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Inhibition of the Biodegradative Threonine Dehydratase from *Serratia marcescens* by α-Keto Acids and Their Derivatives

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Abstract: Biodegradative threonine dehydratase was purified to homogeneity from *Serratia marcescens* ATCC 25419 by streptomycin sulfate treatment, Sephadex G-200 gel filtration chromatography followed by AMP-Sepharose 4B affinity chromatography. The molecular weight of the purified enzyme was 118,000 by fast protein liquid chromatography using superose 6-HR. The enzyme was determined to be a homotetrameric protein with subunit molecular weights of 30,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme was inhibited by α-keto acids and their derivatives such as α-ketobutyrate, pyruvate, glyoxlyate, and phosphoenol pyruvate, but not by α-aminobutyrate and α-hydroxybutyrate. The inhibition of the enzyme by pyruvate and glyoxylate was observed in the presence of AMP. The inhibitory effect of glyoxylate was decreased at high enzyme concentration, whereas the inhibition by pyruvate was independent of the enzyme concentration. The kinetics of inhibition of the enzyme by pyruvate and glyoxylate revealed a noncompetitive and mixed-type inhibition by the two inhibitors with respect to L-threonine and AMP, respectively.

Key words: α-Keto acids, biodegrative threonine dehydratase, inhibition, *Serratia marcescens*.

Threonine dehydratase [L-threonine hydro-lyase (deaminating), EC 4.2.1.16.], also known as threonine deaminase, catalyzes the dehydration of L-threonine and yields α-ketobutyrate and ammonia (Umbarger, 1973). Enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium* have two forms of threonine dehydratase: biosynthetic and biodegradative enzymes (Umbarger, 1978). The biosynthetic enzyme is inhibited by L-isoleucine in a feedback manner (Umbarger and Brown, 1957), whereas the biodegradative enzyme is activated allosterically by AMP and is not inhibited by L-isoleucine (Wood and Gunsalus, 1949). Biodegradative threonine dehydratase has been investigated extensively in *E. coli* and *S. typhimurium* (Shizuta *et al.*, 1969; Shizuta *et al.*, 1973; Bhadra and Datta, 1978; Kim and Datta, 1982). Feldman and Datta (1975) reported a catabolite inactivation of the enzyme from *E. coli*, in which the enzyme was rapidly inactivated by several intermediary metabolites, such as α-ketobutyrate, pyruvate, and glyoxylate, in both intact cells and in purified enzyme preparation. The biodegradative threonine dehydratase from *S. typhimurium* was also subjected to catabolite inactivation (Bhadra and Datta, 1978). A detailed investigation of the catabolite inactivation by pyruvate or glyoxylate indicated that each influences the enzyme activity by two distinct and mutually exclusive mechanisms. 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 (Feldman and Datta, 1975; Bhadra and Datta, 1978). The glyoxylate-mediated loss of catalytic activity, on the other hand, is accompanied by conformational changes in the subunits due to covalent binding of glyoxylate to the tetramer (Park and Datta, 1979a; Park and Datta, 1981). Although many studies on biodegradative threonine dehydratases from *E. coli* and *S. typhimurium* have been carried out, no information is available on the enzymatic properties and inhibition patterns of biodegradatative threonine dehydratase by various metabolites from any *Serratia* strain.

In this report, the inhibition by α-keto acids and their derivatives and the kinetic properties of the inhibition of the purified biodegradative threonine dehydratase from *Serratia marcescens* ATCC 25419 are described.

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Materials and Methods

Bacterial strain and growth conditions

The bacterial strain used in this study was *Serratia marcescens* ATCC 25419. Cells (on brain heart infusion (BHI) media supplemented with pyridoxine HCl) were grown anaerobically for 24 h in a culture flask at 37℃.

Materials

5′-AMP Sepharose 4B, Sephadex G-200, streptomycin sulfate, potassium phosphate, AMP, dithiothreitol (DTT), phenylmethyl sulfonylfluoride (PMSF), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), L-threonine, α-ketobutyrate, α-aminobutyrate, α-hydroxybutyrate, α-ketoglutarate, pyruvate, glyoxylate, phosphoenol pyruvate (PEP), oxalate, citrate, fumarate, succinate, L-isoleucine, L-serine, L-valine, L-methionine, L-histidine, L-arginine, homoserine, N,N,N,N-tetramethylenediamine (TEMED), ammonium persulfate, acrylamide, N,N-methylene-bis-acylamide, coomassie brilliant blue R-250, sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, USA) BHI was purchased from Difco Laboratories (Detroit, USA). All other chemicals were reagent grade and commercially available.

