Mechanisms of Interaction of *Escherichia coli* Threonine Synthase with Substrates and Inhibitors

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ABSTRACT: Threonine synthase (TS), the last enzyme of the threonine biosynthetic pathway, catalyzes L-threonine formation from L-homoserine phosphate (HSerP; K_m = 0.5 mM, V = 440 min^{-1}) and DL-vinylglycine. Furthermore, TS catalyzes β-elimination reactions with L-serine (K_m = 150 mM, V = 4.7 min^{-1}), DL-3-chloroalanine, L-threonine, and L-*allo*-threonine as substrates to yield pyruvate or α-ketobutyrate, while L-alanine, L-2-aminobutanoic acid, and L-2-amino-5-phosphonopentanoic acid are substrates for half-transamination reactions to form the pyridoxamine form of the enzyme and the corresponding α-keto acid. Spectral analyses of all these reactions revealed the transient formation of strongly absorbing long-wavelength chromophores (λ_{max} = 440–445 nm), implying the accumulation of the corresponding pyridoxaldimine *p*-quinonoidal intermediates. HSerP turnover was competitively inhibited by L-3-hydroxyhomoserine phosphate **1** (K_i = 0.050 mM), L-2,3-methanohomoserine phosphate **2** (K_i = 0.010 mM), L-2-amino-3-[(phosphonomethyl)thio)]propanoic acid **5** (K_i = 0.011 mM) and DL-*E*-2-amino-5-phosphono-4-pentenoic acid **10** (K_i = 0.54 mM). **5** and **10** induced the formation of long-wavelength quinonoidal chromophores (λ_{max} = 458 and 460 nm, $\epsilon \approx$ 47 000 and 30 000 M^{-1} cm^{-1}), while incubation with either **1** or **2** induced only minor spectral changes. DL-2-Amino-3-[(phosphonomethyl)amino)]propanoic acid inactivated TS (K_i = 0.057 mM, k_{inact} = 1.44 min^{-1}) with 1:1 stoichiometry, transient formation of a 450-nm chromophore, and finally bleaching of any absorbance at wavelengths longer than 320 nm. *Z*-2-Amino-5-phosphono-3-pentenoic acid **8** is the unusual amino acid found in the peptide antibiotics of the plumbemicin and rhizocticin families. Racemic **8** irreversibly inhibited TS (K_i = 0.1 mM, k_{inact} = 1.50 min^{-1}) with 1:1 stoichiometry and the concomitant formation of a 482-nm chromophore ($\epsilon \approx$ 30 000 M^{-1} cm^{-1}). DL-*E*-2-Amino-5-phosphono-3-pentenoic acid was a less potent irreversible inhibitor of TS (K_i = 0.4 mM, k_{inact} = 0.25 min^{-1}), inducing absorption maxima at 462 and 500 nm. The acetylenic amino acid DL-2-amino-5-phosphono-4-pentynoic acid **12** bound to TS (K_D = 0.38 mM) forming a quinonoidal chromophore (λ_{max} = 452 nm, $\epsilon \approx$ 30 000 M^{-1} cm^{-1}), but inhibition of the enzyme by **12** could not be detected under assay conditions even at high inhibitor concentrations. Mechanisms consistent with these observations are proposed.

Escherichia coli threonine synthase (TS;[1] EC 4.2.99.2), the last enzyme of the threonine biosynthetic pathway, catalyzes the formation of L-threonine from the substrate L-homoserine phosphate (HSerP). In this pyridoxal 5′-phosphate- (PLP-) dependent β,γ-replacement reaction (Scheme 1) substrate HSerP forms a Schiff base with the enzyme-bound cofactor PLP **I**, followed by α-proton abstraction to yeld an α-carbanion equivalent stabilized as the pyridoxaldimine *p*-quinonoid **II**. This first deprotonation is followed by the stereospecific removal of the β-*pro*-*S*-hydrogen atom (Fuganti, 1979) and subsequent nonhydrolytic elimination of the γ-substituent as inorganic phosphate (Flavin & Kono, 1960). The γ-methylene group of the resulting β,γ-unsaturated pyridoxal *p*-quinonoid of vinylglycine **III** picks up a proton from the solvent (Flavin & Slaughter, 1960), thus yielding the PLP derivative of *E*-aminocrotonate **IV**. The addition of water and finally reverse transaldimination yields L-threonine with retention of configuration (Fuganti, 1979).

Our recent finding (Laber and Pohlenz, unpublished experiments) that *Z*-2-amino-5-phosphono-3-pentenoic acid, the unusual amino acid found in the peptide antibiotics of the Plumbemicin and Rhizocticin families, is an irreversible inhibitor of TS, initiated us to study the TS reaction in more detail. Since the enzyme-bound cofactor as well as certain PLP-derived reaction intermediates are known to have distinctive UV–visible absorption maxima, their spectrophotometric detection was used as a probe for the identification of kinetically competent intermediates in the TS reaction. The interaction of TS with its substrate HSerP and with alternate substrates, undergoing either β-elimination or half-transamination reactions, is described in this paper.

Also, we report herein the kinetic and spectroscopic evaluation of a number of previously prepared HSerP analogues (Harde et al., 1994), which acted as reversible and irreversible inhibitors of TS. These studies allowed us to gain further insight into the TS reaction mechanism.

MATERIALS AND METHODS

Materials. Pyruvate kinase and lactate dehydrogenase from rabbit muscle were purchased from Boehringer-Mannheim. Alkaline phosphatase, Type VII-S, from bovine intestinal mucosa, and DL-2-amino-5-phosphonopentanoic acid were from Sigma. D- and L-2-Amino-5-phosphonopentanoic acid were from Tocris Neuramin (Bristol, England), and L-*threo*-2-amino-3-chlorobutanoic acid was purchased from Calbiochem. DL-*Z*-2-Amino-5-phosphono-3-pentenoic acid **8**, DL-*E*-2-amino-5-phosphono-3-pentenoic acid **9**, and DL-*E*-2-amino-5-phosphono-4-pentenoic acid **10** were prepared as

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[1] Abbreviations: HSerP, L-homoserine phosphate; μkat, a reaction rate of 1 μmol s^{-1}; PEP, phosphoenolpyruvate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; TS, threonine synthase.

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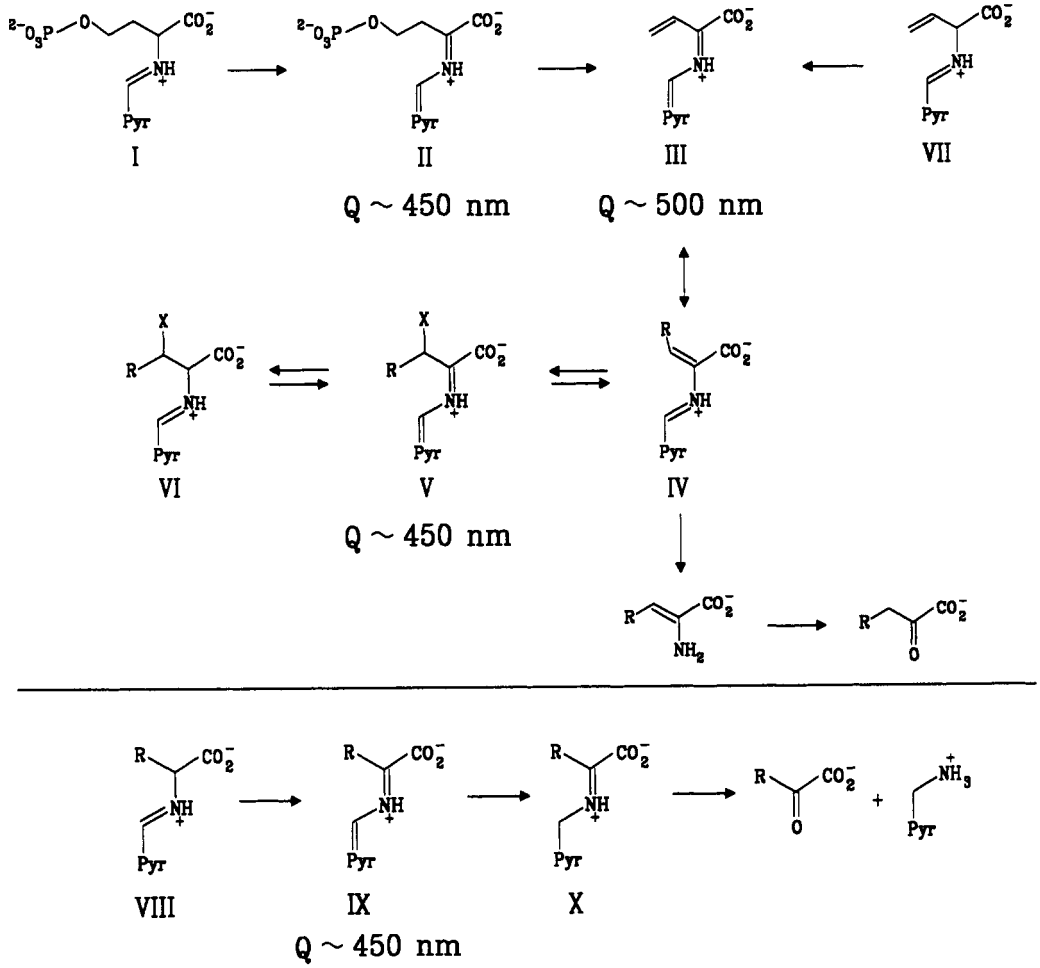
Scheme 1: Postulated Mechanisms for the Reactions Catalyzed by Threonine Synthase[a]



[a] (Above) Reactions of HSerP, vinylglycine, and substrates undergoing a deamination reaction (X = Cl, OH; R = H, CH_3, CH_2CH_3). The first step in each reaction is the formation of an aldimine Schiff base followed by the loss of the α-hydrogen of the amino acid. The resulting quinonoid structures undergoe tautomerization to yield the α,β-unsaturated aldimine **IV**, an intermediate in each of the reactions. With HSerP or vinylglycine as the substrate, β-addition of water to **IV** yields threonine (X = OH, R = CH_3). For deamination reactions, hydrolysis of **IV** yields the corresponding α-keto acid and NH_4^+. (Below) Half-transamination reaction (R = CH_3, CF_3, CH_2CH_2OH, CH_2CH_3, $CH_2CH_2CH_2PO_3^{2-}$). Inorganic phosphate, ammonia, and water, the byproducts of the reactions, are omitted for clarity.

described previously (Nachev, 1988; Allgeier et al., 1989; Angst et al., 1987). The synthesis of compounds **5**–**7**, **11**, and **12** (Table 2) is described elsewhere (Harde et al., 1994).

