Catabolite Inactivation of Biodegradative Threonine Dehydratase of *Escherichia coli*†

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ABSTRACT: Incubation of *Escherichia coli* cells with glucose, pyruvate, and certain other metabolites led to rapid inactivation of inducible biodegradative threonine dehydratase. Analysis with several mutant strains showed that pyruvate, and not a metabolite derived from pyruvate, was capable of inactivating enzyme, and that glucose acted indirectly after being converted to pyruvate. Some other α-keto acids such as oxaloacetate and α-ketobutyrate (but not α-ketoglutarate) were also effective. Inactivation of threonine dehydratase by pyruvate was also observed with purified enzyme preparations. The rates of enzyme inactivation increased with increased concentrations of pyruvate and decreased with increased levels of AMP. Increasing protein concentrations lowered the rates of enzyme inactivation. Dithiothreitol had a large effect on the maximum extent of inactivation of the enzyme by pyruvate; high concentrations of AMP and DTT almost completely counteracted the effect of pyruvate. Gel filtration data showed that pyruvate influenced the oligomeric state of the enzyme by altering the association–dissociation equilibrium in favor of dissociation; the Stokes' radius of the pyruvate-inactivated enzyme was 32 Å as compared to 42 Å for the untreated enzyme. Reassociation of the dissociated form of the enzyme was achieved by removal of excess free pyruvate by dialysis against buffer supplemented with AMP and DTT. Incubation of threonine dehydratase with [^{14}C]pyruvate revealed apparent covalent attachment of pyruvate to the enzyme. Strong protein denaturants such as guanidine, urea, and sodium dodecyl sulfate failed to release bound radioactive pyruvate; the molar ratio of firmly bound pyruvate was approximately 1 mol/150,000 g of protein. Pretreatment of the enzyme with *p*-chloromercuribenzoate and 5,5′-dithiobis(2-nitrobenzoate) (Nbs_2) did not reduce the binding of [^{14}C]pyruvate suggesting no active site SH was involved in the pyruvate–enzyme linkage. Titration of active and pyruvate-inactivated enzyme with Nbs_2 indicated that the loss in enzyme activity was not due to oxidation of essential sulfhydryl groups on the enzyme. Based on these data we propose that the mechanism of enzyme inactivation by pyruvate involves covalent attachment of pyruvate to the active oligomeric form of the enzyme followed by dissociation of the oligomer to yield inactive enzyme.

Multiple modes of regulation of a given enzyme are often designed to meet the exacting physiological needs of a particular microorganism growing in a variety of external environments. One example of such an enzyme is the biodegradative threonine dehydratase (EC 4.2.1.16) of *Escherichia coli* which is subject to several different types of regulatory control. First, it is an inducible enzyme and is synthesized when the organism is grown anaerobically in a medium containing tryptone and yeast extract; the enzyme cannot be detected when the cultures are maintained aerobically (Wood and Gunsalus, 1949; Umbarger and Brown, 1957). Second, the induced synthesis of this enzyme is prevented if glucose is added to the culture medium (Wood and Gunsalus, 1949; Umbarger and Brown, 1957); analyses of the "glucose effect" revealed that the synthesis of this enzyme is under catabolite repression control (Shizuta and Hayaishi, 1970). Third, the activity of this enzyme is modulated by the allosteric effector AMP, which causes oligomerization of the protomeric species and decreases the K_m for L-threonine (Hirata et al., 1965; Phillips and Wood, 1965; Whanger et al., 1968; Gerlt et al., 1973). Finally, inhibition of enzyme activity by the reaction product α-ketobutyrate in the absence of AMP (Shizuta et al., 1973) appears to be an additional means of regulation of the dehydration reaction.

Several years ago, Chapman (1967) observed a rapid loss of enzyme activity when a large number of metabolites including glucose were added individually to growing cultures of *E. coli* B. In cell-free extracts glucose did not inactivate the enzyme; however, pyruvate inactivated the enzyme to a variable extent ranging from 3 to 41%; attempts to reactivate the enzyme were unsuccessful (Chapman, 1967). In this article we present evidence to confirm Chapman's initial findings and extend the observations to demonstrate that threonine dehydratase activity of *E. coli* is rapidly inactivated by several intermediary metabolites, especially pyruvate, in both intact cells and in purified enzyme preparations. Glucose, by itself, had no effect on enzyme activity. The mechanism of enzyme inactivation appears to include apparent covalent attachment of pyruvate and dissociation of the enzyme. This new mode of regulation of enzyme activity by cellular metabolites, designated here as *catabolite inactivation,* supplements the other regulatory mechanisms elucidated thus far for the control of biodegradative threonine dehydratase in *E. coli.*

Experimental Section

Reagents. All ingredients of bacterial growth media were obtained from Difco Laboratories. [1-^{14}C]Pyruvate and [U-^{14}C]pyruvate (specific activity 8–11 Ci/mol) were pur-

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chased from Amersham/Searle and New England Nuclear. All other chemicals, of highest purity commercially available, were bought from Sigma Chemical Co., J. T. Baker Chemicals, Mann Research Chemicals, or Calbiochem.

Bacterial Strains. Escherichia coli K12 (ATCC 14948) was obtained from Dr. Dale L. Oxender. The following K12 mutant strains were obtained as indicated: A-10 (*AceF*), lacking lipoic reductase transacetylase (Henning et al., 1966), was from Dr. L. P. Hager; DF 44 *pgi zwf*-27, containing inactive glucose-6-phosphate dehydrogenase and phosphoglucose isomerase (Fradkin and Fraenkel, 1971), was from Dr. D. G. Fraenkel; AB 257 pC-1, lacking component I of 3′,5′-cyclic AMP phosphodiesterase (Monard et al., 1970), was from Dr. H. V. Rickenberg. Starting from A-10 as the parental strain, a double mutant (DF-9), lacking both lipoic reductase transacetylase and phosphoenol pyruvate synthetase (*AceF pps*), was isolated following the method described by Brice and Kornberg (1967).