Enzyme assay and protein determination

The activity of biodegradative threonine dehydratase was measured colorimetrically using the method of Friedemann and Haugen (1943), as modified by Bhadra and Datta (1978). The amount of protein was determined by the method of Lowry *et al.* (1951).

Enzyme purification

Biodegradative threonine dehydratase was purified by a procedure similar to that described for the enzyme from *S. typhimurium* (Bhadra and Datta, 1978) with some modifications. Throughout the purification procedure, the temperature was maintained between 0 and 4℃. Solid streptomycin sulfate was added slowly to 37 ml of crude extract, stirring until 3% (1.11 g/ml) saturation was achieved. The pellet was discarded after centrifugation at 15,000×g for 30 min, and the supernatants were pooled. The solution was dialyzed twice for 16 h against 100 volumes of standard buffer (50 mM potassium phosphate pH 6.8, 1 mM AMP, 1 mM DTT, and 1 mM PMSF). The streptomycin sulfate fraction was applied to a Sephadex G-25 column (3.2×18 cm) which had been equilibrated with the standard buffer. The column was then eluted with the same buffer at a flow rate of 6 ml/h. Fractions (3 ml each) containing enzyme activity were pooled. The enzyme solution (721.6 mg/41 ml) was lyophilized and dissolved in a minimum volume of standard buffer. The enzyme solution obtained from the previous step was applied to a Sephadex G-200 column (3.4×94 cm), which was pre-equilibrated with the standard buffer and eluted with the same buffer at a flow rate of 3 ml/h. Fractions (3 ml each) containing enzyme activity were pooled. The enzyme solution (109 mg/61 ml) was dialyzed twice for 16 h against 100 volumes of AMP-free standard buffer (50 mM potassium phosphate pH 6.8, 1 mM DTT, and 1 mM PMSF). The dialyzed sample was concentrated by ultrafiltration under 5 ml and applied to an AMP-Sepharose affinity column (1.5×6 cm) which was equilibrated with the same buffer. The column was eluted with the 10 mM AMP at a flow rate of 6 ml/h, and 1.2 ml fractions were collected.

Electrophoresis

Nondenaturing disc polyacrylamide gel electrophoresis (PAGE) of native enzyme in 10% acrylamide gel was performed according to the method of Laemmli (1970). The gel system consisted of 10% acrylamide resolving gel (pH 8.8) and 4% stacking gel. Denaturing PAGE on 12.5% acrylamide running gel was performed in the presence of SDS by the method of Laemmli (1970). Protein was stained with 0.125% coomassie brilliant blue R-250. Activity staining of the gel was done as described by Feldberg and Datta (1970). The gel was incubated with 100 mM potassium phosphate buffer (pH 8.0) containing 20 mM L-threonine, 1 mg/ml NBT and 1 μg/ml PMS for 3 h at 37℃ in the dark. After a purple color developed at the enzyme band, the gel was rinsed with 50% methanol and 10% acetic acid.

Molecular weight

The molecular weight of the enzyme was also estimated by fast protein liquid chromatography (FPLC) using superose 6-HR following Andrews' procedure (1965). Molecular weight markers were thyroglobulin (669,000), β-amylase (200,000), lactate dehydrogenase (140,000) and BSA (66,000).

To determine the subunit molecular weight, SDS-PAGE on 12.5% acrylamide running gel was performed by the method of Laemmli (1970). Molecular weight markers were phosphorylase b (97,400), BSA (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), and lysozyme (14,400).

Inhibitory effects of various metabolites on enzyme activity

Purified enzyme (5 μg) was incubated for 15 min at 37℃ with metabolites in 100 mM potassium phos-

phate buffer (pH 8.0). The final concentration of the metabolites was 25 mM. An aliquot of incubation mixture was assayed for enzyme activity under standard assay condition. Inhibitory effect is presented as percent activity remaining compared to the control activity. Control enzyme activity which was not treated with metabolite is considered to be 100%.