Synthesis of L-*Homoserine Phosphate.* Phosphorylation of L-homoserine was performed at 37 °C in 100 mL of 2.5 mM HEPES, 8 mM $MgCl_2$, 5 mM ATP, 100 mM L-homoserine, 100 mM PEP, and 0.04% NaN_3. The solution was adjusted to pH 8.0 and the reaction was started by the addition of 2.5 μkat of pyruvate kinase and 1.4 μkat of homoserine kinase. The reaction progress was monitored by determining the pyruvate formed with lactate dehydrogenase (Lamprecht & Fritze, 1984). Ater about 30 h, when >95% of PEP was consumed, the pH was adjusted to about 10 with KOH and the solution was applied to a 500-mL Bio-Rad AG 1X8 anion-exchange column (200–400 mesh, Cl^- form). After being washed with 700 mL of water, the column was eluted with 4 L of 12 mM HCl, and HSerP was detected by reaction with ninhydrin (Rosen, 1957). Ninhydrin-positive fractions were concentrated by rotary evaporation to about 10 mL. After neutralization with KOH, the HSerP concentration was determined after alkaline phosphatase treatment by determination of the amount of liberated inorganic phosphate (Lanzetta et al., 1979). Overall yields were about 85%.

Synthesis of Analogues of Homoserine Phosphate. HSerP analogues were prepared by enzymatic phosphorylation of the corresponding homoserine analogues with homoserine kinase essentially as described for the synthesis of HSerP. Specificity of homoserine kinase for L-amino acids was assumed.

L-*3-Hydroxyhomoserine Phosphate* **1**. To 10 mL of 16 mM HEPES, pH 7.6, 160 mM KCl, 8 mM $MgCl_2$, 10 mM ATP, 10 mM DL-3-hydroxyhomoserine (Hamel & Painter, 1953), and 0.04% NaN_3 were added 0.1 μkat of homoserine kinase, and the reaction mixture was incubated at 37 °C for 16 h. After the pH was adjusted to about 10, the solution was diluted to 50 mL with water and applied to an 80-mL Bio-Rad AG 1X8 anion-exchange column, and **1** was eluted and quantified as described for HSerP (49% yield). Independent determination of the concentration of **1** with the ninhydrin assay (Rosen, 1957) confirmed that only a single hydroxyl group was phosphorylated.

L-*2,3-Methanohomoserine Phosphate* **2**. To 25 mL of 16 mM HEPES, pH 7.6, 160 mM KCl, 8 mM $MgCl_2$, 4.8 mM ATP, 28 mM PEP, 30 mM DL-2,3-methanohomoserine (Bland et al., 1988), and 0.04% NaN_3 were added 3.3 μkat of pyruvate kinase and 0.2 μkat of homoserine kinase, and the solution was incubated at 37 °C for 8 h. After the pH was adjusted to about 2 with HCl,the solution was applied to an 80-mL column of Dowex 50WX8 cation exchanger (100–200 mesh, H^+-form). After extensive washing with water, the column was eluted with a linear gradient of 0–1 M HCl. **2** was detected by measuring the inorganic phosphate liberated by alkaline

phosphatase treatment, pooled, and treated as described above (21% yield).

L-*α-Methylhomoserine Phosphate* **3**. The phosphorylation reaction was performed at pH 7.6 in 20 mL of 10 mM HEPES, 100 mM KCl, 8 mM $MgCl_2$, 5 mM ATP, 18 mM PEP, 35 mM DL-α-methylhomoserine, 0.04% NaN_3, 0.7 μkat of pyruvate kinase, and 0.7 μkat of homoserine kinase. After 6 h at 37 °C the reaction was complete and **3** was purified as described for **1** (42% yield).

L-*2-Amino-5-hydroxy-2,3-methanopentanoic acid 5-O-Phosphate* **4**. The phosphorylation reaction was performed at pH 8.0 in a 20-mL solution of 2.5 mM HEPES, 8 mM $MgCl_2$, 5 mM ATP, 10 mM PEP, 10 mM DL-2-amino-5-hydroxy-2,3-methanopentanoic acid, 0.04% NaN_3, 1.7 μkat of pyruvate kinase, and 1.4 μkat of homoserine kinase. After a 96-h incubation at 37 °C, **4** was purified as described for **1** (10% yield).

Enzyme Purification. Cloning, overexpression, and purification of *E. coli* homoserine kinase and TS will be described elsewhere.

Enzyme Assays. Threonine synthase activity was measured at 37 °C by adding about 0.1 μg of purified TS to a solution of 50 mM HEPES pH 8.0, 100 mM $(NH_4)_2SO_4$, 0.1 mM PLP, 1 mM HSerP, and 24 μg of bovine serum albumin in a total volume of 100 μL. After 10 min the reaction was terminated by the addition of malachite green reagent solution and the inorganic phosphate liberated from HSerP was quantified (Lanzetta et al., 1979).

For inactivation experiments TS was preincubated at 30 °C in 100 μL of 50 mM HEPES, pH 8.0, containing 150 mM KCl and varying concentrations of inhibitor. At predetermined time intervals 10-μL aliquots were transfered to 490 μL of a solution of 50 mM HEPES, pH 8.0, containing 150 mM KCl, 0.1 mM PLP, and 5 mM HSerP. After 15 min of incubation at 37 °C, the reaction was terminated by the addition of 500 μL of double-concentrated malachite green reagent solution and inorganic phosphate was determined (Lanzetta et al., 1979).

Deamination reactions were followed by measuring α-keto acid formation at 340 nm and 37 °C in a NADH-dependent coupled spectrophotometric assay. The reaction mixture contained 100 mM HEPES, pH 8.0, 100 mM $(NH_4)_2SO_4$, 0.1 mM PLP, 0.3 mM NADH, 240 μg bovine serum albumin, 50 μg of lactate dehydrogenase, 150 μg of threonine synthase, and 5–150 mM of substrate in a total volume of 1 mL.

Titration with Inhibitors. Spectrophotometric titration studies were performed such that small amounts of inhibitor in 50 mM potassium phosphate, pH 7.2, containing 150 mM KCl were successively added to homogeneous TS in the same buffer to reach the required inhibitor concentrations. Titration curves were constructed from steady-state absorbance spectra recorded 5 min after each addition of inhibitor and were corrected for changes in volume and for the initial absorbance of the solution before addition of inhibitor.

Spectral Studies. Steady-state absorbance spectra were recorded at 30 °C with a Kontron Uvikon 930 spectrophotometer using an optical path of 1.0 cm and 0.4–2.6 mg/mL homogenous TS in 50 mM potassium phosphate, pH 7.2, containing 150 mM KCl.

Protein Determination. Protein was determined by the method of Bradford (1976) with bovine serum albumin as the standard. For spectral studies or titration experiments the concentration of homogenous TS was calculated on the basis of an extinction coefficient at 280 nm of 34 974 M^{-1} cm^{-1} and a subunit molecular weight of 47 060.

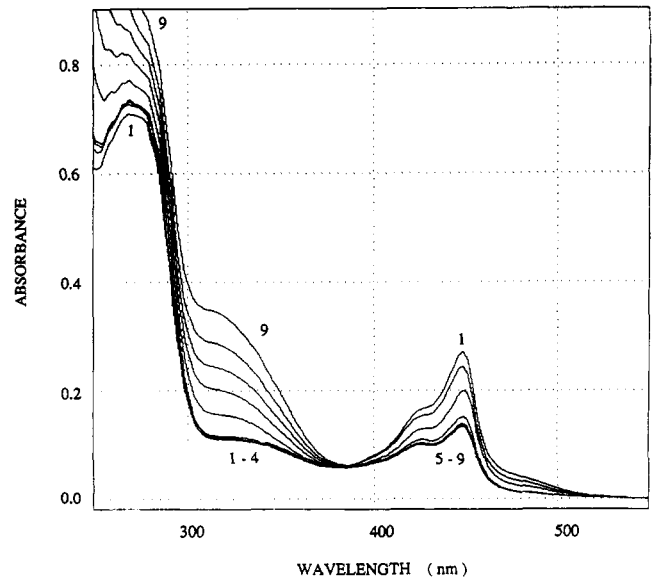


FIGURE 1: Absorbance spectra for the reaction of 20.0 μM threonine synthase with 40 mM L-homoserine phosphate in 50 mM potassium phosphate buffer, pH 7.2, containing 0.15 M KCl. Spectra were collected 0.2 (1), 2.7 (2), 4 (3), 8 (4), 120 (5), 240 (6), 360 (7), 495 (8), and 675 (9) min after mixing.