Cell Growth. Stock cultures were maintained on nutrient agar stabs. Large scale cultures were grown anaerobically at 37° in tryptone–yeast extract medium as described by Umbarger and Brown (1957) supplemented with 50 mg of pyridoxine/l. of medium.

Enzyme Assay. Threonine dehydratase activity in intact cells was measured by the colorimetric assay method for α-keto acids (Friedemann and Haugen, 1943) as modified by Datta (1966). Cells were harvested by centrifugation and washed twice in 100 m*M* potassium phosphate buffer (pH 8.0) containing 3 m*M* AMP. The cell pellet was resuspended in one-tenth the original volume of the buffer and toluene was added to a final concentration of 5%. After mixing vigorously for 30 sec, an aliquot of toluene-treated cells was incubated at 37° for 5 min in an assay mixture containing 10 μmol of L-threonine, 3 μmol of AMP, 1 μmol of L-isoleucine, and 100 μmol of potassium phosphate (pH 8.0) in a final volume of 1 ml. One unit of enzyme is defined as the amount producing an increase in absorbance at 540 nm of 1.00/hr over that observed for a blank reaction mixture containing all components except the cell suspension which was added back after stopping the reaction with trichloroacetic acid. The amino acid L-isoleucine was included to ensure inhibition of activity of the biosynthetic threonine dehydratase, if present. In some cases, the enzyme activities of cell extracts and purified preparations were also measured colorimetrically as described above; appropriate dilutions of enzyme replaced toluene-treated cells. All values reported with this procedure were within the linear range of the assay.

An alternative method of assaying enzyme activity was based on the findings of Feldberg and Datta (1970) that threonine dehydratase can reduce ferricyanide during the dehydration reaction. The reaction mixture, in a final volume of 1 ml, contained the following: 100 μmol of potassium phosphate buffer (pH 8.0), 10 μmol of L-threonine, 3 μmol of AMP, and 1.25 μmol of potassium ferricyanide. Reduction of ferricyanide at 37° was followed by decrease in absorbance at 420 nm immediately after enzyme addition. The rate of ferricyanide reduction was linear for 1–2 min and then decreased rapidly to a near-zero rate after the reduction of 0.45 μmol of ferricyanide (Feldberg and Datta, 1970). The initial rates as calculated from the decrease in absorbance at 420 nm in the first minute were proportional to enzyme concentrations. One unit of enzyme is defined as the amount required to produce a change in absorbance at 420 nm of 1.00/min. The presence of pyruvate or low concentrations of DTT[1] occasionally carried with the enzyme sample did not interfere with the activity assay; with DTT, the reduction of ferricyanide was instantaneous and the enzyme-catalyzed decrease in absorbance was determined after this initial reduction of ferricyanide.

Stokes' Radius. Stokes' radius was determined on calibrated Sephadex G-200 columns as described by Siegel and Monty (1966). The location of the enzyme markers were established by assaying for their activities (Feldberg and Datta, 1971).

Binding of [^{14}C]Pyruvate. Two independent methods were used to determine binding of [^{14}C]pyruvate to the enzyme. In the first method, enzyme samples that had been incubated with radioactive pyruvate under appropriate conditions were heated at 100° for 10 min. The heated samples were diluted in 10 m*M* potassium phosphate buffer (pH 8.0) and dialyzed exhaustively against a large excess of the same buffer until the solution outside the dialysis bag contained no significant radioactivity over that of the background. The entire content of the dialysis bag was used for radioactivity measurements.

In an alternative procedure, the enzyme sample treated with [^{14}C]pyruvate was precipitated with 50% Cl_3CCOOH at 4° in the presence of 0.5 mg of bovine serum albumin. The suspension was collected on a Whatman GF/C filter paper, washed thoroughly by suction with 5% Cl_3CCOOH, dried, and counted. In all cases the counts retained by the filter paper in the blank reaction mixture without the enzyme were subtracted.

For counting aqueous solutions, Bray's solution was used; for dry filter paper, Omnifluor dissolved in toluene was used as the scintillation mixture.

Sulfhydryl Titration. Titration of reactive sulfhydryl groups with Nbs_2 was performed according to the general method of Ellman (1959). The buffer used was 100 m*M* potassium phosphate (pH 8.0) containing 3 m*M* AMP.

Protein Determination. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the reference protein.

Enzyme Purification. Threonine dehydratase of *E. coli* K12 was purified according to the method described by Shizuta et al. (1969). The last DEAE-Sephadex step was omitted since no significant purification was achieved over the previous fractionation step using hydroxylapatite.

Polyacrylamide gel electrophoresis of 150 μg of protein in the presence of sodium dodecyl sulfate and staining with Coomassie Blue according to the method of Weber and Osborn (1969) revealed a major protein band and a minor band amounting to less than 10% of the total protein. Between 3 and 4 additional bands were barely visible when viewed under fluorescent light. Based on these observations, the enzyme appeared to be about 90% pure.

Results

Enzyme Inactivation in Intact Cells. In *E. coli* K12 threonine dehydratase activity per milliliter of culture reached a maximum when the cells grown anaerobically at 37° in tryptone–yeast extract medium reached stationary phase of growth. Thereafter, the enzyme level fell slowly reaching 60% of the induced level after about 24 hr although the cell number remained constant during this period. The results given in Figure 1A demonstrate that addition of glucose or

[1] Abbreviations used are: DTT, dithiothreitol; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate).

