 2-amino-3-ketobutyrate ligase is at the active site.

Threonine metabolism in eukaryotes and prokaryotes has been known to occur via three pathways, one initiated by threonine dehydratase, another by threonine aldolase, and a third by threonine dehydrogenase. Recent studies (1–3) have shown that the threonine dehydrogenase-initiated route is probably the most important of the three; it accounts for 87% of the L-threonine degraded in the liver of normally fed rats (4), it is the only pathway for L-threonine degradation that is detected in chicken liver (5), and it assumes the predominant role in *Escherichia coli* (6) and other microorganisms (7). Threonine dehydrogenase (EC 1.1.1.103) catalyzes the NAD^+-dependent oxidation of L-threonine to 2-amino-3-ketobutyrate (or 2-amino-3-oxobutanoate).

2-Amino-3-oxobutanoate glycine-lyase (CoA-acetylating) (EC 2.3.1.29), also called 2-amino-3-ketobutyrate ligase, aminoacetone synthase, or aminoacetone synthetase, catalyzes the second step in the threonine dehydrogenase-initiated pathway; by this reaction, glycine and acetyl CoA are formed via a CoA-dependent cleavage of 2-amino-3-ketobutyrate. The conversion of threonine to glycine by the coupled action of these two enzymes accounts for the observation that *glyA* (serine hydroxymethyltransferase gene) mutants can utilize threonine for growth (8–10). In cells that contain a functional *glyA* gene, these coupled reactions provide a highly efficient alternate route for serine biosynthesis (6). The genes for threonine dehydrogenase and 2-amino-3-ketobutyrate ligase make up the *tdh* operon which is located near minute 81 of the *E. coli* genetic map (11). The *tdh* operon is one among a multigene family that is regulated by L-leucine, mediated by the leucine-responsive regulatory protein (Lrp)[4] (12–14).

In an earlier paper (15), we reported the purification of 2-amino-3-ketobutyrate ligase to homogeneity from extracts of a mutant of *E. coli* K-12 that we forced to grow

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[4] Abbreviations used: Lrp, leucine-responsive regulatory protein; PTH, phenylthiohydantoin; PTC, phenylthiocarbamoyl; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate.

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on L-threonine as sole carbon source; this mutant has constitutively derepressed levels of both threonine dehydrogenase and the ligase. 2-Amino-3-ketobutyrate ligase from *E. coli* is an 84,000-Da protein and consists of two apparently identical subunits. It is a pyridoxal 5′-phosphate-dependent enzyme and is strictly specific for glycine. The primary structure of the *kbl* (2-amino-3-ketobutyrate CoA ligase) gene and that of its protein product are known (16).

We are attempting to map active-site amino acid residues in both threonine dehydrogenase (17–19) and this ligase in efforts to elucidate how these two enzymes are tightly coupled or may even form a catalytic complex that allows for efficient passage of the unstable 2-amino-3-ketobutyrate to the ligase so that it is cleaved rather than spontaneously decarboxylated. In first studies with the *E. coli* ligase, we isolated and sequenced the active-site peptide containing the lysine residue that forms a Schiff base with pyridoxal 5′-phosphate (20). We now report that an arginine residue (or residues), located in a peptide corresponding to amino acid residues 349–378 in the native protein, is also a participant in the active site of this enzyme.

MATERIALS AND METHODS

Enzymes and chemicals. Homogeneous samples of 2-amino-3-ketobutyrate ligase were prepared from extracts of mutant *E. coli* SBD-76 cells as reported earlier (15). The purity of all samples was established by polyacrylamide gel electrophoresis, both with and without SDS. The specific activity of enzyme preparations used in these studies was in the range of 2.5 to 2.8 units/mg of protein; a unit of ligase activity is defined as that amount which catalyzed the formation of 1 μmol of CoA/min at 25°C. Molar enzyme concentrations are based on the ligase being a homodimer with a relative molecular mass of 84 kDa (15). *Staphylococcus aureus* V8 endoproteinase was a product of Boehringer Mannheim Biochemicals.