HPLC Analysis of PLP and PMP. After spectra collection the reaction mixture was concentrated to about 100 μL with a Centricon 10 microconcentrator and heated to 100 °C for 5 min to denature the enzyme. The solution was mixed with 10 μL of 4 N HCl and with 1 volume of the mobile phase consisting of 6 mM sodium 1-heptanesulfonic acid in 67 mM KH_2PO_4, adjusted to pH 2.6 with H_3PO_4. After centrifugation, an aliquot of the supernatant was injected onto a Knauer Lichrosorb RP-18 10-μm 250- × 4-mm column which was eluted isocratically at a flow rate of 1 mL/min. PLP and PMP were detected at 254 nm and had retention times of 5.1 and 7.3 min, respectively.

Analysis of α-Keto Acids. α-Keto acids were quantified by reaction with 2,4-dinitrophenylhydrazine (Friedemann & Haugen, 1943). Individual α-keto acids were identified after derivatization with *o*-phenylenediamine. Derivatives were separated by reversed-phase HPLC on a Merck Hibar RT Lichrospher RP-18 5-μm 250- × 4-mm column in an elution gradient from 10 to 40% acetonitrile in 25 mM MOPS/NaOH, pH 7.1, at 1 mL/min flow rate. Detection of the derivatives was achieved by their fluorescence at 415 nm with excitation at 333 nm (Hayashi et al., 1983; Qureschi, 1977).

Amino Acid Analysis. Amino acids were analyzed after precolumn derivatization with *o*-phthaldialdehyde (Jones & Gilligan, 1983).

RESULTS

Reaction with L-Homoserine Phosphate. L-Homoserine phosphate is an excellent substrate for TS (K_m = 0.5 mM; V = 9.3 μmol min^{-1} mg^{-1} or 440 min^{-1}). In the absence of substrates the spectrum of TS shows absorbance maxima at 333 and 416 nm, beside the protein absorption at 279 nm (pH 7.2). Spectra taken about 10 s after mixing with HSerP show a sharp increase in absorbance at 448 nm and a broad shoulder extending from 460 to 510 nm (Figure 1). Both maxima decrease in intensity with time, and after the absorbance at 448 nm has finally reached a constant value the absorbance in the region of 300–350 nm and below 280 nm, which is characteristic for of α-keto acids, starts to increase slowly. Thus, upon prolonged incubation the threonine formed is slowly

Table 1: Substrates of Threonine Synthase Undergoing Elimination or Half-Transamination Reactions[a]

substrate	product	λ_{max} (nm)	$\epsilon(\lambda_{max})$ (M^{-1} cm^{-1})
L-homoserine phosphate	threonine	448	13000
L-serine	pyruvate	449	34000
L-threonine	α-ketobutyrate	448	12000
L-*allo*-threonine	α-ketobutyrate	453	16000
DL-3-chloroalanine	pyruvate	448	4000
L-*threo*-2-amino-3-chlorobutanoic acid	α-ketobutyrate		
DL-3-hydroxynorvaline	α-ketovalerate		
DL-vinylglycine	threonine	448	6000
L-alanine	PMP	454	10000
L-2-aminobutanoic acid	PMP	455	700
L-homoserine	PMP	455	5000
L-norvaline	PMP		
DL-*O*-methylserine	PMP		
DL-allylglycine	PMP		
DL-3,3,3-trifluoroalanine	PMP	440	22000
DL-2-amino-5-phosphonopentanoic acid	PMP	455	23000

[a] The extinction coefficient of transient chromophores were derived from the highest intensity observed.

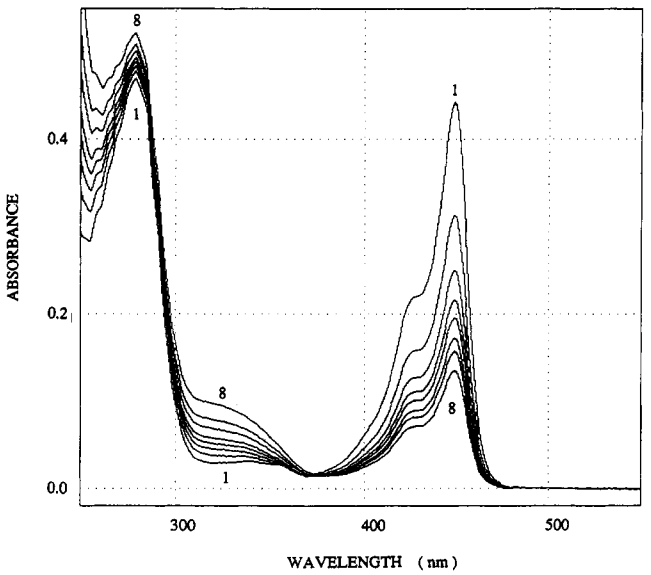


FIGURE 2: Absorbance spectra for the reaction of 13.4 μM threonine synthase with 50 mM L-serine in 50 mM potassium phosphate buffer, pH 7.2, containing 0.15 M KCl. Spectra were collected 0.3 (1), 3 (2), 6 (3), 9 (4), 12 (5), 18 (6), 24 (7), and 36 (8) min after mixing.

deaminated to α-ketobutyrate. When the experiment is repeated at 4 °C or at pH 8.0, identical spectra are observed.

β-Elimination Reactions. In addition to the deamination of L-threonine (see above), TS catalyzes the deamination of L-serine, L-*allo*-threonine, DL-3-chloroalanine, L-*threo*-2-amino-3-chlorobutanoic acid, and 3-hydroxynorvaline (Table 1). Spectra recorded after mixing of TS with L-serine (Figure 2) show a new pronounced maximum at 449 nm. Over a 100-min incubation period the 449-nm peak decreases in intensity with a concomitant increase in short-wavelength absorbance caused by pyruvate, the product of the deamination reaction. D-Amino acids are not used as substrates.

Pyruvate was identified by HPLC analysis as the primary product of serine and 3-chloroalanine deamination. α-Ketobutyrate was found to be the primary product of threonine, *allo*-threonine, and *threo*-2-amino-3-chlorobutanoic acid deamination.

Kinetic constants for the deamination of L-serine were determined as K_m = 150 mM and V = 0.1 μmol min^{-1} mg^{-1} (4.7 min^{-1}). No kinetic constants were determined for the other substrates, since either the reactions are too slow or saturating substrate concentrations could not be achieved.

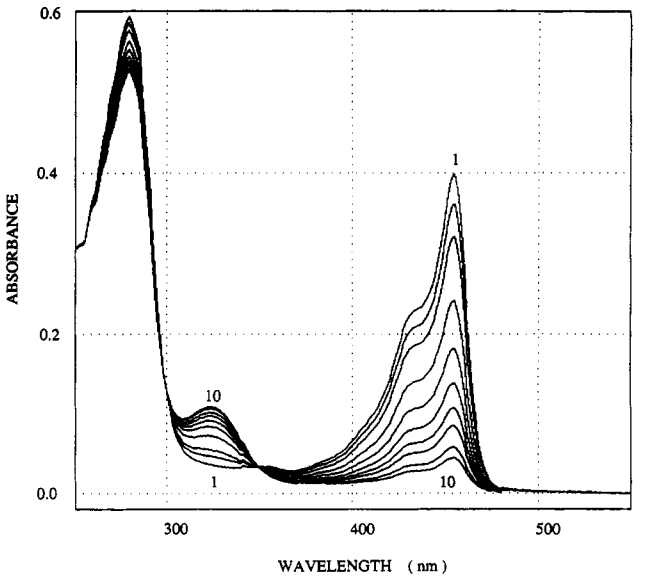


FIGURE 3: Absorbance spectra for the reaction of 15.1 μM threonine synthase with 10 mM DL-2-amino-5hphosphonopentanoic acid in 50 mM potassium phosphate buffer, pH 7.2, containing 0.15 M KCl. Spectra were collected 0.3 (1), 27 (2), 50 (3), 110 (4), 170 (5), 230 (6), 290 (7), 350 (8), 470 (9), and 590 (10) min after mixing.

However, from the rate of absorbance increase in the 300–350-nm region the velocities of the deamination reactions were judged to decrease in the order 3-chloroalanine ≥ serine > *allo*-threonine ≫ threonine ≥ *threo*-2-amino-3-chlorobutanoic acid ≫ 3-hydroxynorvaline.

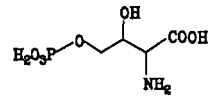
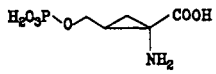
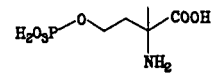
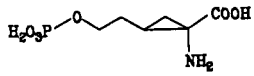
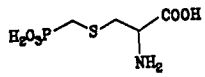
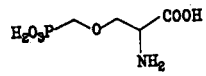
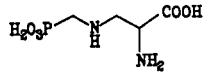
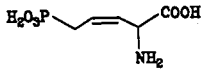
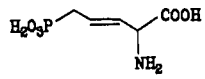
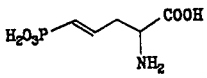
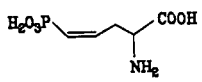
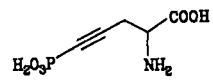
Reaction with DL-*Vinylglycine.* Vinylglycine is a substrate for TS and is rapidly converted to threonine. After mixing of TS with 20 mM vinylglycine, a maximum at 448 nm develops slowly. This is in contrast to the time course observed during substrate turnover and deamination reactions, where the high-wavelength chromophore has already reached its maximum intensity immediately after mixing. After longer incubation, an increase in absorbance in the region of 300–350 nm and below 280 nm, characteristic for α-keto acid formation, is also observed.