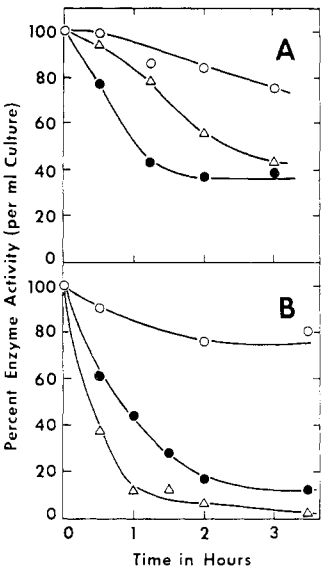

FIGURE 1: Inactivation of threonine dehydratase in intact cells by glucose and pyruvate. (A) Aliquots of cells obtained from stationary phase of growth in tryptone–yeast extract medium were exposed to 11 m*M* glucose or 50 m*M* pyruvate and incubated further at 37° without shaking. Samples were withdrawn at various time periods and threonine dehydratase activities of washed cells were measured after toluene treatment as described in the Experimental Section. (B) The protocol was the same as in (A) except that a resting cell suspension was used (see text). (○) Untreated control; (△) 11 m*M* glucose; (●) 50 m*M* pyruvate.

pyruvate to fully induced cells obtained from stationary phase of growth resulted in a significant loss of enzyme activity. The enzyme activity was also abolished when the culture was vigorously aerated (not shown). A small decrease in enzyme activity could be seen in the untreated control. The kinetics of disappearance of enzyme activity in the presence of glucose or pyruvate using resting cell suspension under noninducing conditions is shown in Figure 1B. In these experiments cells from stationary phase of growth were harvested, washed, and resuspended in 100 m*M* potassium phosphate buffer (pH 8.0) and starved at 37° for 2 hr prior to addition of glucose or pyruvate. Greater than 80% of the enzyme activity disappeared in about 2 hr in the presence of glucose or pyruvate, whereas, only 20% reduction in activity was seen in the untreated cells. A comparison of the data in Figure 1A and B indicates that cells obtained from stationary growth phase showed slower rate of enzyme inactivation and the maximum extent of enzyme inactivation was smaller. The difference presumably reflects the intracellular concentrations of metabolites that counteract the effects of glucose and pyruvate.

The rapid decrease in enzyme activity described above could not be accounted for by enzyme dilution due to increased cellular growth and decreased rate of enzyme synthesis by catabolite repression caused by glucose or pyruvate. No further cell division and enzyme synthesis occurred in untreated cells. With stationary phase cells, a maximum increase in cell density in the presence of glucose or pyruvate was about 50%, whereas, no increase in cell number was observed with starved cells. In the latter case, greater than 80% of the enzyme activity was lost in 2 hr (Figure 1B). Addition of chloramphenicol (100 μg/ml) and actinomycin D (10 μg/ml) to *both* control and glucose- or pyruvate-treated cultures, that caused inhibition of macromolecular synthesis, did not influence the loss of enzyme activity by these metabolites.

Several other observations summarized below provided evidence that loss of enzyme activity upon glucose or pyruvate addition was due to specific inactivation of threonine dehydratase and not due to decreased synthesis or loss of activity of inducible enzymes in general. For example, addition of cAMP (1 m*M*), which reversed catabolite repression by glucose (cf. Shizuta and Hayaishi, 1970), did not alter the glucose-mediated enzyme inactivation kinetics; inactivation of enzyme by glucose or pyruvate was also observed with *E. coli* AB 257 pC-1 that lacks component I of the cAMP phosphodiesterase and is thus resistant to catabolite repression (Monard et al., 1970). Furthermore, using identical experimental conditions, glucose-dependent loss of enzyme activity was not observed with three other catabolite repressible enzymes, β-galactosidase, D-serine deaminase, and tryptophanase of *E. coli* K12.

Inactivation of threonine dehydratase in intact cells by glucose or pyruvate was not prevented by simultaneous addition of pyridoxal phosphate (1 mg/ml), 20 m*M* AMP, 5 m*M* dithiothreitol, or phenylmethanesulfonyl fluoride (300 μg/ml). Various attempts to reactivate enzyme that has been inactivated in vivo were also unsuccessful. For example, enzyme activity was not regained when cells treated with pyruvate were washed and suspended in fresh medium containing tryptone–yeast extract in the presence of chloramphenicol (to prevent a new round of enzyme induction) suggesting apparent irreversible nature of enzyme inactivation in vivo.

Effects of Additional Metabolites in Intact Cells. A large number of metabolites were tested for their ability to cause enzyme inactivation in resting cell suspensions. The data presented in Table I, experiment I revealed that, in addition to glucose and pyruvate, several other metabolites such as α-ketobutyrate, oxaloacetate, glyoxalate, phosphoenol pyruvate (class I compounds), as well as the L isomers of the amino acids threonine, serine, cysteine, and glycine (class II compounds) caused significant enzyme inactivation. The compounds listed in class III caused very little loss in enzyme activity. To rule out the possibility that the lack of enzyme inactivation by the class III compounds was not due to lack of transport inside the cells, starved cells were pretreated with Tris-EDTA buffer according to the method of Shizuta and Hayaishi (1970) to overcome any permeability barrier; identical results were obtained with or without the Tris-EDTA pretreatment.

Since both glucose and pyruvate can be further metabolized by a variety of cellular enzymes in intact cells, several mutant strains of *E. coli* K12, selected for their inability to metabolize glucose and pyruvate (see Experimental Section), were employed to identify the precise nature of the inactivating metabolite. Using resting cell suspensions of the appropriate mutant strains we found that glucose and pyruvate caused enzyme inactivation in strains A-10 (*Ace F*, lacking functional pyruvate dehydrogenase) and DF-9 (*Ace F, pps*), a double mutant lacking both pyruvate dehydrogenase and phosphoenol pyruvate synthase. With the strain DF 44 (*pgi, zwf* 27), containing inactive phosphoglucose isomerase and glucose-6-phosphate dehydrogenase, pyruvate showed normal inactivation kinetics, whereas, glucose was unable to cause enzyme inactivation. These data strongly suggest that pyruvate, and not a metabolite derived from pyruvate, was capable of causing enzyme inactivation, and that glucose acted indirectly after being converted to pyruvate (also, see below).