HPLC-grade methanol and acetonitrile were obtained from Burdick and Jackson Laboratories, Inc., whereas phenyl[2-^{14}C]glyoxal (27 mCi/mmol) was from Research Products International Corp. The following materials were purchased from the companies noted: 5,5′-dithiobis(2-nitrobenzoic acid), phenylisothiocyanate, triethylamine, and PTH-amino acid standards from Pierce Chemical Co.; acetyl CoA and pyridoxal · HCl from Sigma Chemical Co.; phenylglyoxal, 1,2-cyclohexanedione, and 2,3-butanedione from Aldrich Chemical Co.; pyridoxal 5′-phosphate from Nutritional Biochemicals Corp.; TFA from J. T. Baker Chemical Co. 4-(Oxyacetyl)phenoxyacetic acid was kindly provided by Drs. P. J. Duerksen-Hughs and K. D. Wilkinson of Emory University (Atlanta, GA). All other chemicals were of the highest purity commercially available.

The HPLC equipment used to obtain peptide maps and to separate PTH-amino acids has been described before (21).

Assay of ligase activity and inactivation studies with dicarbonyl reagents. 2-Amino-3-ketobutyrate ligase activity was measured spectrophotometrically at 412 nm as described earlier for Assay I (15). Unless indicated otherwise, inactivation studies were carried out by incubating the enzyme (12–15 μM) in the dark with a given dicarbonyl reagent in 50 mM potassium phosphate buffer (pH 7.0) at 25°C. Aliquots (5–10 μl) of the reaction mixture were removed at varying time intervals and mixed with 1 ml of the assay mixture, and the increase in absorbance at 412 nm was monitored.

Protection of ligase activity against inactivation by phenylglyoxal. An aliquot of the enzyme (12–15 μM) was first incubated for 30 min at 25°C alone or with acetyl CoA (2 mM), pyridoxal 5′-phosphate (3 mM), glycine (100 and 200 mM), or L-threonine (10 and 50 mM) in 50 mM potassium phosphate buffer (pH 7.8). Phenylglyoxal (2 mM, final concentration) was then added to the incubation mixture and aliquots were subsequently removed at fixed time points over a period of 20 to 30 min for assay of ligase activity. Controls consisted of the enzyme alone or the enzyme plus acetyl CoA, pyridoxal 5′-phosphate, glycine, or threonine in the absence of any phenylglyoxal.

Coenzyme-dependent reactivation of native and phenylglyoxal-treated apoligase. Resolution of the holoenzyme was accomplished by dialyzing it against buffer containing 5 mM hydroxylamine as previously described (15). The apoligase (20 nmol) so prepared was first incubated for 50 min at 25°C in 50 mM potassium phosphate buffer (pH 7.0) either alone or with 10 mM phenylglyoxal. Pyridoxal 5′-phosphate (100 μM, final concentration) was then added to the incubation mixtures and aliquots were subsequently removed at varying time intervals for measuring both ligase activity and absorptivity at 428 nm.

Determination of the nature and number of essential amino acids modified in the ligase. Two methods were used to confirm the usual reaction specificity of phenylglyoxal with amino acid residues in proteins and to determine the number of residues modified; they were comparative amino acid analyses of the native and the phenylglyoxal-inactivated ligase and second, radioactive incorporation measurements with [^{14}C]phenylglyoxal.

For amino acid analyses, an aliquot of the enzyme (0.2 mg) was completely inactivated by reaction with 10 mM phenylglyoxal for 40 min at 25°C in 50 mM potassium phosphate buffer (pH 7.0). An aliquot (50 μl) of this reaction mixture and a comparable amount of the native ligase treated in the same manner without phenylglyoxal were hydrolyzed for 24 h at 110°C in 1 ml of 6 N HCl containing 20 μl of mercaptoacetic acid.

For quantitative radioactive measurements, 2-amino-3-ketobutyrate ligase (10.5 nmol) was incubated at 25°C with 8 mM phenyl[2-^{14}C]glyoxal (sp act = 2175 dpm/nmol) in 50 mM potassium phosphate buffer (pH 7.0); total volume = 0.8 ml. At specific times, aliquots (60 μl) of the incubation mixture were removed and immediately mixed with 1 ml of ice-cold 10% trichloroacetic acid, and the mixtures were then centrifuged for 15 min at 5000 rpm. The precipitates were subsequently washed several times with ice-cold 10% trichloroacetic acid and finally briefly with cold ethanol before they were dried under vacuum. The solid residues remaining were dissolved in a small volume of 0.1 N NaCl and the levels of radioactivity in the resultant solutions determined by liquid scintillation counting techniques, making use of a Packard Model 3300 spectrometer and Safety Solve scintillation mixture.

