Chemical Modification of the Biodegradative Threonine Dehydratase from *Serratia marcescens* with Arginine and Lysine Modification Reagents

Byung Bum Choi and Soung Soo Kim*

Department of Biochemistry, College of Science and Bioproducts Research Center, Yonsei University, Seoul 120-749, Korea

(Received September 30, 1994)

Abstract: Biodegradative threonine dehydratase purified from *Serratia marcescens* ATCC 25419 was inactivated by the arginine specific modification reagent, phenylglyoxal (PGO) and the lysine modification reagent, pyridoxal 5'-phosphate (PLP). The inactivation by PGO was protected by L-threonine and L-serine. The second order rate constant for the inactivation of the enzyme by PGO was calculated to be 136 $M^{-1}min^{-1}$. The reaction order with respect to PGO was 0.83. The inactivation of the enzyme by PGO was reversed upon addition of excess hydroxylamine. The inactivation of the enzyme by PLP was protected by L-threonine, L-serine, and α-aminobutyrate. The second order rate constant for the inactivation of the enzyme by PLP was 157 $M^{-1}min^{-1}$ and the order of reaction with respect to PLP was 1.0. The inactivation of the enzyme by PLP was reversed upon addition of excess acetic anhydride. Other chemical modification reagents such as N-ethylmaleimide, 5,5'-dithiobis (2-nitrobenzoate), iodoacetamide, sodium azide, phenylmethyl sulfonylfluoride and diethylpyrocarbonate had no effect on the enzyme activity. These results suggest that essential arginine and lysine residues may be located at or near the active site.

Key words: biodegradative threonine dehydrates, chemical modification, *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). Biodegradative threonine dehydratase has been investigated extensively in *Escherichia coli* and *Salmonella typhimurium* (Shizuta *et al.*, 1969; Bhadra and Datta, 1978; Kim and Datta, 1982). The enzyme activity is allosterically stimulated by AMP and is inhibited by α-keto acids, pyruvate and glyoxylate (Wood and Gunsalus, 1949; Feldman and Datta, 1975; Bhadra and Datta, 1978). Recently, the *E. coli tdc* gene which encodes biodegradative threonine dehydratase was cloned (Goss *et al.*, 1985) and the complete amino acid sequence of the protein was deduced from the nucleotide sequence of the gene (Datta *et al.*, 1987). With the tryptic peptide of borohydride-reduced enzyme from *E. coli*, the pyridoxal phosphate-bound lysine residue involved in the dehydration reaction was determined to be Lys-58 (Datta *et al.*, 1987), and with the photoreactive AMP analog, 8-azido-AMP, 13-residues from Thr-230 to Arg-242 were found to be involved in binding AMP (Patil and Datta, 1988). The glyoxylate binding site of this *E. coli enzyme* was the heptapeptides from Ser-33 to Arg-39 (Patil and Datta, 1989). However, little is known about the residues present in the active site of this enzyme from *Serratia marcescens*. In this communication, we describe the results of chemical modification studies with the purified biodegradative threonine dehydratase from *S. marcescens* ATCC 25419. We present evidences that both arginine and lysine residues are in or near the active site of *S. marcescens* enzyme.

*To whom correspondence should be addressed.
Tel: 82-2-361-2698, Fax: 82-2-361-2698.

Materials and Methods

Bacterial strain and culture conditions

The bacterial strain used in this study was *Serratia marcescens* ATCC 25419. It was obtained from Professor H. D. Braymer of Louisiana State University. Stock cultures were maintained on brain heart infusion (BHI) agar slants. Cells (5 l of BHI media supplemented with 0.05 g of pyridoxine HCl per liter/batch) were grown anaerobically for 24 h in a 5 l culture flask at 37℃ on an incubator. The cells were harvested by centrifugation (10,000×g, 30 min) when the optical density of the culture at 660 nm was 1.00. They were then

stored at −70℃.