Inactivation of Purified Enzyme. When purified threo-

Table I: Inactivation of Threonine Dehydratase in Intact Cells and in Purified Preparations by Various Metabolites.[a]

	Metabolite[b]	Percent Enzyme Inactivation: Intact Cells (Expt I)	Purified Enzyme (Expt II)
Class I	Pyruvate	90	80
	Oxaloacetate	90	39
	α-Ketobutyrate	64	65
	Phosphoenol pyruvate	28	47
	Glyoxalate	20	73
Class IIA	Glucose	80	<10
	L-Cysteine	80	<10
	Glycine	59	<10
	L-Tryptophan	50	
Class IIB	L-Serine	99	85
	L-Threonine	70	75
	D-Serine		<10
	D-Threonine		<10
Class III	Acetate citrate, fumarate Malate, succinate, glycerol, L-glutamate, L-aspartate, L-phenylalanine, L-tyrosine, L-alanine, L-lysine, L-methionine, L-homoserine, L-valine, L-leucine, L-isoleucine, α-ketoglutarate, D-lactate	<10	<10

[a] For experiment I, resting cell suspensions (see legend of Figure 1) were incubated for 4 hr at 37° in 100 m*M* potassium phosphate buffer, pH 8.0, with or without metabolites. For the compounds listed under class III the cells were pretreated with Tris-EDTA buffer for 2 min at 37° (Shizuta and Hayaishi, 1970). Threonine dehydratase activity was assayed using toluene-treated cells (see Experimental Section). For experiment II, samples of purified enzyme (170 μg/ml) in 100 m*M* potassium phosphate buffer (pH 8.0) containing 3 m*M* AMP were incubated for 20 min at 37° with or without metabolites. An aliquot of incubation mixture was assayed for enzyme activity by the ferricyanide method. During assay the final concentration of the metabolite was reduced to 0.25 m*M*; at this concentration none of the compounds reduced enzyme activity when added directly to the assay mixture. In both experiments the percent enzyme inactivation was calculated relative to a control sample without any added metabolite. [b] In expt I, 50 m*M* each, except phosphoenol pyruvate which was 20 m*M*. In expt II, 25 m*M* each, except L-serine which was 5 m*M*.

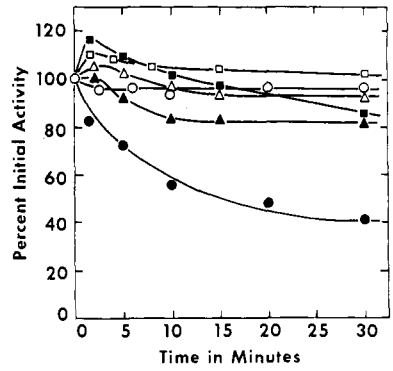

FIGURE 2: Inactivation of purified threonine dehydratase by pyruvate and the effects of AMP and DTT on enzyme inactivation. Aliquots of purified enzyme at a final concentration of 100 μg/ml in 100 m*M* potassium phosphate buffer (pH 8.0) were incubated at 25° in the presence or absence of pyruvate with AMP and DTT as indicated. At the times indicated 5 μl of the incubation mixture was withdrawn and enzyme activity was determined by the ferricyanide method; during assay pyruvate concentration was 0.05 m*M*. Open symbols, no pyruvate; closed symbols, 10 m*M* pyruvate; circles, 3 m*M* AMP; triangles, 3 m*M* AMP plus 5 m*M* DTT; squares, 15 m*M* AMP.

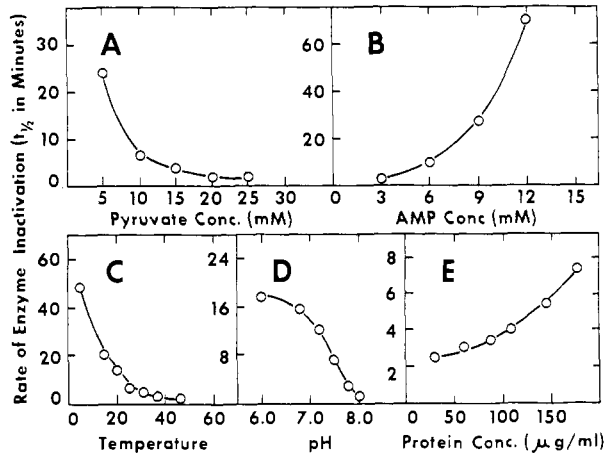

FIGURE 3: Influence of various parameters on the rate of enzyme inactivation by pyruvate. Aliquots of purified threonine dehydratase were incubated with or without pyruvate under a variety of experimental conditions as indicated and the kinetics of enzyme inactivation were followed using the standard assay conditions (see Experimental Section and legend to Figure 2). The rates of enzyme inactivation are expressed as time required in minutes to obtain 50% loss of enzyme activity ($t_{1/2}$) relative to a control sample that was treated in identical manner except without pyruvate. Unless otherwise specified, all incubation mixtures contained 100 m*M* potassium phosphate buffer (pH 8.0), 3 m*M* AMP, 20 m*M* pyruvate, and 170 μg/ml of purified enzyme; the temperature of incubation was 37°.

nine dehydratase was incubated at 25° with 10 m*M* pyruvate in 100 m*M* potassium phosphate buffer (pH 8.0) containing 3 m*M* AMP for various time periods and aliquots were withdrawn for assaying enzyme activity, almost 60% of the enzyme activity was lost in 30 min (Figure 2). During assay, pyruvate concentration was reduced by a factor of 100–200; control experiments revealed that up to 0.5 m*M* pyruvate had no effect on threonine dehydratase activity when added directly to the assay mixture.

The results given in Figure 2 also demonstrate that high concentration of AMP (15 m*M*) or addition of 5 m*M* DTT to the enzyme solution containing 3 m*M* AMP during treatment with pyruvate prevented enzyme inactivation to a significant extent.