Inhibition of enzyme actvity by pyruvate and glyoxylate in the presence of various levels of AMP

Stock enzyme solution (100 μg/ml) was exhaustively dialyzed for 16 h at 4℃ against 100 volumes of 50 mM potassium phosphate buffer (pH 8.0) containing 1 mM DTT and 1 mM PMSF to remove AMP. The effect of different concentrations of pyruvate and glyoxylate on the activity of AMP-free enzyme (5 μg) was assayed in the presence of various levels of AMP and 50 mM L-threonine. Percent inhibition was calculated relative to a control sample without pyruvate or glyoxylate at each AMP concentration. Data were plotted as reciprocal velocity versus reciprocal AMP concentration with pyruvate or glyoxylate.

Inhibition of enzyme activity by pyruvate and glyoxylate at various concentrations of L-threonine

The effect of different concentrations of pyruvate and glyoxylate on the activity of AMP-free enzyme solution (0.83 μM) was assayed in the presence of various concentrations of L-threonine and 3 mM AMP. Data were plotted as reciprocal velocity versus reciprocal L-threonine concentration with pyruvate or glyoxylate.

Results and Discussion

Purification of biodegradative threonine dehydratase

Nondenaturing PAGE of the purified biodegradative threonine dehydratase from *S. marcescens* revealed a single protein band which was coincident with the band from activity staining (Fig. 1). The next step, streptomycin sulfate treatment, resulted in 1.2-fold purification with 86.2% recovery of the total activity and the next step, Sepadex G-200 column, gave a 6.7-fold purification. The final affinity chromatography step resulted in a 50-fold overall purification with 15.6% recovery of the original enzyme activity. The molecular weight of the purified enzyme from *S. marcescens* was estimated to be 118,000 by FPLC using superose 6-HR (Fig. 2) and 120,000 by native gradient PAGE (data not shown). The enzyme was found to be composed of four identical subunits with subunit molecular weights of 30,000, as estimated by SDS-PAGE (Fig. 3), indicating that the enzyme from *S. marcescens* is also a homotetrameric protein. The native molecular weights of enzymes from *E. coli* and *S. typhimurium* were report-

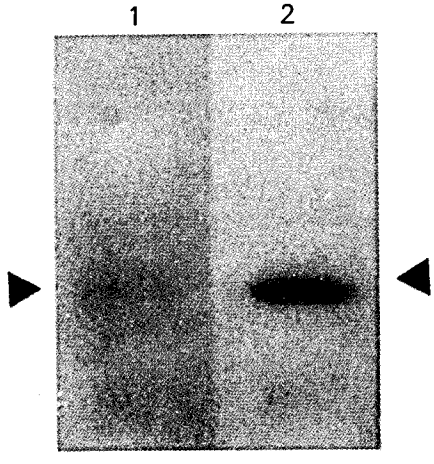

Fig. 1. Nondenaturing PAGE of the purified biodegradative threonine dehydratase.
Lane 1 : activity staining; Lane 2 : purified enzyme (3 μg).

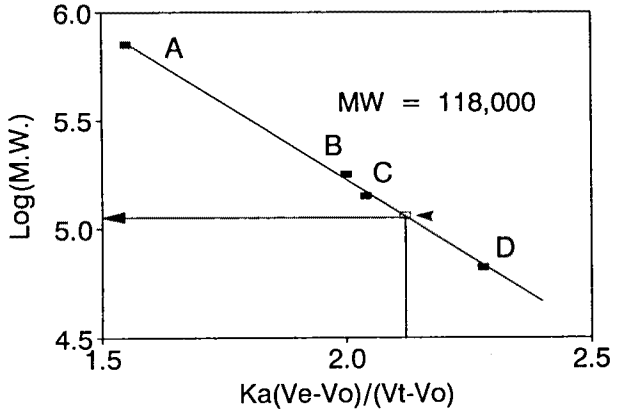

Fig. 2. Determination of the molecular weight of the biodegradative threonine dehydratase by FPLC superose 6-HR. A, thyroglobulin (669,000); B, β-amylase (200,000); C, lactate dehydrogenase (140,000); D, BSA (66,000). The position of threonine dehydratase is indicated by an arrow.