Amino acid analyses. Prior to analysis, enzyme samples were dialyzed exhaustively against glass-redistilled water and then lyophilized. Analyses were usually performed on a Beckman Model 120C automatic amino acid analyzer equipped with a microbore column; half-cystine residues were determined as *S*-sulfocysteine after treating the acid hydrolysate with dithiothreitol and sodium tetrathionate (22). Tryptophan was measured by hydrolyzing the protein with 4 M methanesulfonic acid (23). On occasion, the amino acids present in protein hydrolysates were also determined by HPLC as PTC-amino acid derivatives using the Waters Pico-Tag system (24).

Peptide isolation and sequencing procedures. 2-Amino-3-ketobutyrate ligase (6 mg) from *E. coli* was incubated at 25°C with 10 mM phenyl[2-^{14}C]glyoxal (sp act = 1 μCi/μmol) in 50 mM potassium phosphate buffer (pH 7.0) for 30 min after which time less than 3% of the original ligase activity could be detected. The pH of the reaction mixture was then decreased to 4.0 by dropwise addition of 2 N HCl and the resulting solution dialyzed exhaustively against 50 mM sodium formate buffer (pH 4.0). The inactive enzyme adduct was subsequently digested with *S. aureus* V8 endoproteinase (25 to 1 mol ratio, enzyme to protease) at 25°C for 24 h. The peptide solution so obtained was first passed through a Gelman Acro LC13 filter and aliquots of the filtrate were then injected directly onto a Waters Novapak C_{18} HPLC column (3.9 mm × 15 cm) at room temperature equilibrated with 0.1% (v/v) of TFA. Peptides were separated and eluted using TFA/acetonitrile solvent mixtures in

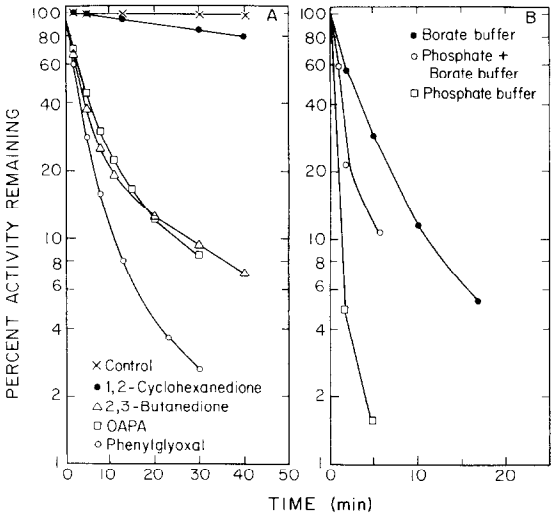

FIG. 1. (A) Effect of various dicarbonyl reagents on *E. coli* 2-amino-3-ketobutyrate ligase activity. The sample of enzyme used was first dialyzed for 8 h at 4°C against 50 mM borate buffer (pH 7.8). The ligase (0.2 mg) was then incubated at 25°C with each reagent (10 mM) indicated in 50 mM borate buffer (pH 7.8); final volume, 0.2 ml. Aliquots (10 μl) were removed at the times indicated, added to cuvettes in a final volume of 1.0 ml, and assayed as indicated in the text. OAPA, 4-(oxyacetyl)phenoxyacetic acid. (B) Effect of buffer on ligase inactivation by phenylglyoxal. Enzyme as in (A) was incubated with 10 mM phenylglyoxal in 50 mM borate buffer (pH 7.8), 50 mM phosphate plus 50 mM borate buffer (pH 7.8), or 50 mM phosphate buffer (pH 7.8). Aliquots were removed and assayed as indicated for (A). Controls in (A) and (B) contained no dicarbonyl reagent.

sequence as follows: 0–25 min linear gradient (0–25%, v/v) of acetonitrile in 0.1% TFA; 25–75 min linear gradient (25–55%) of acetonitrile in 0.1% TFA. Fractions (1 ml) were collected and the presence of radioactivity therein was determined by liquid scintillation counting.

Those fractions containing the radioactivity of interest were pooled and further purified by HPLC by passage through a Waters Novapak C_{18} analytical column using a linear gradient of 28–35% (v/v) acetonitrile in 0.1% TFA over a period of 60 min. The purified, radioactive peptide so obtained was concentrated to dryness and sequenced manually by the thin-film and the partitioning method described by Tarr (25).