Several factors were found to be crucial for the pyruvate-dependent inactivation of the purified threonine dehydratase. The results are summarized in Figure 3. It is clear that increasing concentrations of pyruvate from 5 to 25 m*M* at a constant level of AMP (3 m*M*) increased the rate of enzyme inactivation by eightfold (Figure 3A), whereas, the rate of inactivation decreased 25-fold when the AMP concentration was raised from 3 to 12 m*M* at 20 m*M* pyruvate (Figure 3B). The inactivation rates increased as the temperature and pH of the incubation mixtures were raised (Figure 3C and D). Increasing protein concentrations lowered the rate of enzyme inactivation (Figure 3E).[2] It should be emphasized that although the rates of enzyme inactivation changed drastically in higher AMP concentrations, the

[2] Based on these observations, experimental conditions were devised to maximally inactivate the enzyme especially in high protein concentrations and in the absence of DTT. Dialysis of threonine dehydratase for 24–48 hr at 4° against 25–50 m*M* pyruvate in 100 m*M* potassium phosphate buffer (pH 8.0) containing 3 m*M* AMP led to greater than 80% enzyme inactivation relative to a control enzyme dialyzed without pyruvate.

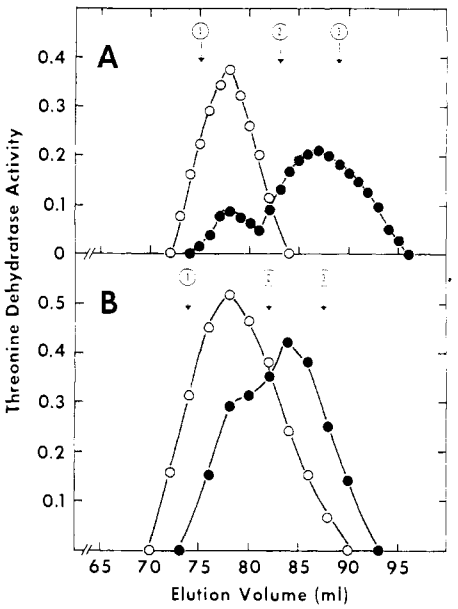

FIGURE 4: Sephadex G-200 elution profiles of purified threonine dehydratase following treatment with or without pyruvate. (A) Closed circles, 680 μg of enzyme inactivated by dialysis against 50 m*M* pyruvate in 100 m*M* potassium phosphate buffer (pH 8.0) containing 3 m*M* AMP for 18 hr at 4° was mixed with the reference proteins and passed through a Sephadex G-200 column (85 × 1.4 cm) equilibrated at 4° with 100 m*M* potassium phosphate buffer (pH 8.0) containing 3 m*M* AMP and 50 m*M* pyruvate. Open circles, a separate aliquot of enzyme treated without pyruvate but otherwise under identical conditions was passaged through a separate calibrated Sephadex G-200 column in 100 m*M* potassium phosphate buffer (pH 8.0) containing 3 m*M* AMP. (B) 1.36 mg of purified enzyme was inactivated by dialysis for 24 hr at 4° against 50 m*M* pyruvate in 100 m*M* potassium phosphate buffer (pH 8.0) containing 3 m*M* AMP. One-half aliquot of the pyruvate-treated enzyme was dialyzed for 24 hr at 4° against 100 m*M* potassium phosphate buffer (pH 8.0) containing 3 m*M* AMP and passaged through a calibrated Sephadex G-200 column (85 × 1.4 cm) equilibrated in the same buffer (B, closed circles). The remaining portion was dialyzed for 24 hr at 4° against 100 m*M* potassium phosphate buffer (pH 8.0) containing 15 m*M* AMP and 1 m*M* DTT and passaged through a separate calibrated Sephadex G-200 column equilibrated in the buffer containing 15 m*M* AMP and 1 m*M* DTT (B, open circles). For all gel-filtration experiments the flow rates were approximately 4 ml/hr and 1-ml fractions were collected. Each column was eluted separately and the results are juxtaposed to facilitate comparison. The positions of the reference enzymes were as follows: (1) yeast alcohol dehydrogenase, molecular weight 142,000, Stokes' radius 45 Å, (2) alkaline phosphatase, molecular weight 80,000, Stokes' radius 37 Å, (3) horseradish peroxidase, molecular weight 40,000, Stokes' radius 30 Å. The profile of the pyruvate-inactivated enzyme was determined by treating the column fractions with 1 m*M* DTT for 15 min at 37° to reactivate the enzyme.

maximum loss of enzyme activity at 15 m*M* AMP (as calculated from the equilibrium value reached during inactivation kinetics) was 65–70% as compared to 80% with 3 m*M* AMP. Dithiothreitol, on the other hand, had a large influence on the maximum extent of enzyme inactivation by pyruvate. At 1 m*M* DTT (in buffer containing 3 m*M* AMP) 55% of enzyme activity was lost, whereas, with 5 m*M* DTT enzyme activity was reduced only 20%.

Effects of Various Metabolites on Purified Enzyme. Several common intermediary metabolites were examined to determine the specificity of the enzyme inactivation process. As summarized in Table I, experiment II, most of the compounds exhibiting significant effect were α-keto acids (α-ketoglutarate being the only exception). Although L-cysteine and glycine were able to inactivate threonine dehydratase in intact cells, these amino acids as well as glucose were not effective with purified enzyme, indicating that these compounds acted indirectly after further metabolism in the cell.

The data given in Table I also reveal that L-threonine and L-serine, the two substrates of threonine dehydratase, caused enzyme inactivation. The effects of L-serine may be explained by the well-known "serine suicide" reaction (McLemore and Metzler, 1968; Phillips and Wood, 1965). It is conceivable, however, that both serine and threonine acted indirectly through their respective dehydration products, pyruvate and α-ketobutyrate. D-Serine and D-threonine were completely ineffective with purified enzyme preparations.