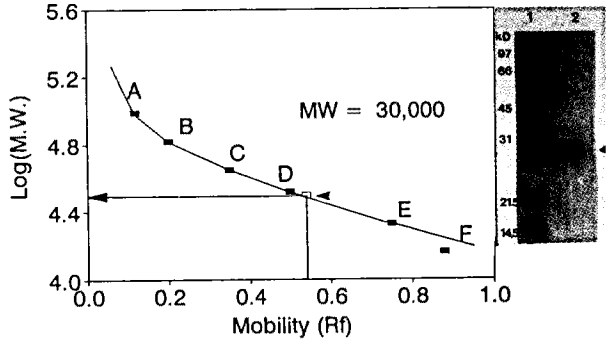

Fig. 3. Determination of the molecular weight of subunit by SDS-PAGE (12.5%). Lane 1 : molecular weight markers; Lane 2 : purified enzyme (6 μg). A, phosphorylase b (97,400); B, BSA (66,000); C, ovalbumin (45,000); D, carbonic anhydrase (31,000); E, trypsin inhibitor (21.500); F, lysozyme (14.400). The position of threonine dehydratase is indicated by an arrow.

Table 1. Effects of various metabolites on the activity of biodegradative threonine dehydratase[a].

Metabolite (25 mM)	Relative activity (%)[b]
Control	100
α-Ketobutyrate	28
α-Aminobutyrate	99
α-Hydroxybutyrate	96
Pyruvate	14
Glyoxylate	13
PEP	25
α-Ketoglutarate	32
Oxalate	91
Citrate	75
Fumarate	89
Succinate	96
L-Threonine	91
L-Serine	93
L-Isoleucine	98
L-Valine	90
L-Methionine	83
L-Histidine	92
L-Arginine	79
Homoserine	81

[a]Enzyme solution was dialyzed for 16 h at 4℃ against 100 mM potassium phosphate buffer (pH 8.0) containing 1 mM DTT and 1 mM PMSF. Purified enzyme (5 μg) was incubated for 15 min at 37℃ with 25 mM metabolites. Enzyme activities were assayed under the standard assay conditions.
[b]Control enzyme activity which was not treated with metabolite is considered as 100%.

ed to be 147,000 and 140,000, respectively, and enzymes from *E. coli* and *S. typhimurium* were composed of four identical polypeptides with subunit molecular weights of 38,000 and 36,000, respectively (Shizuta *et al.*, 1969; Bhadra and Datta, 1978). Thus, the native molecular weight and the subunit molecular weight of biodegradatative threonine dehydratase from *S. marcescens* are slightly less than the weights of enzymes isolated from *E. coli* and *S. typhimurium*. The purified enzyme solution was used for all other studies.

Effects of various metabolites on the enzyme activity

The purified enzyme from *S. marcescens* was inhibited considerably by most α-keto acids and their derivatives (Table 1). The extent of inhibition by α-ketobutyrate, pyruvate, glyoxylate, PEP, and α-ketoglutarate were 72, 86, 87, 75, and 68%, respectively. However, α-aminobutyrate, α-hydroxybutyrate, and oxalate were not inhibitory. Among other intermediary metabolites, citrate and fumarate decreased the enzyme activity by 25 and 11%, respectively, but succinate was not inhibitory. Among amino acids tested, L-methionine, L-arginine, and homoserine decreased the enzyme activity approximately 20%, whereas the other amino acids were not inhibitory. The inhibition of the *S. marcescens* biodegradative threonine dehydratase by α-ketoglutarate is interesting because the activity of *E. coli* K-12 and *S. typhimurium* enzymes was not inhibited by α-ketoglutarate (Feldman and Datta, 1975; Bhadra and Datta, 1978).

Table 2. Inhibition of enzyme activity by pyruvate and glyoxylate at varying levels of AMP[a].

AMP Conc. (mM)	Inhibition by pyruvate (%)		Inhibition by glyoxylate (%)	
	10 mM	20 mM	10 mM	20 mM
0.1	55	60	53	78
0.5	52	58	38	72
1.0	51	54	31	70

[a]Enzyme solution was exhaustively dialyzed at 4℃ against 100 mM potassium phosphate buffer, pH 8.0, to remove AMP. Inhibition of AMP-free enzyme (5 μg) by pyruvate and glyoxylate was assayed colorimetrically in the presence of various levels of AMP. Percent inhibition is calculated relative to a control sample without pyruvate or glyoxylate at each AMP concentration.