RESULTS

Inactivation studies with various dicarbonyl reagents. As is shown in Fig. 1A, incubation of *E. coli* 2-amino-3-ketobutyrate ligase with 10 mM 1,2-cyclohexanedione, 2,3-butanedione, 4-(oxyacetyl)phenyoxyacetic acid, or phenylglyoxal in 50 mM borate buffer (pH 7.8) results in a time-dependent loss of enzymatic activity. Phenylglyoxal was found to be the most effective of the modifiers tested, causing >95% inactivation within 20 min. Figure 1B compares the rates of inactivation caused by 10 mM phenylglyoxal in borate as opposed to phosphate buffer; as can be seen, the rate is considerably faster and the extent greater in phosphate buffer. As a consequence, all subsequent inactivation studies were carried out with phenylglyoxal in phosphate buffer.

Loss of 2-amino-3-ketobutyrate ligase activity is also dependent on the concentration of dicarbonyl reagent used; Fig. 2 shows a semilogarithmic plot of the rates of inactivation caused by varying concentrations of phenylglyoxal in 50 mM potassium phosphate buffer (pH 7.0). The rates were found not to be linear suggesting that more than one amino acid residue of different reactivity is quite likely being modified during the process of inactivation. The reaction order (n) with respect to the concentration of phenylglyoxal was determined from the slope of a plot of log ($1000/t_{1/2}$) versus the log of reagent concentration (mM). In this type of plot, a straight line was obtained giving a value of 1.30 ± 0.10 (possible slopes within the error bars ranged from 0.74 to 1.88).

With *E. coli* 2-amino-3-ketobutyrate ligase being a homodimeric protein (15), the possibility was considered that the loss of ligase activity on treatment with phenylgloxal might be due to dissociation of the enzyme into its subunits. To test this, a control sample of the enzyme and a sample inactivated 85% by reaction with phenylglyoxal were first dialyzed extensively against phosphate buffer (50 mM, pH 7.0). These samples were then individually applied to and eluted from a column of Sephadex G-100 that had been calibrated previously with proteins of known molecular weight. The two ligase samples were found to have identical elution profiles with the expected relative levels of ligase activity and an elution volume equal to that for a protein with $M_r \cong 84{,}000$, demonstrating that phenylglyoxal inactivation is not a consequence of subunit dissociation.

It has been reported (26–28) that certain dicarbonyl reagents used for selective modification of arginine residues are photosensitive, causing a loss of enzymatic ac-

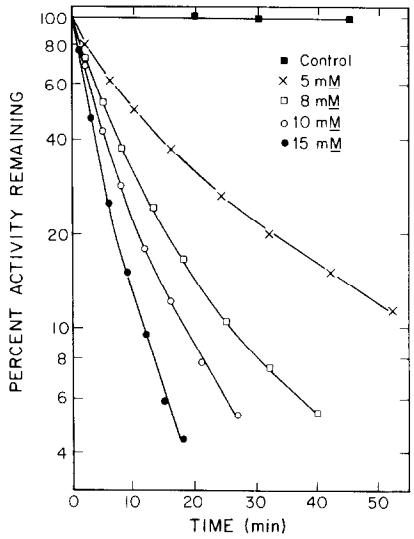

FIG. 2. Inactivation of *E. coli* 2-amino-3-ketobutyrate ligase by varying concentrations of phenylglyoxal. The enzyme (0.2 mg) was incubated in 50 mM potassium phosphate buffer (pH 7.0) at 25°C with the concentrations of phenylglyoxal listed. At the time points plotted, a 10-μl aliquot was removed, added to cuvettes in a final volume of 1.0 ml, and assayed as indicated in the text.

tivity because of light-induced alterations in protein structure rather than chemical reaction with an essential residue. This possibility was avoided in the results presented here by routinely carrying out the inactivation reaction in vessels protected from light.

Protective effect of substrates, substrate analogs, or coenzyme against inactivation. The effect that compounds added to the reaction mixture might have on the extent of inactivation caused by phenylglyoxal was tested. It was found that the presence of either acetyl CoA (2 mM) or pyridoxal 5'-phosphate (3 mM) gives >50% protection. When preincubated with the ligase, glycine also provides some protection against inactivation (~40 to 60%) but only when present at high concentrations (100 and 200 mM, respectively). Since the unstable 2-amino-3-ketobutyrate per se is not available for use, threonine was tested as an analog; whereas 10 and 50 mM L-threonine showed good protection (~50 and 75%, respectively) no significant effect was seen when the same concentrations of D-threonine were tested.