Dissociation of Threonine Dehydratase by Pyruvate. The enzyme from *E. coli* is known to exist in various oligomeric forms depending on the protein concentration as well as on the presence of AMP and DTT in the buffer solution (Hirata et al., 1965; Whanger et al., 1968; Gerlt et al., 1973). The effects of higher levels of AMP, DTT, and especially high enzyme concentration in counteracting pyruvate-mediated loss in enzyme activity led us to suspect an alteration in the oligomeric state of the enzyme by pyruvate. The results of gel filtration on Sephadex G-200 of native and pyruvate-inactivated enzymes (Figure 4A) revealed that the untreated enzyme eluted as one peak having Stokes' radius of 41 Å corresponding to an approximate molecular weight of 116,000. The pyruvate-treated enzyme that had lost 80% of its activity eluted as one major peak with Stokes' radius of 32 Å corresponding to an apparent molecular weight of about 65,000, and a minor peak at a position coinciding with the native enzyme. The species with a Stokes' radius of 32 Å was completely inactive; the elution profile could only be detected after incubating the column fractions with 1 m*M* DTT for 15 min at 37° prior to activity assay. Identical elution profiles of pyruvate-treated enzyme were observed when the gel filtrations were carried out with or without pyruvate in the eluting buffer.

The data displayed in Figure 4B show that pyruvate-inactivated enzyme having a Stokes' radius of 32 Å can be reassociated and reactivated by AMP and DTT. The elution profile represented by open circles reveals that the combination of 15 m*M* AMP and 1 m*M* DTT were able to reassociate completely the enzyme dissociated by pyruvate and restored the Stokes' radius of 41 Å from 32 Å. The shoulder seen on the trailing edge of the elution profile from the enzyme dialyzed against 3 m*M* AMP (closed circles, Figure 4B) suggested that a small fraction of the protein was able to reassociate in low concentration of AMP in the absence of DTT. Alternatively, this may arise from incomplete dissociation of the enzyme by pyruvate (cf. Figure 4A). These results are consistent with the notion that pyruvate influenced the oligomeric state of the enzyme by altering the association–dissociation equilibrium in favor of dissociation.

Binding of [^{14}C]Pyruvate. As mentioned above, the pyruvate-treated enzyme remained inactive and dissociated after passage through Sephadex G-200 equilibrated with 100 m*M* potassium phosphate buffer (pH 8.0) containing 3 m*M* AMP with or without 25 m*M* pyruvate (cf. Figure 4); the apparent irreversible nature of the inactivation process strongly suggested that pyruvate may be bound very tightly, or even covalently, to the enzyme. In preliminary experiments inactivation of the enzyme by [^{14}C]pyruvate and subsequent gel filtration on Sephadex G-25 in pyruvate-free buffer showed that both enzyme activity and a significant

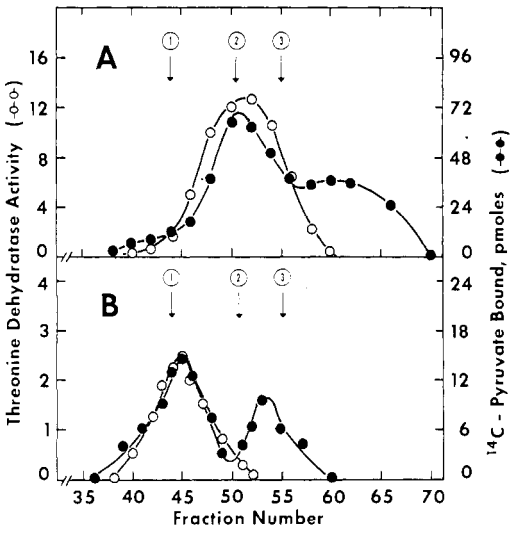

FIGURE 5: Binding of [^{14}C]pyruvate to threonine dehydratase. (A) Purified threonine dehydratase (500 μg) was incubated for 18 hr at 4° with 25 mM [1-^{14}C]pyruvate (specific activity 1.14 Ci/mol) in 100 mM potassium phosphate buffer (pH 8.0) containing 3 mM AMP. The mixture was dialyzed for 24 hr at 4° against a large excess of 100 mM potassium phosphate buffer (pH 8.0) containing 3 mM AMP and 25 mM nonradioactive pyruvate, and applied on a Sephadex G-200 column (58 × 0.8 cm) equilibrated in the above buffer. Threonine dehydratase activities of the column fractions were determined after treating aliquots with 1 mM DTT for 15 min at 37°. The amount of [^{14}C]pyruvate bound was calculated from the radioactivity associated with the fractions as determined by the filter assay (see Experimental Section). The profiles seen in (B) were obtained by concentrating a portion of the pooled fractions numbered 38–58 from (A), dialyzing for 48 hr at 4° against 100 mM potassium phosphate buffer (pH 8.) containing 15 mM AMP and 1 mM DTT, and passaging through a Sephadex G-200 column equilibrated with the same buffer.

fraction of radioactivity eluted in the exclusion volume. The results illustrated in Figure 5A demonstrate that following gel filtration on Sephadex G-200 the profile of radioactivity was coincident with the profile of the enzyme having a Stokes' radius of 32 Å. A fraction of radioactivity also appeared as a shoulder on the leading edge, although no enzyme activity (with or without incubation with DTT) was detected in these fractions suggesting that some [^{14}C]pyruvate may be associated with a lower molecular weight species due to further dissociation of the enzyme.

Since the pyruvate-mediated dissociated form of threonine dehydratase can be reassociated by removal of excess pyruvate by dialysis against AMP and DTT (see Figure 4B) the question arose whether pyruvate was still bound to the reassociated form of the enzyme. The results shown in Figure 5B indicate that following dialysis against 15 mM AMP and 1 mM DTT the reassociated enzyme eluted in the position corresponding to a species having a Stokes' radius of 42 Å. The profile of radioactivity also shifted to the higher molecular weight form suggesting pyruvate was still bound to the oligomeric form of the enzyme. A fraction of radioactivity eluted at a position corresponding to the dissociated enzyme species is presumably due to incomplete oligomerization of the protein.