Half-Transamination Reactions. In the presence of L-alanine the transient formation of a 454-nm chromophore is observed in the spectrum of TS, paralleled by the rapid formation of a 325-nm chromophore. At the end of the experiment no long-wavelength absorbance remaines visible and denaturation of the enzyme yields all (101%) of the cofactor as PMP. Thus, alanine undergoes a half-transamination reaction.

Besides L-alanine, DL-3,3,3-trifluoroalanine, L-2-aminobutanoic acid, L-norvaline, DL-allylglycine, L-homoserine, DL-*O*-methylserine, and DL-2-amino-5-phosphonopentanoic acid are substrates for the half-transamination reaction (Table 1). However, complete conversion of the cofactor to the PMP form takes up to 24 h.

Addition of 10 mM DL-2-amino-5-phosphonopentanoic acid to TS results in the spectral changes shown in Figure 3. An apparent equilibrium constant K_D = 0.14 mM was determined for the formation of the 455-nm chromophore by titration of TS. However, under standard assay conditions no inhibition of enzyme activity is observed. When TS is subjected to gel filtration immediately after the addition of DL-2-amino-5-phosphonopentanoic acid, the long-wavelength absorbance is lost and the original TS spectrum is restored. Incubation of TS with DL-2-amino-5-phosphonopentanoic acid at pH 8.5 produces the 455-nm chromophore, while incubation at pH 6.0 fails to change the TS spectrum. When each enantiomer

Table 2: Structures, Inhibition Constants, and Absorbance Maxima of Inhibitors of Threonine Synthase[a]

structure	no.	K_i (mM)	k_{inact} (min^{-1})	λ_{max} (nm)	$\epsilon(\lambda_{max})$ (M^{-1} cm^{-1})
	**1**	0.05	*b*		
	**2**	0.01	*b*		
	**3**	*c*			
	**4**	*c*			
	**5**	0.011	*b*	459	47000
	**6**	*c*		449	2000
	**7**	0.057	1.44	450	17000
	**8**	0.10	1.50	482	40000
	**9**	0.40	0.25	462, 500	31000 (462 nm)
	**10**	0.54	*b*	460	30000
	**11**	*c*		462	9000
	**12**	*c*		452	30000

[a] Inhibition constants for compound **7** were derived by measuring the loss of absorbance of the 450-nm chromophore at pH 8.0. All other inhibition constants were derived from enzyme activity measurements. The extinction coefficients of transient chromophores were derived from the highest intensity observed. [b] Competitive inhibition. [c] No inhibition under standard assay conditions and 10 mM inhibitor concentration.

of 2-amino-5-phosphonopentanoic acid is separately tested against TS, only the L-enantiomer produces the effects described above for the racemic mixture. The corresponding D-enantiomer shows no activity at all.

The spectral changes in the presence of DL-3,3,3-trifluoroalanine show a unique time course since the long-wavelength chromophore is not fully developed immediately after mixing but appears slowly, reaching its maximum intensity after about 20 min.

Homoserine Phosphate Analogues. In their study of the interaction of aspartate and aspartate-derived antimetabolites with the enzymes of the threonine biosynthetic pathway, Shames et al. (1984) showed that L-*threo*-3-hydroxyhomoserine was phosphorylated by homoserine kinase to L-*threo*-3-hydroxyhomoserine phosphate **1**, an inhibitor of TS. To further characterize the mode of inhibition, we prepared **1**, together with its analogues **2**, **3**, and **4**. **1** and **2** act as competitive inhibitors of TS, while **3** and **4** do not significantly interfere with homoserinephosphate turnover (Table 2). Both **1** and **2** cause a slight decrease in absorbance of TS at 416 nm and a slight increase at about 300–310 nm, but no long-wavelength chromophore is observed. In the presence of **3** and **4** the spectrum remains unchanged.

2-Amino-3-[(phosphonomethyl)thio-, -oxo-, and -amino]-propanoic Acid. L-2-Amino-3-[(phosphonomethyl)thio]propanoic acid **5** is reported to be a slow-binding inhibitor of TS and it has been shown that the enzyme catalyzes the exchange of the α-proton and the stereospecific exchange of a single β-proton of **5** with solvent-derived deuterium (Ash et al., 1987). In our hands **5** competitively inhibited HSerP turnover (Table 2), but time dependency of the inhibition could not be detected. Immediately after mixing with **5** the spectrum of TS is dominated by a new chromophore at 459 nm, which does not change during incubation times up to 24 h. An apparent dissociation constant of K_D = 15 μM was determined by titration of Ts with **5**, which is close to the K_i value. When TS treated with **5** was denatured, 79% of the cofactor was recovered; 88% as PLP and 12% as PMP.

DL-2-Amino-3-[(phosphonomethyl)oxo]propanoic acid **6**, which is the oxygen analogue of inhibitor **5**, does not inhibit TS (Table 2).

When TS is preincubated with DL-2-amino-3-[(phosphonomethyl)amino]propanoic acid **7** in the absence of HSerP, the enzyme activity decreases as a function of time and inhibitor concentration. Loss of activity follows pseudo-first-order kinetics, but under the experimental conditions used, saturation of the inactivation reaction could not be obtained. The rate constant for inactivation was determined as $k = 1.3 \times 10^3$ M^{-1} s^{-1}.

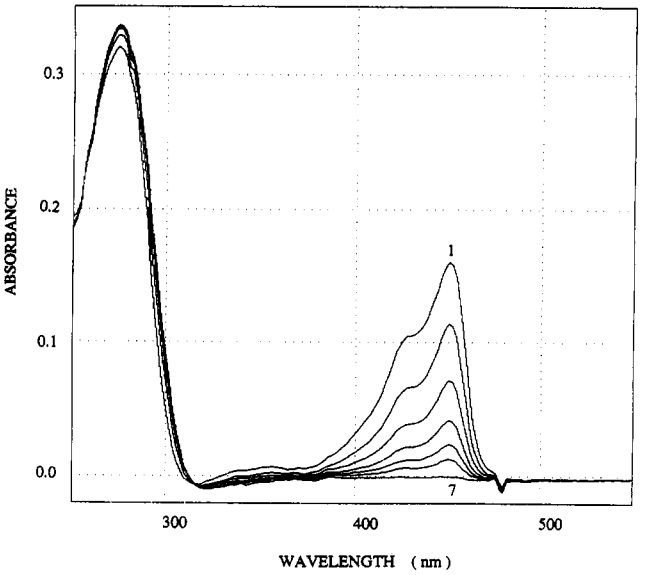


FIGURE 4: Absorbance spectra for the reaction of 9.2 μM threonine synthase with 5 mM DL-amino-3-[(phosphonomethyl)amino]propanoic acid **7** in 50 mM potassium phosphate buffer, pH 7.2, containing 0.15 M KCl. Spectra were collected 0.3 (1), 0.6 (2), 0.9 (3), 1.2 (4), 1.5 (5), 1.8 (6) and 3.0 (7) min after mixing.

Spectra of the enzyme taken 15 s after mixing with 5 mM **7** show a new absorbance maximum at 450 nm (Figure 4). The intensity of this chromophore decreases with time, and after 3 min no absorbance remains at wavelengths longer than 320 nm. Single-wavelength experiments were performed at 450 nm and high inhibitor concentrations to determine the kinetic constants for the decay of this chromophore. Loss of absorbance followed pseudo-first-order and saturation kinetics with $K_i = 57\ \mu M$ and $k_{inact} = 1.44\ min^{-1}$ (Table 2). From these values the rate constant for inactivation at low inhibitor concentration was calculated as $4.2 \times 10^2\ M^{-1}\ s^{-1}$, close to the value obtained by inactivation kinetics.

The data presented here suggest that **7** is an enzyme-activated irreversible inhibitor of TS. For this type of inhibitor an important indicator of potency is the partitioning ratio, the number of turnovers per inactivation event. The partitioning ratio was determined kinetically by titration of TS activity with **7** (Figure 5). Complete inhibition of TS by **7** was achieved at a ratio of 2 mol of racemic inhibitor/mol of enzymic sites. Since it seems that TS does not interact with D-amino acids (see above), one molecule of the L-enantiomer of **7** is sufficient to irreversibly inactivate TS. Thus, turnover of **7** does not take place and the partitioning ratio is 0.

When TS treated with **7** was denatured, only 30% of the cofactor was recovered: 88% as PLP and 12% as PMP. However, a new peak with a retention time differing from those of both PLP and PMP was observed. Although this derivative presumably accounts for the remaining 70% of missing cofactor, an accurate determination of its concentration was not possible due to the lack of an appropriate extinction coefficient.

2-Amino-5-phosphono-3-pentenoic Acids. DL-*Z*-2-Amino-5-phosphono-3-pentenoic acid **8** is an irreversible inhibitor of TS (Table 2). Spectra of TS recorded after mixing with **8** show an increase in absorbance at 482 nm (Figure 6). After the 482-nm chromophore has reached its maximum intensity, the final spectrum does not change during incubation times up to 24 h. Comparison of this spectrum with other TS spectra (see for example Figure 3) reveals that the 482-nm peak is unusually broad at its long-wavelength side. Spectral band-shape analysis (Metzler et al., 1973) revealed that this

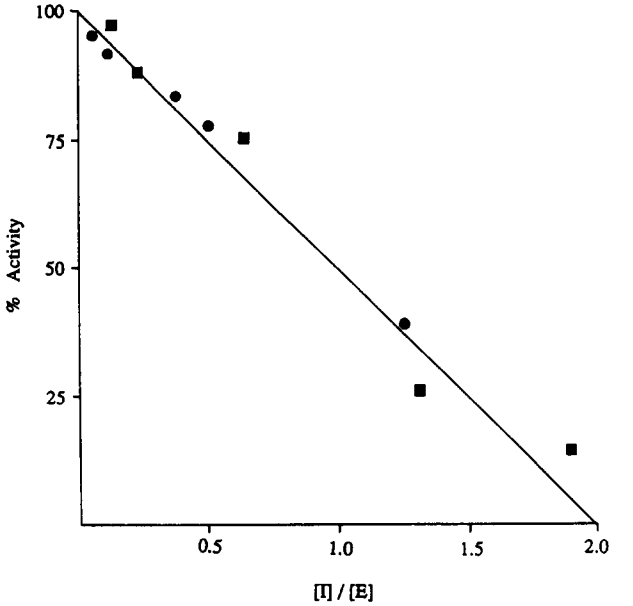


FIGURE 5: Titration of threonine synthase with DL-2-amino-3-[(phosphonomethyl)amino]propanoic acid **7** (○) and DL-*Z*-2-amino-5-phosphono-3-pentenoic acid **8** (■). Threonine synthase (20.0 μM) and **7** (1–25 μM) or threonine synthase (39.25 μM) and **8** (5–75 μM) were preincubated for 2.5 and 5 h at 20 °C, respectively, and aliquots were assayed for enzyme activity.