Kinetic values of partially inactivated ligase. The enzyme used in these studies was treated with phenylglyoxal in the usual manner except that a limited extent of inactivation was accomplished by adding a threefold excess of L-arginine (relative to the initial concentration of phenylglyoxal) to the reaction mixture after a fixed interval of time. Thereafter, the mixture was dialyzed against 50 mM phosphate buffer (pH 7.0) to remove excess reagents and dialyzable reaction products. Reaction rates were measured using a constant level of acetyl CoA and varying concentrations of glycine; K_m and V_{max} values were calculated from the experimental data by Lineweaver–Burk plots (29) using a computer-assisted linear least-squares fit. Under the experimental conditions used, native enzyme was determined to have a K_m = 12 mM for glycine and a V_{max} = 2.90 μmol of CoA formed min^{-1} mg^{-1}, whereas the corresponding values found for inactivated enzyme retaining 58% of the original level of activity were 13 mM and 1.68 μmol min^{-1} mg^{-1}, respectively.

Reactivation of native and phenylglyoxal-inactivated apoligase by pyridoxal 5'-phosphate. Previous studies showed that the enzymatic activity of the apoenzyme form of *E. coli* 2-amino-3-ketobutyrate ligase could be fully recovered by incubation with pyridoxal 5'-phosphate but not by pyridoxal or pyridoxamine 5'-phosphate (15). Since pyridoxal 5'-phosphate gave protection against inactivation of the native ligase by phenylglyoxal, we examined factors affecting reactivation of the apoligase. Under the experimental conditions reported here, good recovery of ligase activity with time in parallel with an increase in 428-nm absorptivity was seen when the native apoenzyme was incubated with pyridoxal 5'-phosphate (Fig. 3). In contrast, when the apoligase was first modified by reaction with 10 mM phenylglyoxal under the usual conditions and then incubated with 100 μM pyridoxal 5'-phosphate for

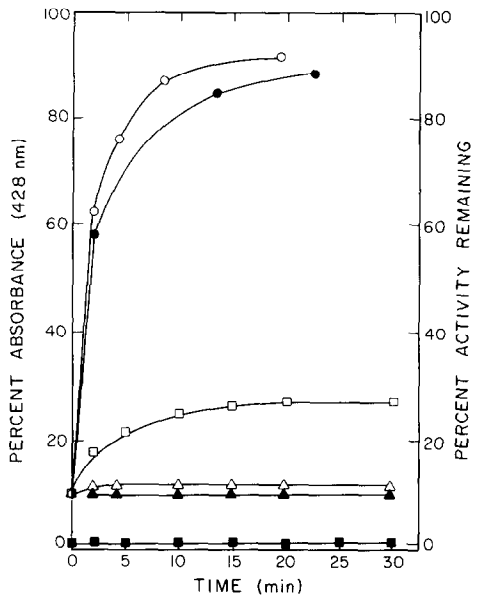

FIG. 3. Time-dependent regeneration of 2-amino-3-ketobutyrate ligase activity and 428-nm absorptivity of native and phenylglyoxal-modified apoenzyme on incubation with pyridoxal 5'-phosphate. Apoligase preparation, its treatment with phenylglyoxal, and subsequent incubation with pyridoxal 5'-phosphate are described under Materials and Methods. Aliquots (10 μl) of the reaction mixture were removed at the times indicated and assayed for both ligase activity (■) and absorptivity at 428 nm (□). Controls contained apoligase not reacted with phenylglyoxal but incubated with either pyridoxal 5'-phosphate (● and ○, ligase activity and 428-nm absorptivity, respectively) or pyridoxal (▲ and △, activity and absorptivity, respectively). The absorbance and specific activity of the holoenzyme were taken as 100%.

30 min, no recovery of enzymatic activity and only a slight increase in absorptivity at 428 nm was seen. Likewise, incubation of the native apoligase with pyridoxal caused no return of either catalytic activity or unique absorptivity.