Materials

5′-AMP Sepharose 4B, Sephadex G-200, streptomycin sulfate, potassium phosphate, AMP, dithiothreitol (DTT), phenylmethyl sulfonylfluoride (PMSF), L-threonine, L-serine, α-aminobutyrate, phenylglyoxal (PGO), 2,3-butanedione, pyridoxal 5′-phosphate (PLP), N-ethylmaleimide (NEM), 5,5′-dithiobis (2-nitrobenzoate) (DTNB), iodoacetamide, sodium azide, diethylpyrocarbonate (DEP), hydoxylamine, 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). One ml reaction mixture contained 100 mM potassium phosphate buffer (pH 8.0), 50 mM L-threonine, 3 mM AMP, 10 mM L-isoleucine (to inhibit the activity of biosynthetic threonine dehydratase, if present) and the enzyme solution. One unit of enzyme activity represents the formation of 1 μmol of α-ketobutyrate per min. The concentration of α-ketobutyrate was determined from a molar extinction coefficient of 4,000 at 540 nm. The specific activity is expressed as unit per mg of protein. 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. The enzyme was purified by streptomycin sulfate treatment, Sephadex G-200 gel filtration chromatography followed by AMP-Sepharose 4B affinity chromatography. The purity of the enzyme preparations was checked routinely by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS.

Chemical modification of arginine and lysine residues of the biodegradative threonine dehydratase

The modifications of arginine and lysine residues of threonine dehydratase were essentially carried out as described elsewhere (Plapp *et al.*, 1971; Shoun *et al.*, 1980; Lundblad and Noyes, 1984). Briefly, modification of the arginine residue of the enzyme was carried out by reacting the enzyme solution (5 μg) with different concentrations of PGO in 50 mM sodium bicarbonate buffer (pH 8.4), or different concentrations of 2,3-butanedione in 50 mM sodium borate buffer (pH 8.8) at 37℃. Modification of the lysine residue of the enzyme was carried out by reacting the enzyme solution (5 μg) with different concentrations of PLP in 50 mM potassium phosphate buffer (pH 7.0) at 37℃. Aliquots of the reaction mixture were removed for the measurement of the residual enzyme activity at time intervals of 2, 4, 6, 8, 10, 12, 14, 16 and 20 min.

Reaction of the biodegradative threonine dehydratase with other chemical modifiers

The purified enzyme solution (0.83 μM) was incubated with given concentrations (0.5 mM and 1 mM) of several modification reagents, NEM, DTNB, iodoacetamide, sodium azide, PMSF and DEP in 50 mM potassium phosphate buffer (pH 7.0) for 15 min at 37℃. Aliquots of the reaction mixture were taken and assayed. Stock solutions of PMSF were prepared by dissolving it in 99% ethanol just before use. The ethanol concentration of the reaction mixture was kept at less than 5% so that it could not affect the enzyme activity.

Protection of the activity of biodegradative threonine dehydratase by L-threonine, L-serine, and α-aminobutyrate

Protection against the inactivation of the enzyme by PGO or PLP was carried out by preincubation of the enzyme (0.83 μM) with 50 mM L-threonine, L-serine, or α-aminobutyrate for 15 min at 37℃ before the addition of each modification reagent. Aliquots of the reaction mixture were removed for the measurement of the enzyme activity at time intervals of 2, 4, 6, 8, 10, 12, 14, 16 and 20 min.

Reactivation of the inactivated biodegradative threonine dehydratase by hydroxylamine

The enzyme solution (0.83 μM) was incubated with 1 mM PGO, and 50 mM α-aminobutyrate for 14 min at 37℃. In this case, α-aminobutyrate was added to the reaction mixture to protect the PLP against modification by hydroxylamine. The PGO-inactivated enzyme was then treated with 25 mM hydroxylamine in 50 mM potassium phosphate buffer (pH 7.0). The residual enzyme activity was measured at time intervals of 2, 6, 8, 12, 16, and 26 min.

Reactivation of the inactivated biodegradative threonine dehydratase by acetic anhydride

The enzyme solution (0.83 μM) was incubated with

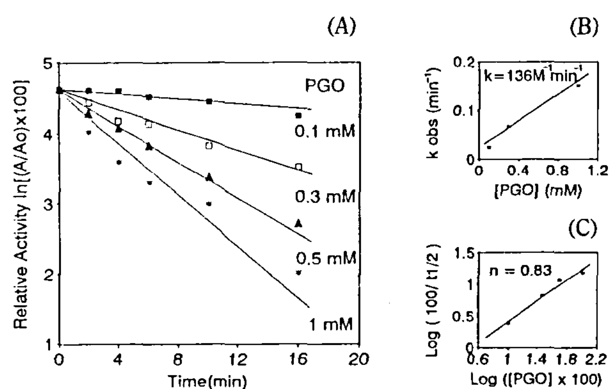

Fig. 1. (A) Inactivation of the biodegradative threonine dehydratase by PGO. The enzyme solution (0.83 μM) was incubated with various concentrations of PGO in 50 mM sodium bicarbonate buffer (pH 8.4) at 37℃, and the remaining activity was determined. (B) Plot of pseudo first-order rate constant (k_{obs}) for the inactivation of threonine dehydratase obtained at various concentrations of PGO. (C) Double-logarithmic plot of the half-times of inactivation of threonine dehydratase at various concentrations of PGO.