A series of experiments to remove enzyme-bound [^{14}C]pyruvate including exhaustive dialysis against large excess of nonradioactive pyruvate, repeated precipitation with ammonium sulfate, treatment with 1 N HCl, and precipitation with Cl_3CCOOH were unsuccessful. Strong protein denaturants such as guanidine, urea, and sodium dodecyl sulfate also failed to release pyruvate attached to the enzyme (Table II, experiment I). The molar ratio of firmly bound pyruvate was approximately 1 mol of pyruvate/150,000 g of enzyme.[3]

Table II: Stability of Pyruvate–Enzyme Complex and Effects of Prior Treatments on Pyruvate Binding.[a]

	moles of Pyruvate Bound/150,000 g of Protein
Expt I. Treatment of Pyruvate–Enzyme Complex	
None	1.25
Guanidine-HCl, 6 M	1.18
Urea, 6 M	1.42
Sodium dodecyl sulfate, 2%	0.83
Heated	1.08
Expt II. Pretreatment of Enzyme	
None	1.44
L-Serine, 0.1 M	0.60
L-Threonine, 0.1 M	1.03
p-Chloromercuribenzoate, 0.125 mM	1.27
5,5′-Dithiobis (2-nitrobenzoate), 0.125 mM	1.08

[a] For experiment I, purified threonine dehydratase (0.7 mg/ml) was incubated at 4° for 48 in 100 mM potassium phosphate buffer (pH 8.0) containing 3 mM AMP and 27 mM [1-^{14}C] pyruvate (specific activity 1.05 Ci/mol). About 80% of enzyme activity was lost at the end of the incubation period. Following enzyme inactivation aliquots of the incubation mixture (112 μg of protein) were treated with various denaturants as indicated and incubated further for 60 min at 37°. One aliquot was heated for 10 min at 100°. For experiment II, aliquots of purified enzyme (112 μg) were incubated with the compounds indicated for 24 hr at 4° prior to addition of [^{14}C]-pyruvate and the incubation continued for 48 hr at 4°. In all cases 100% of the enzyme activity was lost prior to pyruvate addition with the exception of L-threonine where the enzyme inactivation was approximately 50%. The amount of pyruvate bound was determined by the filter technique described in the Experimental Section.

The amino acid residue(s) involved in the binding of pyruvate has not been established as yet. Preincubation of threonine dehydratase with large molar excess of p-chloromercuribenzoate or 5,5′-dithiobis(2-nitrobenzoate), that resulted in complete inactivation of the enzyme, did not influence pyruvate binding (Table II, experiment II) suggesting active site -SH groups were not involved in the attachment of pyruvate to the protein.

In addition to pyruvate, the amino acids L-threonine and L-serine were able to inactivate threonine dehydratase both in intact cells as well as in purified enzyme preparations (see Table I). If the enzyme was inactivated with L-threonine prior to addition of [^{14}C]pyruvate, a slight reduction in pyruvate binding was observed (Table II, experiment II). On the other hand, serine-inactivated enzyme bound approximately half the amount of radioactive pyruvate. These results may be taken as evidence for different binding sites on the enzyme for threonine and pyruvate,[4] and that serine and pyruvate may have overlapping binding sites. However, it is also possible that serine may bind to the enzyme at site(s) distinct from that of pyruvate, and that enzyme containing bound serine exists in a different conformational state that is not capable of reacting with pyruvate. Thus,

[3] Although it appears highly likely, we cannot claim that the firm binding of pyruvate represents a covalent linkage with the enzyme since the chemical adduct has not been isolated and identified.

[4] If the inactivation of threonine dehydratase is mediated by α-ketobutyrate, one of the products of the dehydration reaction, this result could also suggest that the binding site(s) of α-ketobutyrate and pyruvate on the enzyme molecule are distinct and nonoverlapping.

the mechanism of enzyme inactivation by threonine or pyruvate appears to be different than that proposed for the "serine suicide" reaction in the following ways: (a) high concentration of pyridoxal phosphate that reversed serine inactivation (McLemore and Metzler, 1968) did not influence enzyme inactivation by pyruvate, (b) addition of excess serine only partially prevented the firm attachment of pyruvate to the enzyme, and (c) excess AMP and DTT decreased the rate and/or extent of enzyme inactivation by threonine and pyruvate, but not by serine (D. A. Feldman and P. Datta, unpublished observation).

Titration of Sulfhydryl Groups with Nbs_2. Since threonine dehydratase of *E. coli* is sensitive to oxidation and requires thiol reagents for enzyme stability, it could be argued that the pyruvate-dependent rapid loss of enzyme activity may actually result from oxidation of essential sulfhydryl groups that might be exposed during dissociation of the oligomeric species. Consequently, a reduction in the number of reactive sulfhydryl groups of the inactive enzyme may be predicted. A comparison of the results of Nbs_2 titration of the untreated and pyruvate-inactivated enzyme, however, showed no loss of titratable sulfhydryl groups from the pyruvate-treated enzyme. In fact, the average value of the ratio of the number of -SH groups on the pyruvate-inactivated enzyme to the untreated active enzyme, in three separate experiments, was 1.5. A slightly greater extent of Nbs_2 reaction with the inactive enzyme may be explained by an increased number of -SH groups being accessible to the thiol reagent due to enzyme dissociation. Additionally, in the presence of 2% sodium dodecyl sulfate, the extent of reaction with Nbs_2 was identical for the active and inactive species indicating the same total number of reactive sulfhydryl groups. We interpret these data to mean that inactivation of threonine dehydratase by pyruvate was not due to oxidation of essential sulfhydryl on the enzyme.