In this context, it was also considered whether the observed loss of some or all of the ligase activity on treatment with phenylglyoxal might possibly be due to dissociation of the holoenzyme. To test this, the levels of activity exhibited by pure holoenzyme and the holoenzyme inactivated 70% by phenylglyoxal were determined after they were first dialyzed against phosphate buffer and then preincubated (30 min at 4°C) with varying concentrations (0 to 100 μM) of pyridoxal 5'-phosphate. The added coenzyme had no effect whatever on the level of activity of either enzyme sample, indicating that phenylglyoxal inactivation is not due to holoenzyme dissociation.

Nature and number of essential amino acids modified in the inactivated ligase. Previous studies (30) showed that phenylglyoxal interacts specifically with the guanidinium group of arginine in a stoichiometric relationship of 2 mol of reagent/mol of arginine. To confirm this specificity of reaction with *E. coli* 2-amino-3-ketobutyrate ligase and to determine the number of residues modified,

amino acid analyses were carried out on native as well as dione-inactivated ligase. The amino acid composition determined for the native enzyme, showing 26.3 arginine residues per subunit, was in excellent agreement for each residue with that reported earlier (15). In contrast, whereas the content of all of the other amino acid residues was the same for the phenylglyoxal-inactivated enzyme, only 24.8 residues of arginine per subunit were found in this sample. Hence, complete inactivation was associated with the loss of 1.5 arginine residues per ligase subunit.

The correlation between the loss of ligase activity and the incorporation of [^{14}C]phenylglyoxal as a function of time is shown in Fig. 4. If it is assumed that the stoichiometry of reaction is as noted earlier, the data again indicate that approximately two arginine residues/ligase subunit are modified in the inactivated enzyme.

Isolation and purification of peptides containing phenylglyoxal-modified residues. *S. aureus* V8 endoproteinase was used to obtain peptide digests of *E. coli* 2-amino-3-ketobutyrate ligase modified by [^{14}C]phenylglyoxal. A representative peptide map of a digest separated by HPLC is shown in Fig. 5A. Fractions eluted at 11 to 13 min contained 88% of the applied radioactivity with the remaining 12% found in fractions at 43 to 46 min. We were surprised that when the fractions containing the first radioactive peak were chromatographed a second time on a Novapak C_{18} column with a different acetonitrile gradient (i.e. 28–35% acetonitrile in 0.1% TFA over 0 to 60 min), the eluted radioactive peak showed no absorptivity at 214 nm (data not shown). We therefore took ligase that had been inactivated by reaction with [^{14}C]phenylglyoxal but was *not* digested with the endoproteinase and subjected it directly to HPLC on the Novapak column under the same experimental conditions. Again in this instance,

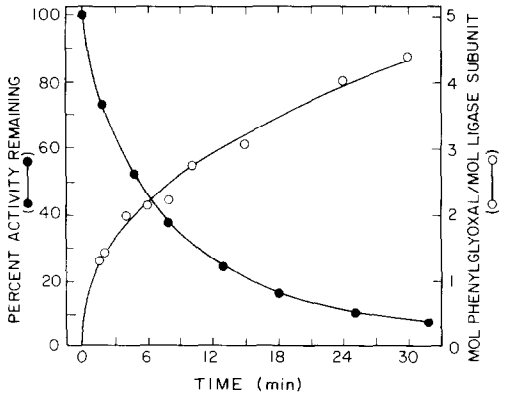

FIG. 4. Correlation of the extent of inactivation of *E. coli* 2-amino-3-ketobutyrate ligase activity by phenylglyoxal with the number of mol of [^{14}C]phenylglyoxal bound to the enzyme. The ligase was inactivated by incubating with phenyl[2-^{14}C]glyoxal. At the times indicated, an aliquot (10 μl) was removed for assaying ligase activity and another (60 μl) for precipitating, washing, and redissolving the protein before measuring the level of radioactivity present. Experimental details are given under Materials and Methods.