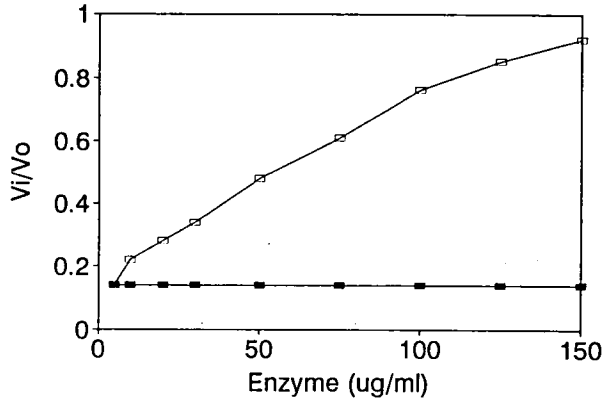

Fig. 4. Effect of enzyme concentration on the inhibition of biodegradative threonine dehydratase by pyruvate and glyoxylate. Enzyme activity was assayed colorimetrically with 1 mM AMP and 50 mM L-threonine, and 25 mM pyruvate or glyoxylate. The data was expressed as the ratio of activity in the presence of pyruvate (V_i) or glyoxylate (V_i) over that in the absence of pyruvate (V_o) or glyoxylate (V_o) at each enzyme concentration used. (■–■); pyruvate, (□–□); glyoxylate.

Inhibition of enzyme activity by pyruvate and glyoxylate

With the enzyme from *S. marcescens*, 10 mM pyruvate inhibited the enzyme activity by 51% at 1 mM AMP (Table 2). In contrast, with the enzyme from *E. coli* W, Shizuta *et al.* (1969) observed that 1 mM AMP completely abolished the inhibitory effect of 5 to 10 mM pyruvate and α-ketobutyrate. The inhibition of the

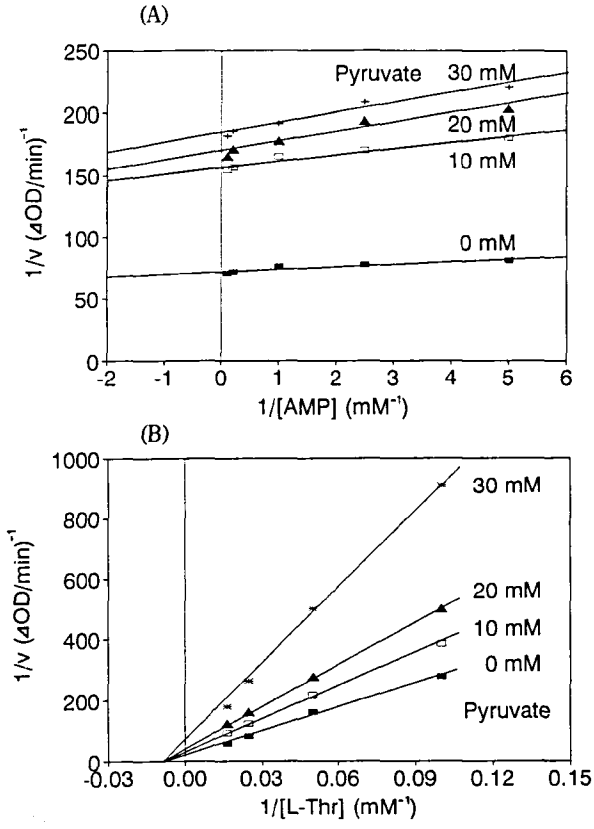

Fig. 5. Double reciprocal plots of activity as a function of pyruvate concentration at different levels of AMP and L-threonine. (A) AMP-free enzyme (5 μg) was assayed colorimetrically with 50 mM L-theonine and various concentrations of AMP and pyruvate. (B) AMP-free enzyme (5 μg) was assayed colorimetrically with 3 mM AMP and various concentrations of L-threonine and pyruvate. Data are plotted as reciprocal initial velocity versus reciprocal AMP concentration (A), or reciprocal L-threonine concentration (B).