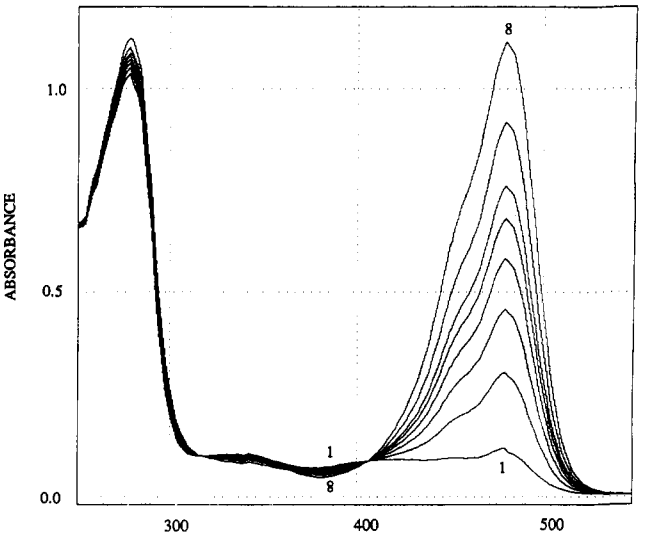


FIGURE 6: Absorbance spectra for the reaction of 29.8 μM threonine synthase with 0.1 mM DL-*Z*-2-amino-5-phosphono-3-pentenoic acid **8** in 50 mM potassium phosphate buffer, pH 7.2, containing 0.15 M KCl. Spectra were collected 0.3 (1), 0.8 (2), 1.4 (3), 2.0 (4), 2.6 (5), 3.2 (6), 4.9 (7), and 11.5 (8) min after mixing.

broadening is caused by the combined absorbance of a major chromophore with an absorption maximum at 482 nm and a minor component with a maximum at about 498 nm. Single-wavelength experiments were performed at 482 nm to determine the concentration dependence and the time constant for formation of this chromophore. The absorbance increase at different concentrations of **8** followed pseudo-first-order kinetics (K_i = 0.33 mM, k_{inact} = 6.3 min^{-1}). When the experiment was performd at pH 7.2, saturation kinetics were not observed and the rate constant for the formation of the 482-nm chromophore was determined to be 44 $M^{-1}\ s^{-1}$ (rate constant for loss of TS activity: 125 $M^{-1}\ s^{-1}$).

When TS, after incubation with **8** to reach the maximum intensity of the 482-nm chromophore, is subjected to gel filtration or extensive dialysis, no change in the absorbance

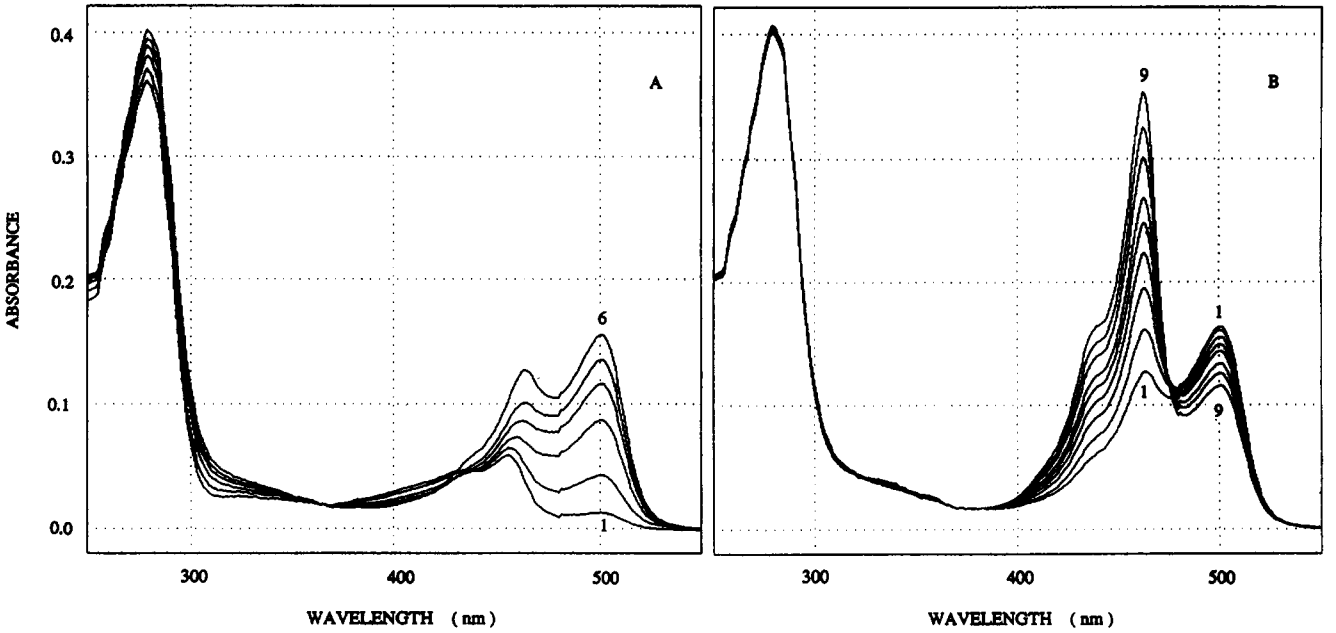


FIGURE 7: Absorbance spectra for the reaction of 10.3 μM threonine synthase with 5 mM DL-*E*-2-amino-5-phosphono-3-pentenoic acid **9** in 50 mM potassium phosphate buffer, pH 7.2, containing 0.15 M KCl. Spectra were collected (panel A) 0.5 (1), 3 (2), 15 (4), 21 (5), and 33 (6) and (panel B) 33 (1), 52 (2), 72 (3), 92 (4), 112 (5), 132 (6), 173 (7), 212 (8), and 283 (9) min after mixing.

spectrum is observed, and the ratio of $\epsilon_{280}/\epsilon_{482}$ remains unchanged.

The stoichiometry of inactivation was determined by titration of TS with **8** (Figure 5). Racemic **8** completely inhibited the activity of TS at a molar ratio of 2 mol of inhibitor/mol of enzyme. Since it seems that TS specifically reacts with L-amino acids, one molecule of the L-enantiomer of **8** is sufficient to inactivate TS. Thus, turnover of **8** does not take place and the partitioning ratio is 0.

Prolonged incubation of inactivated TS with either 100 mM sodium cyanoborohydride or 20 mM sodium borohydride did not change the spectrum. When the inactivated enzyme was denatured by dilution into 6 M guanidine hydrochloride buffered to pH 7.2 with 0.1 M potassium phosphate, the 482-nm absorbance was lost with a half-time of 1.5 min. Mapping of tryptic peptides by reversed-phase HPLC did not reveal any differences between inactivated TS and an untreated control. When TS treated with **8** was denatured, 67% of the cofactor was recovered: 87% as PLP and 13% as PMP.

DL-*E*-2-Amino-5-phosphono-3-pentenoic acid **9** is an irreversible inhibitor of TS, but in comparison with **8**, significantly higher inhibitor concentrations and longer preincubation times are required for inactivation (Table 2). In the presence of **9** there is a fast increase in absorbance of TS at 500 nm, followed by a slower absorbance increase at 462 nm (Figure 7). While the 500-nm chromophore reaches a maximum and then starts to decline, the 462-nm chromophore constantly increases in intensity. The time course for increase and decrease of the 500-nm chromophore followed first-order kinetics with rate constants of 0.06 min^{-1} and 0.002 min^{-1}, respectively. The intensity increase of the 462-nm chromophore could not be described by first-order kinetics.

Gel filtration of TS incubated with 5 mM of **9** for 3 h does not induce a change of the absorption spectrum. When the inactivated enzyme is denatured, both the 462- and 500-nm absorbances are lost immediately.

2-Amino-5-phosphono-4-pentenoic Acids. DL-*E*- and DL-*Z*-2-amino-5-phosphono-4-pentenoic acid **10** and **11** were synthesized as analogues of **8** and **9** to study the effects of a shift of the double bond from the 3- to the 4-position on the ability of these compounds to act as inhibitors of TS. While **10** is a reversible competitive inhibitor, **11** does not inhibit the enzyme (Table 2). Immediately after mixing of TS with **10**, a new absorbance maximum at 460 nm is observed, which does not undergo significant changes, even upon prolonged incubation. An apparent equilibrium constant for its formation of 0.23 mM was obtained by titration. When TS treated with **10** was denatured, only 16% of the cofactor was recovered: 61% as PLP and 39% as PMP. Mixing of TS with 10 mM **11** induces the very slow development of a chromophore at 462 nm.