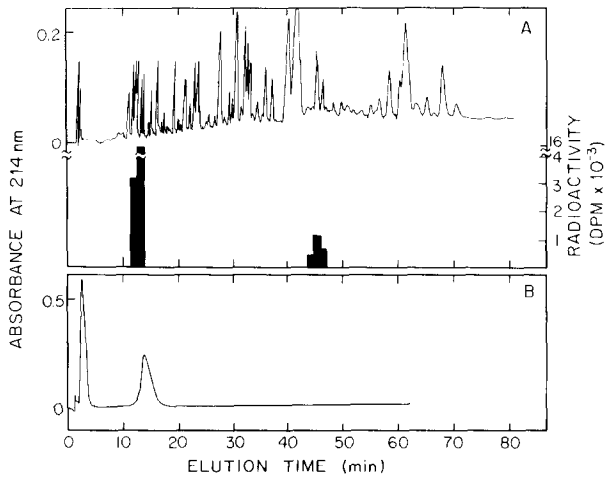

FIG. 5. Peptide maps of *E. coli* 2-amino-3-ketobutyrate ligase after digestion with *Staphylococcus aureus* V8 endoproteinase. (A) Ligase inactivated approximately 97% by reaction with phenyl[2-^{14}C]glyoxal. (B) Rechromatography of the second radioactive peptide eluting at 43 to 46 min in peptide map (A). The solid histograms indicate the amount of radioactivity found in the collected fractions. Experimental details are given under Materials and Methods and in the text.

the major radioactive peak (eluting at 12 to 13 min containing 80% of applied radioactivity) showed no 214-nm absorptivity (data not shown). This result suggests that the early-eluting, major radioactive peak seen in Fig. 5A is likely due to the presence of free phenylglyoxal which is leached from any bound form of the reagent under HPLC experimental conditions. The second radioactive peak in Fig. 5A (eluting at 43 to 46 min) was therefore pooled and rechromatographed on the Novapak C_{18} column using a linear gradient over 60 min of 28–35% acetonitrile in 0.1% aqueous TFA. As can be seen in Fig. 5B, a fairly broad uv-absorbing peak containing most of the applied radioactivity was eluted between approximately 13 and 15 min. These fractions were pooled and the sample was subjected to amino acid analysis as well as sequence determination. Although the purity of the eluted peak was suspect because of its shape, the very clean PTH–amino acid chromatograms we obtained throughout the manual Edman degradation procedure excluded the possibility of this peak being contaminated by other peptides. The broadness of the peak is most likely due to slow leaching during chromatography of free phenylglyoxal from the peptide–dione adduct.

Amino acid composition and sequence determination of the radioactive peptide. The radioactive peptide isolated as shown in Fig. 5B was hydrolyzed with 6 N HCl containing 1% phenol and its composition determined by HPLC as PTC-amino acid derivatives using the Waters Pico-Tag system. The results are shown in Table I; when calculated to the nearest whole integer, a total of 30 amino acid residues was found including two arginines. These two residues were detected as PTC-arginine rather than

TABLE I

Amino Acid Composition of the [^{14}C]Phenylglyoxal-Arginine Peptide of *E. coli* 2-Amino-3-ketobutyrate Ligase

Amino acid[a]	Residue/peptide[b]	Residues in enzyme sequence 349 to 378[c]
Glu/Gln	3.90 (4)	3
Ser	0.90 (1)	1
Gly	2.73 (3)	3
His	0.82 (1)	1
Arg	2.00 (2)	2
Thr	2.40 (2)	3
Ala	3.50 (3)	3
Pro	2.33 (2)	3
Tyr	1.70 (2)	2
Val	3.30 (3)	3
Met	0.74 (1)	1
Ile	2.60 (3)	2
Phe	1.76 (2)	2
Lys	0.70 (1)	1
Total	30	30

[a] Determined by HPLC as PTC-amino acid derivatives using the Waters Pico-Tag system (24).

[b] The first values listed are from amino acid analyses; data are the average of duplicate samples hydrolyzed and analyzed separately. The nearest whole integer numbers are given in parentheses.

[c] From Aronson *et al.* (16).

PTC-phenylglyoxal-arginine peaks since the Pico-Tag procedure is carried out under highly alkaline conditions. Manually, we were able to determine the sequence of the first 17 amino acid residues of this peptide by the thin-film and partitioning method of Tarr (25); it had the primary structure Gly–Ile–Tyr–Val–Thr–Gly–Phe–Phe–Tyr–Pro–Val–Val–Pro–Lys–Gly–Gln–Ala. From the known amino acid sequence for *E. coli* 2-amino-3-ketobutyrate ligase, as deduced from DNA sequencing (16), and the known specificity of the V8 endoproteinase for cleaving peptide bonds involving the carboxyl group of glutamic acid, it can be determined that this 17-amino-acid sequence corresponds to residues 349 to 365 in the primary structure of the ligase. Consequently, the complete 30-amino-acid peptide as isolated represents the stretch from residues 349 to 378 in the intact enzyme. The amino acid composition as found for the isolated peptide is consistent with this conclusion.