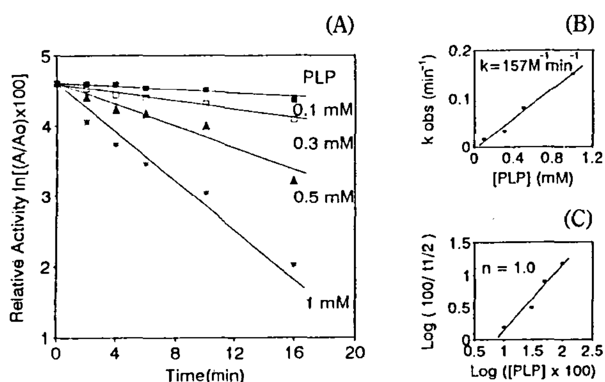

Fig. 2. (A) Inactivation of the biodegradative threonine dehydratase by PLP. The enzyme solution (0.83 μM) was incubated with various concentrations of PLP in 50 mM potassium phosphate buffer (pH 7.0) at 37℃, and the remaining activity was determined. (B) Plot of pseudo first-order rate constant (k_{obs}) for the inactivation of threonine dehydratase obtained at various concentrations of PLP. (C) Double-logarithmic plot of the half-times of inactivation of threonine dehydratase at various concentrations of PLP.

1 mM PLP for 12 min at 37℃. The PLP-inactivated enzyme was then treated with 20 mM acetic anhydride in 50 mM potassium phosphate buffer (pH 7.0). The residual enzyme activity was measured at time intervals of 2, 4, 6, 8, 12, 16, and 20 min.

Results and Discussion

Inactivation of the biodegradative threonine dehydratase by phenylglyoxal

Fig. 1A shows the time course for the inactivation of the purified threonine dehydratase (0.83 μM) incubated with various concentrations of PGO. The biodegradative threonine dehydratase results in a rapid loss of activity. The rate of inactivation was proportional to the concentration of PGO and the incubation time. Loss of activity followed pseudo first order kinetics. A semi-log plot of the residual enzyme activity versus incubation time shows a linear inactivation pattern. From this plot, the second order rate constant for inactivation was determined to be 136 $M^{-1}min^{-1}$ (Fig. 1B). The reaction order with respect to PGO was determined to be 0.83 from a double logarithmic plot of the reciprocal of the half-time of inactivation against the reagent concentration (Fig. 1C). These results suggest that the inactivation may be due to the reaction of one arginine

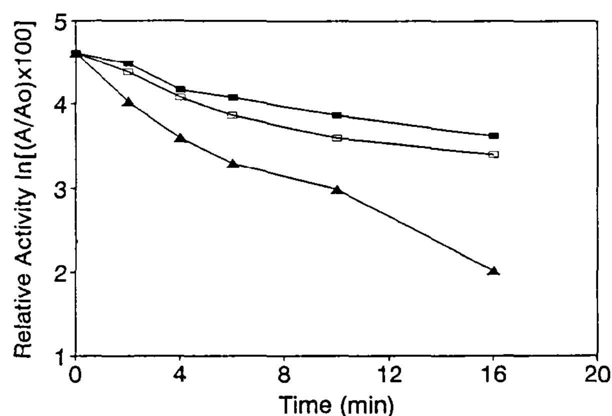

Fig. 3. Effect of substrates on the rate of inactivation by PGO of the biodegradative threonine dehydratase. The enzyme solution (0.83 μM) was preincubated with 50 mM L-threonine or L-serine prior to the addition of 1 mM PGO, and the remaining activity was determined. Activity in the presence of L-threonine (■-■) and L-serine (□-□). Activity in the presence of PGO only (▲-▲).