DL-*2-Amino-5-phosphono-4-pentynoic Acid.* Acetylenic amino acids are well-known inactivators of various PLP-dependent enzymes (Silverman, 1988). To take advantage of this observation, we designed **12** as a potential irreversible inhibitor. However, **12** failed to inhibit TS activity even at high concentrations (Table 2). Mixing of TS with **12** results in the immediate formation of a new absorbance maximum at 452 nm (Figure 8), whose intensity decreases with time and is paralleled by the formation of a new chromophore at 331 nm. However, the intensity of this chromophore is too high to be caused by PMP (see Figure 3). An equilibrium constant for the formation of the 452-nm chromophore of 0.38 mM was determined by titration experiments. When TS treated with **12** was denatured, 85% of the cofactor was recovered: 51% as PLP and 49% as PMP.

DISCUSSION

Reaction with Homoserine Phosphate and Deamination Reactions. Absorption maxima of 400–430 and 310–340 nm for the protonated and unprotonated forms, respectively, for the Schiff base formed between PLP and an ϵ-NH_2 group of a lysyl residue have been reported (Morino & Snell, 1967). Since the maximum at 333 and 416 nm in the spectrum of the purified TS are pH-dependent, they can be assigned to the unprotonated and protonated forms of the Schiff base. Upon the addition of HSerP, serine, 3-chloroalanine, threonine, or *allo*-threonine to TS, the most prominent change in the spectrum was the formation of a new chromophore in 448–453-nm range (Figures 1 and 2). This chromophore is assigned to a quinonoidal reaction intermediate on the basis of the following criteria (Davis & Metzler, 1972; Karube &

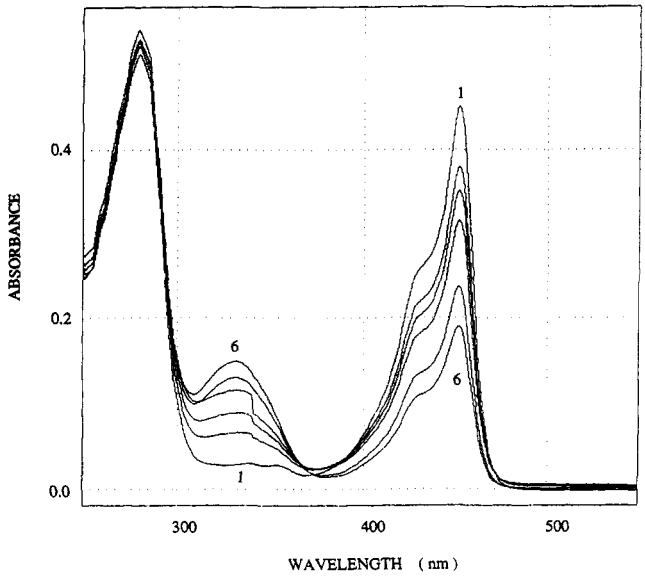


FIGURE 8: Absorbance spectra for the reaction of 10.3 μM threonine synthase with 5 mM DL-2-amino-5-phosphono-4-pentynoic acid **12** in 50 mM potassium phosphate buffer, pH 7.2, containing 0.15 M KCl. Spectra were collected 0.3 (1), 57 (2), 95 (3), 145 (4), 250 (5), and 335 (6) min after mixing.

Matsushima, 1977; Kallen et al., 1985): (a) The absorption spectra of quinonoidal species are characterized by long-wavelength bands that are much narrower than other PLP absorption bands; (b) there is at least one prominent shoulder of about one-third the amplitude of the major peak located about 27 nm from the absorption maximum on the high-energy side; (c) their extinction coefficients are estimated to range from 20 000 to 50 000 M^{-1} cm^{-1}.

Thus, for deamination reactions the 448–453-nm chromophore is caused by the quinonoid **V** (Scheme 1). Accumulation of **V** implies that elimination of the β-substituent is rate-limiting, as it is in the tryptophanase reaction (Morino & Snell, 1967). The velocity of the deamination reaction was found to be highly dependent on the length of the carbon chain of the corresponding substrate. The highest reaction rate was obtained with three-carbon substrates, i.e., serine and 3-chloroalanine. However, deamination is at least 100-fold slower than HSerP turnover. Four-carbon substrates are deaminated much more slowly, and increasing the chain length to five carbon atoms gave an extremely slow reaction rate. The deamination of *allo*-threonine was much faster than the deamination of threonine. This may be explained if one assumes *anti*-elimination. Deamination of *threo*-2-amino-3-chlorobutanoic acid did not produce any long-wavelength chromophore, indicating that α-proton abstraction was slower than chloride elimination.

During HSerP turnover the 448-nm chromophore is either caused by intermediate **II** or **V**, or both (Scheme 1). Intermediate **III** must be excluded, since the 448-nm chromophore is also observed during the deamination of serine and 3-chloroalanine, which both lack a γ-carbon and are thus unable to form **III**. Discrimination between **II** or **V** as the major absorbing species in the initial phase of HSerP turnover is not possible on the basis of spectral data. At the end of longer incubation periods the 448-nm absorbance is most likely caused by **V**, since α-ketobutyrate is thought to start accumulating due to the buildup of threonine in the incubation mixture. However, accumulation of the 448-nm chromophore proves that either elimination of inorganic phosphate, and therefore accumulation of **II**, or the final step of the reaction, i.e., conversion of the quinonoid **V** to product, is rate-limiting.

Reaction with Vinylglycine. The conversion of vinylglycine to threonine proves that the pyridoxal *p*-quinonoid of vinylglycine (**III**, Scheme 1) is an intermediate in the TS reaction. On the basis of these results the time course of the spectral changes in the presence of vinylglycine can be interpreted as follows. Turnover occurs without the appearance of a long-wavelength chromophore. Upon longer incubation, part of the threonine formed is deaminated to α-ketobutyrate. In the course of this deamination reaction intermediate **V** accumulates, reflected by the increase in absorbance at 448 nm. This reaction sequence would explain the slow onset of the appearance of the 448-nm chromophore. The initial absence of any long-wavelength chromophore argues against the transient accumulation of the pyridoxal *p*-quinonoid of vinylglycine.

Half-Transamination Reactions. Incubation of TS with alanine, 2-aminobutanoic acid, norvaline, allylglycine, or 2-amino-5-phosphonopentanoic acid, i.e., amino acids which do not possess a leaving group in the β-position, produced an absorption maximum at 325 nm. Upon denaturation of the enzyme, the cofactor was recovered as PMP. The likely reaction mechanism (Scheme 1) is analogous to the pathway used by aminotransferases for the first half of enzymatic transamination reactions. The transient accumulation of the quinonoid intermediate **IX** causes the absorbance maximum at about 450 nm. Subsequent protonation of **IX** at C-4′ of the coenzyme yields **X**, and after hydrolysis PMP and stoichiometric amounts of the corresponding α-keto acid. Note that besides TS many other PLP-dependent enzymes carry out transamination as a side reaction (Miles, 1985).

The velocity of the half-transamination reaction was highest with alanine and decreased with increasing length of the amino acid side chain. This decrease in velocity was paralleled by a decrease in the intensity of the transiently formed long-wavelength chromophore. However, this does not hold for 2-amino-5-phosphonopentanoic acid, an isosteric analogue of HSerP. Although it was, as expected, a slow substrate, it gave a long-wavelength chromophore of highest intensity. The doubly negatively charged phosphonate, in correct spatial orientation, apparently introduces favorable interactions with the active site, thereby greatly accelerating binding and/or α-proton abstraction. α-Proton abstraction was shown to be rapidly reversible by the loss of the long-wavelength chromophore upon gel filtration. The existence of an isosbestic point between the 455- and 325-nm peaks (Figure 3) indicates that the quinoid intermediate is directly converted into an intermediate with tetrahedral geometry at C-4′ of PLP, in accordance with the proposed reaction sequence (Scheme 1).

Interestingly, incubation of TS with 3,3,3-trifluoroalanine did not lead to enzymic inactivation, as described for several PLP-dependent enzymes catalyzing β-elimination reactions (Silverman, 1988). Neither was inactivatioon observed upon incubation of TS with 3-chloroalanine, a well-known mechanism-based irreversible inhibitor of various PLP-dependent transaminases and racemases (Silverman, 1988). For the latter two compounds the postulated inactivation mechanism is Michael addition of an active-site nucleophile to the PLP-bound enamine intermediate that is formed by halide elimination. Therefore, we conclude either that a properly orientated nucleophile is unavailable in the active site or that the short lifetime of the enamine intermediate prevents inactivation.

In summary, TS catalyzes not only the synthesis of threonine from HSerP but, depending on the nature of the substrate, also deamination and half-transamination reactions. In all of these reaction pathways the transient appearance of a

chromophore in the 440–455-nm region has been observed, presumably due to the absorbance of the corresponding pyridoxaldimine *p*-quinonoid. Thus, stabilization of a kinetically competent quinonoidal intermediate is a prominent feature in TS catalysis.