Discussion

In the past few years at least three different examples of glucose "inactivation" of enzyme activity in intact cells have been reported. They are malate dehydrogenase (Ferguson et al., 1967) and fructose 1,6-diphosphatase (Gancedo, 1971) from yeast, and isocitrate lyase from *Chlorella pyrenoidosa* (John et al., 1970). With malate dehydrogenase the inactivation was prevented by cycloheximide, sodium azide, or by chilling the cells at 0°. The inactivation by glucose of fructose 1,6-diphosphatase was not prevented by cycloheximide. Fructose and mannose also caused inactivation but 2-deoxyglucose was ineffective. More recently Waindle and Switzer (1973) have shown that aspartate transcarbamylase activity of *Bacillus subtilis* disappeared rapidly from the stationary phase cells prior to sporulation, provided some metabolic energy generating source, e.g., ATP was available. Inhibitors of oxidative phosphorylation and electron transport interrupted enzyme inactivation. The inactivation was decreased in mutant strains lacking enzymes of the tricarboxylic acid cycle. In all cases described above, enzyme inactivation was not observed in cell-free extracts. The data presented in this article on the catabolite inactivation of threonine dehydratase in *E. coli* clearly demonstrate that pyruvate and certain keto acids were among the principal metabolites that led to enzyme inactivation; the effects of glucose and some amino acids that were effective in intact cells but not with purified threonine dehydratase could be explained by their metabolic conversion to these keto acids by cellular enzymes. These various examples of "glucose inactivation" have led us to believe that the phenomenon of catabolite inactivation as a metabolic regulatory device may be more general than suspected heretofore.

Several facts have emerged from the experiments on the effects of pyruvate on purified threonine dehydratase that may suggest a mechanism of enzyme inactivation. (a) The standard buffers employed in the pyruvate binding assays contained 3 m*M* AMP; in the presence of 15 m*M* AMP although the rate of inactivation was decreased, the maximum loss of enzyme activity was 65%. Since Whanger et al. (1968) and Gerlt et al. (1973) reported that high concentrations of AMP caused oligomerization of threonine dehydratase, we can conclude that pyruvate was able to bind enzyme oligomers. (b) Since pyruvate was still bound to the enzyme that was partially reassociated and reactivated (cf. Figure 5B), it is likely that the binding of pyruvate per se may not yield inactive enzyme. (c) The data presented in Figures 4 and 5 clearly show that pyruvate-inactivated enzyme remained dissociated and inactive following removal of excess pyruvate by gel filtration in phosphate buffer containing 3 m*M* AMP. Thus once pyruvate is bound on the enzyme the association-dissociation equilibrium is perturbed resulting in the formation of stable inactive enzyme species having a Stokes' radius of 32 Å. (d) Titration with Nbs_2 of the active and pyruvate-treated inactive threonine dehydratase revealed that loss of enzyme activity was not due to oxidation of essential -SH groups on the enzyme. Additionally, [^{14}C]pyruvate binding experiments showed that no active site sulfhydryl groups were involved in the attachment of pyruvate to the protein. (e) The inactive dissociated form of the enzyme corresponding to a molecular weight of about 65,000 can be reassociated and reactivated following removal of excess pyruvate under certain experimental conditions. For example, dialysis of pyruvate-inactivated enzyme against phosphate buffer containing 3 m*M* AMP and 1 m*M* DTT yielded 70% active enzyme exhibiting a molecular weight of about 120,000. Similar results were seen with 15 m*M* AMP plus 1 m*M* DTT (cf. Figure 4B). In the *absence* of DTT but with AMP concentrations ranging from 3 to 15 m*M*, the enzyme remained inactive; however, the apparent molecular weights reflected the concentrations of AMP present during dialysis and gel filtration, and varied from 62,000 (at 3 m*M*) to 120,000 at 15 m*M* (Feldman, 1973). Thus, the reassociated enzyme appears to exist in two distinct oligomeric forms; high concentrations of AMP alone yield inactive enzyme oligomers, whereas, active oligomers were produced when both AMP and DTT were present. Although the role of DTT in forming active oligomers is not understood, however, it is clear from a variety of data reported here and elsewhere (see Whanger et al., 1968; Gerlt et al., 1973) that the dissociation-reassociation of threonine dehydratase does not always involve oxidation-reduction of enzyme sulfhydryl groups.

These cumulative data led us to propose that inactivation of threonine dehydratase by pyruvate involves binding of pyruvate to the active oligomeric form of the enzyme followed by dissociation of the oligomer to yield inactive enzyme. Thus, pyruvate alters the association-dissociation equilibrium of the enzyme in favor of a dissociated form, and during this process the modifier became attached very tightly to the enzyme. One explanation for the observed stoichiometry of pyruvate binding is that apparent covalent attachment of one molecule of pyruvate to any one of the subunits of the enzyme oligomer is sufficient to trigger a

conformational change that leads to dissociation of the active tetramer; further binding of pyruvate is prevented due to this altered state of the native enzyme.

The results summarized above clearly demonstrate that the mechanism of catabolite inactivation of threonine dehydrastase by pyruvate and other keto acids in the *presence* of AMP is distinct from the allosteric inhibition of enzyme activity by the reaction product α-ketobutyrate observed in the *absence* of AMP (Shizuta et al., 1973). The inhibition by α-ketobutyrate is unique in the following ways: the effect is reversed either by removal of the inhibitor, or by addition of AMP; the inhibitor behaves competitively with AMP, and it does not bind covalently to the enzyme. It would appear, therefore, that these two regulatory mechanisms are mutually exclusive, and that the adenine nucleotide may play a decisive part in specifying which of the two mechanisms may operate at a given metabolic state.

The physiological significance of catabolite inactivation of threonine dehydratase may be rationalized in the context of the proposed role of this inducible enzyme to generate ATP under anaerobic conditions through metabolic conversion of threonine to propionate (Tokushige et al., 1963). Addition of glucose to the culture medium would lead to increased production of pyruvate and ATP and thus decrease the need for anaerobic degradation of threonine. The increased concentration of pyruvate in the cell would shut off threonine catabolism by inactivating threonine dehydratase. It is envisaged that control of *E. coli* threonine dehydratase activity by catabolite inactivation may function as an important regulatory device to reinforce the other mechanisms such as catabolite repression of enzyme synthesis by glucose and reversible allosteric modulation of enzyme activity by AMP and α-ketobutyrate.

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