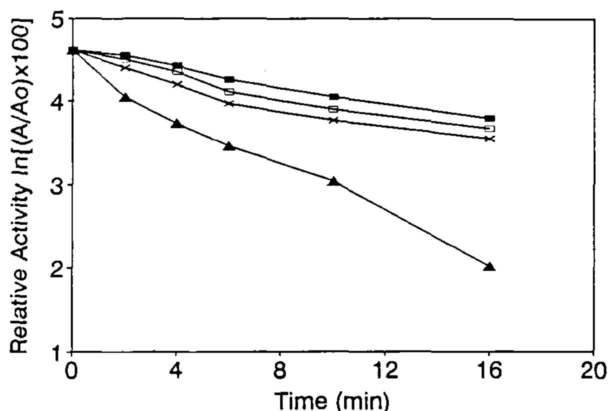

Fig. 4. Effect of substrates on the rate of inactivation by PLP of the biodegradative threonine dehydratase. The enzyme solution (0.83 μM) was preincubated with 50 mM L-threonine, L-serine, or α-aminobutyrate prior to the addition of 1 mM PLP, and the remaining activity was determined. Activity in the presence of L-threonine (■-■), L-serine (□-□) and α-aminobutyrate (X—X). Activity in the presence of PLP only (▲-▲).

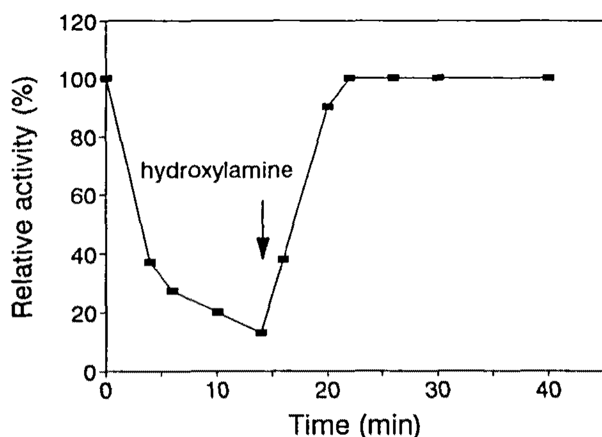

Fig. 5. Reactivation of the biodegradative threonine dehydratase by hydroxylamine. The enzyme solution (0.83 μM) was incubated with 1 mM PGO and 50 mM α-aminobutyrate for 14 min at 37℃ prior to the addition of 25 mM hydroxylamine, and the remaining activity was determined.

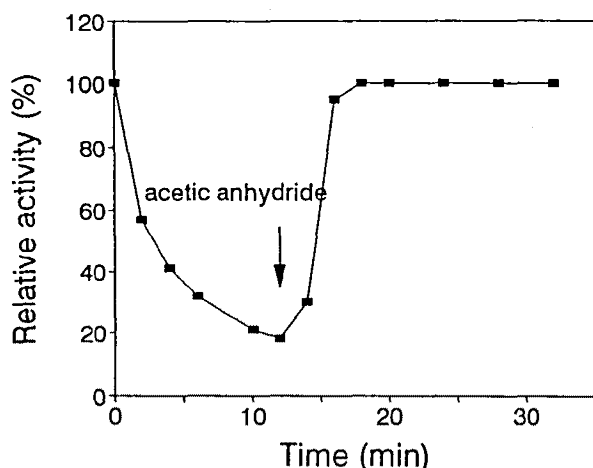

Fig. 6. Reactivation of the biodegradative threonine dehydratase by acetic anhydride. The enzyme solution (0.83 μM) was incubated with 1 mM PLP for 12 min at 37℃ prior to the addition of 20 mM acetic anhydride, and the remaining activity was determined.

residue per active threonine dehydratase molecule.

Inactivation of the biodegradative threonine dehydratase by PLP

The time course for the inactivation of the enzyme (0.83 μM) by various concentrations of PLP is shown in Fig. 2A. Loss of activity followed pseudo first order kinetics. A semi-log plot of the residual activity versus time yields a linear mode of inactivation pattern. The second order rate constant of 157 $M^{-1}min^{-1}$ was determined from the slope of the linear relationships between the k_{obs} values and the PLP concentrations (Fig. 2B). A double logarithmic plot of the reciprocal of the half-time of inactivation against reagent concentration yielded a reaction order of 1.0 with respect to the inhibitor (Fig. 2C), suggesting that there may be one essential lysine residue per active molecule.

Table 1. Effects of modification reagents on the activity of biodegradative threonine dehydratase.

Reagents	Conc. (mM)	Relative activity (%)[a]
Control	–	100
NEM	1	97
DTNB	1	100
Iodoacetamide	1	98
Sodium azide	1	95
PMSF	1	96
DEP	1	105
PGO	0.5	15
	1	8
2,3-Butanedione	0.5	11
	1	5
PLP	0.5	25
	1	11

[a] Inhibitory effect is presented as % activity remaining compared to the control activity. Control enzyme activity which was not treated with modification reagent is considered as 100%.