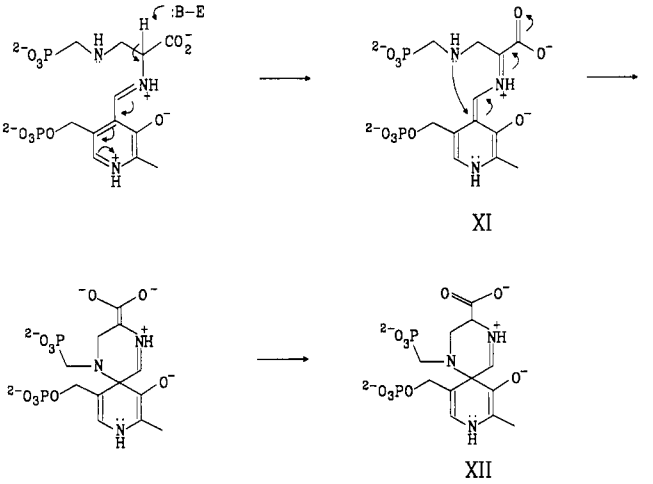
The absorbance maxima observed in the 440–455-nm range are thought to be caused by the same type of reaction intermediate, i.e., **II**, **V**, or **IX** (Scheme 1). The different location of these maxima may simply indicate a change in the environment of the quinonoid complex induced by the substituent of the bound amino acid. For example, it has been shown for tryptophan synthase that the maxima of quinonoid absorption bands are very sensitive to substituents separated by a methylene group from the highly conjugated π-system. Absorption maxima ranging from 450 nm (CN^-) to 464 nm (β-mercaptoethanol) to 476 nm (indole) and 494 nm (2,3-dihydro-L-tryptophan) have been observed (Brzovic et al., 1992).

Homoserine Phosphate Analogues. Compound **1** was a competitive inhibitor of TS with a K_i value 10-fold lower than the K_m of HSerP. The absence of any long-wavelength chromophore in the presence of **1** suggests that α-proton abstraction does not occur. Compounds **2**, **3**, and **4** do not possess an α-proton and therefore were not expected to be substrates or inhibitors of TS. To our surprise compound **2** did competitively inhibit TS. Since **3** was not an inhibitor but has the same chain length as **2**, it must be concluded that the conformational bias of the cyclopropane ring is of major importance for binding. The lack of activity by **4** indicates that the correct length of the carbon chain of a putative inhibitor is an essential prerequisite for activity.

2-Amino-2-[(phosphonomethyl)thio- and -amino]propanoic Acid. Compound **5** was reported to be a slow-binding inhibitor of TS (Ash et al., 1987). We were able to confirm inhibition but could not detect any time dependence. In our hands **5** was a reversible competitive inhibitor. In the presence of **5** the spectrum of TS was dominated by an intense chromophore at 459 nm, which did not change upon prolonged incubation. Therefore, neither deamination nor half-transamination occurs. Formation of the external aldimine and α-proton abstraction are fast reactions and the resulting quinonoidal species causes the 459-nm maximum. Since it has been shown that TS catalyzes the stereospecific exchange of a single β-proton of **5** with solvent-derived deuterium (Ash et al., 1987), the catalytic sequence proceeds one step further. However, the high intensity of the 459-nm chromophore and its stability proves that the equilibrium for β-proton abstraction lies far on the side of the quinonoidal intermediate.

Compound **7** was a time-dependent inhibitor of TS. The inactivation reaction was saturable and proceeded with the initial formation of a 450-nm chromophore, which rapidly decomposed, and finally gave an enzyme species devoid of any absorbance at wavelengths longer than 320 nm. Turnover of **7** did not take place and one inhibitor molecule was sufficient to inactivate TS. A plausible inactivation mechanism has to explain the following observations: (i) At low inhibitor concentrations the 450-nm chromophore disappeared with a rate constant which is approximately equivalent to the value measured for loss of enzyme activity. Thus, the pyridoxaldimine *p*-quinonoid of **7** (**XI**, Scheme 2) is an intermediate in the inactivation process. (ii) The inactivated enzyme does not absorb above 320 nm and therefore there is no additional double bond in conjugation with the pyridine ring of PLP. (iii) A modified cofactor was released upon denaturation of the inactivated enzyme. Thus, **7** is transformed by TS into a species, which does not act as a Michael acceptor for an active-site nucleophile to give a covalently modified enzyme (Silverman, 1988), or if it does, it does so reversibly.

Scheme 2: Mechanism of Inactivation of Threonine Synthase by DL-2-Amino-3-[(phosphonomethyl)amino]-propanoic Acid **7**



Formation of covalently modified PLP is characteristic of inactivation by the so-called enamine mechanism (Likos et al., 1982; Ueno et al., 1982). However, the absorption maxima of these modified cofactors are in the range of 330–336 nm, sometimes shifting to 455–458 nm upon prolonged incubation (Bhattcharjee & Snell, 1990; Bolkenius et al., 1990; Likos et al., 1982; Ueno et al., 1982). We are not aware of examples where this mechanism gives products with $\lambda_{max} < 320$ nm.

An inactivation mechanism involving β-elimination of (aminomethyl)phosphonic acid from **XI** (Scheme 2) seems unlikely. β-Elimination would yield the PLP derivative of aminoacrylate, an intermediate that is also formed upon deamination of serine or 3-chloroalanine. In these cases pyruvates formation is observed and no inactivation occurs.

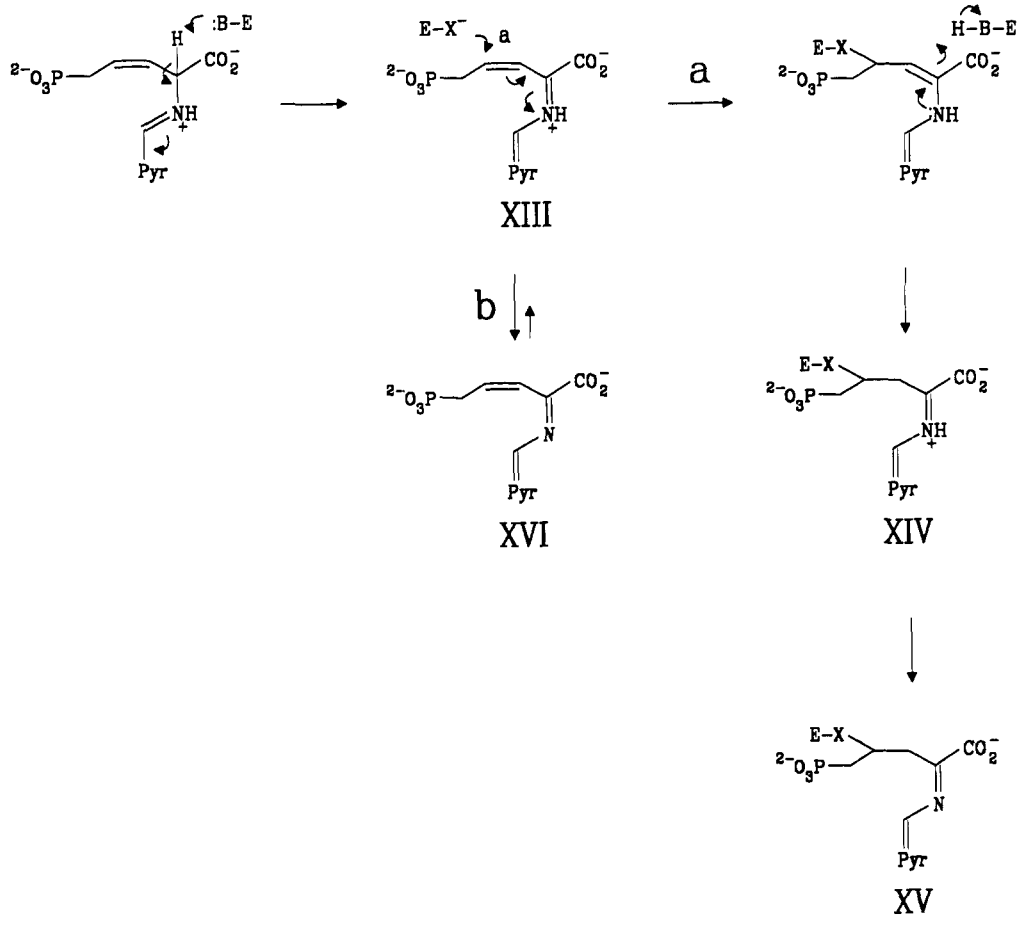
Therefore we propose the reaction mechanism shown in Scheme 2. After formation of the quinonoidal intermediate **XI** (λ_{max} = 450 nm), the free electron pair of the side-chain nitrogen attacks C-4 of the pyridine ring to give the spirocyclic PLP derivative **XII**, which does not have conjugated double bonds and therefore is not expected to absorb at wavelengths longer than 320 nm.

2-Amino-5-phosphono-3-pentenoic Acids. *Z*-2-Amino-5-phosphono-3-pentenoic acid **8** is the unusual amino acid found in all members of the di- and tripeptide antibiotics of the rhizocticin group, produced by *Bacillus subtilis* (Loeffler et al., 1990; Kugler et al., 1990), and of the plumbemicines, produced by *Streptomyces plumbeus* (Park et al., 1976, 1977). Both rhizocticines and plumbemicines are thought to interfere with threonine or threonine-related metabolism, most likely due to inhibition of TS by **8**, which is liberated by peptidases from these antibiotics after uptake into the target cell.

Incubation of TS with **8** resulted in the time-dependent loss of enzyme activity, which was paralleled by the formation of a new chromophore at 482 nm and could not be reversed by gel filtration or dialysis against external PLP. Turnover of **8** did not take place and one inhibitor molecule was sufficient to inactivate each subunit of TS.

The quinonoidal 482-nm chromophore formed concomitant with enzyme inactivation was unusually broad on its long-wavelength side, probably due to the presence of a second chromophore of lower intensity with an absorbance maximum at about 498 nm. Since the 482-nm peak did not show any deformation on the long-wavelength side during its formation, nor splitting upon prolonged incubation, it probably does not

Scheme 3: Mechanism of Inactivation of Threonine Synthase by DL-*Z*-2-Amino-5-phosphono-3-pentenoic Acid **8** and DL-*E*-2-Amino-5-phosphono-3-pentenoic Acid **9**[a]



[a] The carbon–carbon bonds are not meant to imply any specific stereochemistry.

represent two independent chemical species. Instead, broadening may be caused by the existence of two rapidly equilibrating intermediates.