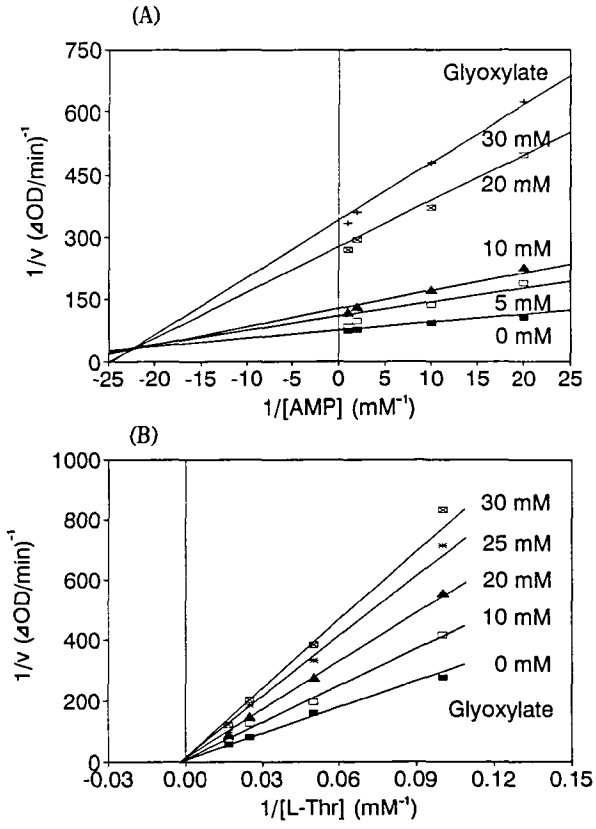

Fig. 6. Double reciprocal plots of activity as a function of glyoxylate concentration at different levels of AMP and L-threonine. (A) AMP-free enzyme (5 μg) was assayed colorimetrically with 50 mM L-theonine and various concentrations of AMP and glyoxylate. (B) AMP-free enzyme (5 μg) was assayed colorimetrically with 3 mM AMP and various concentrations of L-threonine and glyoxylate. Data are plotted as reciprocal initial velocity versus reciprocal AMP concentration (A), or reciprocal L-threonine concentration (B).

enzyme from *S. marcescens* by pyruvate and glyoxylate was observed in the presence of AMP, just as in the case of enzymes from *E. coli* K-12 and *S. typhimurium* (Feldman and Datta, 1975; Bhadra and Datta, 1978).

When the enzyme activity was assayed at various levels of AMP with different concentrations of glyoxylate, marked inhibition was observed regardless of AMP concentrations (Table 2). 10 mM Glyoxylate inhibited the enzyme activity by 53% at 0.1 mM AMP and by 31% at 1 mM AMP. At 1 mM AMP, 10 and 20 mM glyoxylate inhibited the enzyme activity by 31 and 70 %, respectively. These results indicate that the inhibition of the enzyme activity by glyoxylate decreases with increased levels of AMP and increases with increased concentrations of glyoxylate.

When the enzyme activity was assayed with glyoxylate, the extent of inhibition was decreased as the enzyme amount was increased from 5 to 150 μg. The inhibition of enzyme activity by pyruvate, on the other hand, was independent of the concentration of enzyme (Fig. 4). These results suggest that the inhibition of enzyme activity by glyoxylate depends on both enzyme and AMP concentration as well as on the level of glyoxylate, whereas the inhibition by pyruvate does not depend on the enzyme concentration.

Kinetic properties of biodegradative threonine dehydratase

To examine the kinetic relationship between pyruvate and AMP, the enzyme activity was measured as a function of AMP concentration in the presence of various concentrations of pyruvate. A Lineweaver-Burk plot of the pyruvate inhibition of the enzyme activity at various AMP levels is shown in Fig. 5A. The inhibition by pyruvate showed a mixed pattern with respect to AMP, indicating that pyruvate and AMP occupy separate sites on the enzyme molecule. Pyruvate inhibition kinetics of enzymes from *E. coli* and *S. typhimurium* with respect to AMP were noncompetitive and mixed, respectively (Park and Datta, 1979b; Bhadra and Datta, 1978).

Table 3. Comparision of kinetic properties of biodegradative threonine dehydratases from three enteric bacteria.