Protein of the biodegradative threonine dehydratase inactivation by substrates

The inactivation of an enzyme activity by chemical modification reagents for certain amino acid residues does not always directly imply that such residues are present at the active site (Cohen, 1970; Nakanishi *et al.*, 1989). However, protection of an enzyme by substrate against inactivation would suggest that the amino acid residues that are protected from modifications are located at or near the active site. When the biodegradative threonine dehydratase (0.83 μM) was preincubated with 50 mM L-threonine or L-serine, the enzyme was protected from inactivation by PGO, as shown in Fig. 3. This result suggests that a single arginine residue is located at or near the active site. Preincubation of the enzyme with 50 mM L-threonine, L-serine, or α-aminobutyrate also protected the enzyme against inactivation by PLP (Fig. 4). The data indicate that a lysine residue is also located at or near the active site of threonine dehydratase.

Reactivation of the inactivated biodegradative threonine dehydratase by hydroxylamine

The inactivation of the enzyme by PGO was reversed upon addition of excess hydroxylamine (25 mM). The enzyme activity was restored to the level of the control enzyme activity without PGO 8 min after hydroxyl-

amine addition (Fig. 5). This result implies that the inactivation of the enzyme by PGO can be attributed to the modification of an arginine residue in the active site.

Reactivation of the inactivated biodegradative threonine dehydratase by acetic anhydride

The inactivation of the enzyme by PLP was reversed upon addition of excess acetic anhydride (20 mM). The enzyme activity was restored to the level of the control enzyme activity without PLP 6 min after acetic anhydride addition (Fig. 6). This result implies that the inactivation of the enzyme by PLP can also be attributed to the modification of a lysine residue in the active site.

Effect of other chemical modifiers on the activity of biodegradative threonine dehydratase

As shown in Table 1, the biodegradative threonine dehydratase was considerably inhibited by 2,3-butanedione in addtion to PGO and PLP (Table 1). The arginine specific reagent 2,3-butanedione inhibited the enzyme activity by 89 and 95% at 0.5 and 1 mM, respectively. However, sulfhydryl-specific reagents such as NEM, DTNB, iodoacetamide and sodium azide, the serine-specific reagent PMSF and the histidine-reacting reagent DEP had no effect on the threonine dehydratase activity at 1 mM concentration (Table 1).

Acknowledgement

This article is offered to commemorate the retirement of professor Chung-No Joo from the Department of Biochemistry, College of Science, Yonsei University.

References

Bhadra, R. and Datta, P. (1978) *Biochemistry* **17**, 1691.

Cohen, L. A. (1970) in *The Enzymes* (Boyer, P. D., ed.), 3rd ed., Vol. I, pp. 148-211, Academic Press, New York.

Datta. P., Goss, T. J., Omnaas, J. R. and Patil, R. V. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 393.

Feldman, D. A. and Datta, P. (1975) *Biochemistry* **14**, 1760.

Friedemann, T. E. and Haugen, G. E. (1943) *J. Biol. Chem.* **147**, 415.

Goss, T. J. and Datta, P. (1985) *Mol. Gen. Genet.* **201**, 308.

Kim, S. S. and Datta, P. (1982) *Biochim. Biophys. Acta* **706**, 27.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.

Lundblad, R. L. and Noyes, C. M. (1984) in *Chemical Reagents for Protein Modification*, Vol. I (Chap. 10) and Vol. II (Chap. 1), CRC Press, Press, Inc. Boca Raton, Florida.

Nakanishi, Y., Isobashi, F., Ebisuno, S. and Sakamoto, Y. (1989) *Biochim. Biophys. Acta* **996**, 209.

Patil, R. V. and Datta, P. (1988) *Eur. J. Biochem.* **177**, 569.

Patil, R. V. and Datta, P. (1989) *J. Bacteriol.* **171**, 3379.

Plapp, B. V., Moore, S. and Stein, W. H. (1971) *J. Biol. Chem.* **246**, 939.

Shizuta, Y., Nakazawa, A., Tokushige, M. and Hayaishi, O. (1969) *J. Biol. Chem.* **244**, 1883.

Shoun, H., Beppu, T. and Arima, K. (1980) *J. Biol. Chem.* **255**, 9319.

Umbarger, H. E. (1978) *Annu. Rev. Biochem.* **47**, 533.

Wood, W. A. and Gunsalus, I. C. (1949) *J. Biol. Chem.* **181**, 171.