DISCUSSION

The primary importance of the threonine dehydrogenase-initiated pathway in threonine metabolism, whereby L-threonine is converted by the two-step, sequential action of threonine dehydrogenase and 2-amino-3-ketobutyrate ligase to 2-amino-3-ketobutyrate and then acetyl CoA plus glycine, has come to the forefront in recent years. The genes for these two enzymes make up the *tdh* operon. The *tdh* operon lies within a 3.6-kb *Eco*RI fragment located at coordinate 81.2 of the *E. coli* genetic map (11); the *kbl* gene is immediately upstream of the *tdh* gene within the same *Eco*RI fragment and both genes are transcribed from a common promoter (6, 11, 31). The operons of a multigene family which includes *tdh,* collectively known as the "leucine regulon," are up- or down-regulated by L-leucine via a leucine-responsive regulatory protein encoded by the *lrp* gene. Lrp transcriptionally regulates the operons containing the *ilvlH, serA, sdaA, oppABCDF,* and *tdh* genes; L-leucine induces the activity of the *tdh* operon about sevenfold.

One phase of our research is to elucidate the properties and structure/function interrelationships of the two gene products of the *E. coli tdh* operon. Past efforts have led to the isolation of both proteins in homogeneous form, and some of the general properties of each have been described (3, 15). So far, we have succeeded in identifying 2 catalytically essential amino acid residues in threonine dehydrogenase, namely, an arginine (19) and a cysteine (18) residue. Other than for identifying lysine 244 as the residue that forms the azomethine (Schiff base) linkage with pyridoxal 5′-phosphate in 2-amino-3-ketobutyrate ligase (20), no other details interrelating structure with catalytic capability are known for this enzyme from *E. coli.* The results presented here establish the presence of an active-site arginine residue (or two) near the C-terminus of this ligase.

While α-dicarbonyl compounds occasionally react with residues in proteins other than arginine (30, 32–35), they are among the more specific reagents currently available for modification of a specific amino acid. *E. coli* 2-amino-3-ketobutyrate ligase is rapidly inactivated by 2,3-butanedione and phenylglyoxal; reaction with the less widely utilized reagent, 4-(oxyacetyl)phenoxyacetic acid, which was uniquely useful for us in other studies with 2-keto-4-hydroxyglutarate aldolase (36), also effectively inactivates this ligase. Appropriate experimental conditions and/or controls rule out the possibility that the observed loss of ligase activity is due to photo-induced nonspecific alteration of the enzyme, ligand-induced dissociation of the enzyme into its subunits, or ligand-induced dissociation of the coenzyme. Kinetic analyses with the native and partially inactivated ligase show that the K_m value for glycine is essentially unchanged whereas V_{max} for the modified enzyme is correspondingly lower; such effects are consistent with an inactivation process in which active and completely inactivated enzyme molecules are present (37). These kinetic results together with the good level of protection against inactivation observed in the presence of substrates and the coenzyme strongly suggest that the arginine residue(s) identified here is within the active center of the enzyme.

E. coli 2-amino-3-ketobutyrate ligase contains a total of 26 arginine residues/subunit (15, 16); of this total, about half are present in the amino acid sequence that makes up the last $\frac{1}{3}$ of the protein molecule at the C-terminal

end. Comparative amino acid analyses of the native and the phenylglyoxal-inactivated enzyme and data correlating the loss of ligase activity with the number of moles of [^{14}C]phenylglyoxal incorporated indicate a stoichiometry of approximately 2 arginine residues modified/subunit in the completely inactivated enzyme. The radioactive peptide isolated by HPLC after protease digestion contained 2 arginines, namely, residues 366 and 368 (the C-terminus is alanine-398). Whether both of these arginine residues are indeed labeled by reaction with [^{14}C]phenylglyoxal could not be determined in this study and whether both are participants in the active site of this enzyme awaits the development of an alternate experimental technique. The long length (30 amino acid residues) of this peptide precluded manual sequencing of it in its entirety, and even if this had been possible the results would probably not be definitive since manual Edman amino acid sequencing techniques involve highly basic conditions which regenerate free phenylglyoxal from the phenylglyoxal–arginine adduct. Selective changing of these 2 arginine residues separately and together by site-directed mutagenesis would probably be most informative.

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