Properties	*E. coli*	*S. typhimurium*	*S. marcescens*[a]
Pyruvate inhibition kinetics with respect to L-threonine	Uncompetitive[b]	Noncompetitive[d]	Noncompetitive
Pyruvate inhibition kinetics with respect to AMP	Noncompetitive[b]	Mixed[d]	Mixed
Glyoxylate inhibition kinetics with respect to L-threonine	Mixed[c]	–	Noncompetitive
Glyoxylate inhibition kinetics with respect to AMP	Competitive[c]	–	Mixed

[a]This study, [b]Park, L. S. and Datta, P. (1979b), [c]Park, L. S. and Datta, P. (1979a), [d]Bhadra, R. and Datta, P. (1978).

The result of the Lineweaver-Burk plot presented in Fig. 5B reveals a kinetic relationship between pyruvate and L-threonine at a fixed AMP concentration. At different concentrations of pyruvate, the inhibition kinetics showed noncompetitive behavior (Fig. 5B). Pyruvate inhibition kinetics of enzymes from *E. coli* and *S. typhimurium* with respect to L-threonine were uncompetitive and noncompetitive, respectively (Park and Datta, 1979 b; Bhadra and Datta, 1978). These results indicate that the enzyme from *S. marcescens*, like the enzyme from *S. typhimurium*, has separate sites for binding of all three ligands, L-threonine, AMP, and pyruvate.

Fig. 6A indicates that glyoxylate is inhibitory to the purified enzyme at all ranges of AMP tested and that the inhibition by glyoxylate has a mixed pattern with respect to AMP. The kinetic relationship between glyoxylate and L-threonine, on the other hand, appeared to be noncompetitive (Fig. 6B). These results are indicative of the presence of separate binding sites for L-threonine and glyoxylate on the biodegradative threonine dehydratase. A kinetic analysis of the enzyme from *E. coli* showed that the inhibition by glyoxylate was mixed with respect to L-threonine, and competitive in terms of AMP (Park and Datta, 1979a). The kinetics of inhibition of the enzyme from *S. typhimurium* by glyoxylate with respect to L-threonine and AMP is not known at the present time. The results of the studies of the kinetic properties of biodegradative threonine dehydratases from the three enteric bacteria are summmarized in Table 3. With respect to the kinetic properties, the enzyme from *S. marcescens* is more similar to the enzyme from *S. typhimurium* than the enzyme from *E. coli*. The subtle differences in the inhibition patterns among the enzymes from the three enteric bacteria may represent minor structural differences among these enzymes, and further structural studies are necessary to clarify this point.

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References

Andrews, P. (1965) *Biochem. J.* **96**, 595.
Bhadra, R. and Datta, P. (1978) *Biochemistry* **17**, 1691.
Feldberg, R. S. and Datta, P. (1970) *Science* **170**, 1414.
Feldman, D. A. and Datta, P. (1975) *Biochemistry* **14**, 1760.
Friedemann, T. E. and Haugen, G. E. (1943) *J. Biol. Chem.* **147**, 415.
Kim, S. S. and Datta, P. (1982) *Biochim. Biophys. Acta* **706**, 27.
Laemmli, U. K. (1970) *Nature* **227**, 680.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
Park, L. S. and Datta, P. (1979a) *J. Biol. Chem.* **254**, 7927.
Park, L. S. and Datta, P. (1979b) *J. Bacteiol.* **138**, 1026.
Park, L. S. and Datta, P. (1981) *J. Biol. Chem.* **256**, 5362.
Shizuta, Y., Kurosawa, A., Ionue, K., Tanabe, T. and Hayaishi, O. (1973) *J. Biol. Chem.* **248**, 512.
Shizuta, Y., Nakazawa, A., Tokushige, M. and Hayaishi, O. (1969) *J. Biol. Chem.* **244**, 1883.
Umbarger, H. E. (1973) *Adv. Enzymol.* **37**, 349.
Umbarger, H. E. (1978) *Annu. Rev. Biochem.* **47**, 533.
Umbarger, H. E. and Brown, B. (1957) *J. Bacteriol.* **73**, 105.
Wood, W. A. and Gunsalus, I. C. (1949) *J. Biol. Chem.* **181**, 171.