Since unmodified PLP was obtained upon denaturation of the inactivated enzyme, inactivation by the enamine mechanism can be excluded. Another possible inactivation mechanism (Scheme 3a) involves the formation of the quinonoidal intermediate **XIII**, followed by attack of an active-site nucleophile to give a covalently modified inactive enzyme. Upon hydrolysis both, **XIV** and **XV**, would be converted to the inhibitor covalently bound to the TS active site and free PLP. This mechanism agrees well with those postulated for the inactivation of various other PLP-dependent enzymes (Silverman, 1988) and would be sufficient to explain the observed irreversible enzyme inactivation. However, this mechanism seems unlikely to us, since none of our other experiments are indicative of the presence of an active-site nucleophile.

Therefore, we suggest an alternative mechanism (Scheme 3b), which does not involve covalent modification of the enzyme, but establishment of an equilibrium between **XIII** (λ_{max} = 498 nm) and **XVI** (λ_{max} = 482 nm). Both intermediates could be highly stabilized by TS due to their structural similarity to the "transition state" occurring during HSerP turnover, i.e., halfway between **II** and **III** (Scheme 1), with the β-proton already abstracted but the γ-substituent not yet eliminated. Binding of **8** and formation of the quinonoidal intermediates is assumed to be rapid, with the equilibrium between the quinonoidal intermediates on the side of **XVI**. The back-reaction to free enzyme and free **8** is assumed to be so slow that it appears irreversible on the time scale of our experiments. Stabilization of the enzyme–inhibitor complex might be supported by a conformational change of the enzyme, since our attempts to reduce the inactivated enzyme with borohydride failed, indicating that borohydride did not have access to the active site.

Isolation of a tryptic peptide containing the modified nucleophile would support mechanism a, which is characterized by covalent modification of the enzyme. We could not verify the existence of such a modified peptide in tryptic peptide maps obtained by reversed-phase HPLC. However, due to the lack of availability of radiolabeled **8**, this experimental result is not sufficient to rule out mechanism a.

Incubation of TS with **9** also resulted in a time-dependent and saturable loss of enzyme activity. However, the inactivation reaction proceeded more slowly and produced completely different spectral changes, characterized by the transient formation of a 500-nm chromophore and a final maximum at 462 nm. Identity of the 500-nm chromophore with **XIII** (Scheme 3) is assumed. The 462-nm absorbance maximum of the second chromophore strongly suggests a pyridoxaldimine *p*-quinonoidal intermediate, i.e., the β,γ-double bond of the amino acid side chain has apparently been lost during enzyme inactivation. Inactivation (Scheme 3a) might be due to the slow attack of an active-site nucleophile on the β,γ-double bond of transiently accumulating **XIII** to give the covalently modified inactive enzyme **XIV** (λ_{max} = 462 nm). This mechanism would account for the irreversible loss of enzyme activity and would explain the observed spectral changes. However, we are well aware of the unsatisfactory nature of this explanation, because it is difficult to see why only **9** should act as a Michael-type acceptor for an active-site nucleophile. Furthermore, it does not explain why **XIV**

accumulates and does not follow the normal catalytic sequence to yield free PLP and a covalently modified enzyme absorbing at about 416 nm. However, it is remarkable the extent to which cis–trans geometry alters the kinetic course of the inactivation reaction and the nature of intermediates and products.

2-Amino-5-phosphono-4-pentenoic Acids. Both **10** and **11** did not show time-dependent enzyme inactivation. Thus, the shift of the double bond from the 3- to the 4-position altered the type of inhibition. **10** was a weak competitive inhibitor of TS and the formation of a 462-nm chromophore suggests that α-proton abstraction and formation of the corresponding quinonoidal intermediate occurs. We have no explanation for the recovery of only 16% of the cofactor upon denaturation of the inhibited enzyme.

2-Amino-5-phosphono-4-pentynoic Acid. **12** did not inhibit TS activity. However the spectral changes induced indicate that **12** binds to the active site and undergoes α-proton abstraction to form the corresponding pyridoxaldimine *p*-quinonoid (λ_{max} = 452 nm). The 452-nm chromophore undergoes a slow transformation to a species with λ_{max} = 331 nm, but the exact nature of this transformation is unclear. Several PLP-dependent enzymes are inactivated by acetylenic substrate analogues after rearrangement of the acetylene to a reactive allene (Silverman, 1988). If one assumes an analogous mechanism for the reaction of TS with **12**, it is of minor importance, since it is too slow for efficient inhibition.

In this report we have presented a wealth of kinetic and spectral data for the reaction of TS with substrates and inhibitors and proposed reaction mechanisms consistent with these observations. However, (i) the elucidation of the structure of the modified cofactor formed upon reaction with **7**, and (ii) the assignment of the three distinct chromophores observed during inactivation of TS with the cis–trans isomers **8** and **9** to chemically distinct reaction intermediates are still required to fully elucidate these reaction pathways.

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REFERENCES

Allgeier, H., Angst, C., Bold G., Duthaler, R., Heckendorn, R., & Togni, A. (1989) European Patent 302 826.

Angst, C., Brundish, D. E., Dingwall, J. G., & Fagg, G. E. (1987) European Patent 233 145.

Ash, D. E., Farrington, G. K., Kumar, A., Ewaskiewicz, J. E., Shames, S. L., & Welder, F. C. (1987) *Fed. Proc. 46*, 2070.

Bhattcharjee, M. K., & Snell, E. E. (1990) *J. Biol. Chem. 265*, 6664-6668.

Bland, J., Shah, A., Bortolussi, A., & Stammer, C. H. (1988) *J. Org. Chem. 53*, 992–995.

Bolkenius, F. N., Knödgen, B., & Seiler, N. (1990) *Biochem. J. 268*, 409–414.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Brzovic, P. S., Kayastha, A. M., Miles, E. W., & Dunn, M. F. (1992) *Biochemistry 31*, 1180–1190.

Davis, L., & Metzler, D. E. (1972) in *Enzymes, 3rd Ed.* (Boyer, P. D., Ed.) Vol. 7, pp 33–74, Academic Press, New York.

Flavin, M., & Kono, T. (1960) *J. Biol. Chem. 234*, 1109–1111.

Flavin, M., & Slaughter, C. (1960) *J. Biol. Chem. 235*, 1112–1118.

Friedemann, T. E., & Haugen, G. E. (1943) *J. Biol. Chem. 147*, 415–442.

Fuganti, C. (1979) *J. Chem. Soc., Chem. Commun.* 337-339.

Hamel, E. E., & Painter, E. P. (1953) *J. Am. Chem. Soc. 75*, 1362–1368.

Harde, C., Neff, K. H., Nordhoff, E., Gerbling, K. P., Laber, B., & Pohlenz, H. D. (1994) *Bioorg. Med. Chem. Lett. 4*, 273–278.

Hayashi, T., Tsuchiya, H., & Naruse, H. (1983) *J. Chromatogr. 273*, 245–252.

Jones, B. N., & Gilligan, J. P. (1983) *J. Chromatogr. 266*, 471–482.

Kallen, R. G., Korpela, T., Martell, A. E., Matsushima, Y., Metzler, D. E., Morozov, Y. V., Ralston, I. M., Savin, F. A., Torchinsky, Y. M., & Ueno, H. (1985) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) pp 37–108, Wiley, New York.

Karube, Y., & Matsushima, Y. (1977) *Chem. Pharm. Bull. 245*, 2568–2575.

Kugler, M., Loeffler, W., Rapp, C., Kern, A., & Jung, G. (1990) *Arch. Microbiol. 153*, 276–281.

Lamprecht, W., & Fritze, H. (1984) in *Methods of Enzymatic Analysis* (Bergmeyer, H., Ed.) 3rd ed. Vol. 6, pp 570–577, Verlag Chemie, Weinheim, Germany.

Likos, J. J., Ueno, H., Feldhaus, R. W., & Metzler, D. E. (1982) *Biochemistry 21*, 4377–4386.

Loeffler, W., Katzer, W., Kremer, S., Kugler, M., Petersen, F., Jung, G., Rapp, C., & Tschen, J. S. M. (1990) *Forum Mikrobiol. 13*, 156–163.

Metzler, D. E., Harris, C. M., Johnson, R. J., Siano, D. B., & Thomson, J. A. (1973) *Biochemistry 12*, 5377–5392.

Miles, E. W. (1985) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) pp 470–500, Wiley, New York.

Morino, Y., & Snell, E. E. (1967) *J. Biol. Chem. 242*, 2800–2809.

Nachev, I. A. (1988) *Tetrahedron 44*, 1511–1522.

Park, B. K., Hirota, A., & Saki, H. (1976) *Agric. Biol. Chem. 40*, 1905–1906.

Park, B. K., Hirota, A., & Saki, H. (1977) *Agric. Biol. Chem. 41*, 573–579.

Qureschi, G. A. (1977) *J. Chromatogr. 400*, 91–99.

Rosen, H. (1957) *Arch. Biochem. Biophys. 67*, 10–15.

Shames, S. L., Ash, D. E., Wedler, F. C., & Villafranca, J. J. (1984) *J. Biol. Chem. 259*, 15331–15339.

Silverman, R. (1988) *Mechanism-Based Enzyme Inactivation; Chemistry and Enzymology*, Vol. 1., pp 15–53 and Vol. 2, pp 157–185, CRC Press, Boca Raton, FL.

Ueno, H., Likos, J. J., & Metzler, D. E. (1982) *Biochemistry 21*, 4387–4393.