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Inhibition of Thiaminase I from *Bacillus thiaminolyticus*. Evidence Supporting a Covalent 1,6-Dihydropyrimidinyl-Enzyme Intermediate†

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ABSTRACT: Thiaminase I from *Bacillus thiaminolyticus* strain Matsukawa et Misawa is completely and irreversibly inhibited by treatment with 4-amino-6-chloro-2-methylpyrimidine. Inhibition is a time-dependent first-order process, exhibiting a half-time of 4 h at an inhibitor concentration of 5 mM. A specific active-site-directed inactivation is supported by protection of the enzymatic activity in the presence of the substrates thiamin and quinoline as well as by the observation that a stoichiometric amount of inorganic chloride is released during inactivation. 4-Amino-5-(anilinomethyl)-6-chloro-2-methylpyrimidine, which resembles the structure of the product of base exchange of thiamin with aniline, inactivates thiaminase approximately 2 orders of magnitude faster. Inactivation is again complete and irreversible and is a time-dependent first-order process, in this case exhibiting saturation at low inhibitor concentrations (K_I = 96 μM). Enzyme inactivation can be explained as the result of displacement of chloride from the chloropyrimidine by a nucleophile at the enzyme active site. The inactivation suggests that the Zoltewicz–Kauffman model of bisulfite-catalyzed thiamin cleavage [Zoltewicz, J. A., & Kauffman, G. M. (1977) *J. Am. Chem. Soc. 99*, 3134–3142], which calls for the reversible nucleophilic addition of catalyst across the 1,6 double bond of thiamin's pyrimidine ring, may be applicable to thiaminase as well.

Thiaminases are enzymes that destroy thiamin by cleaving the vitamin between the pyrimidinylmethyl group and the thiazole (Fujita, 1954; Murata, 1965, 1982). Thiaminase I (EC 2.5.1.2) catalyzes a base exchange reaction in which the (4-amino-2-methyl-5-pyrimidinyl)methyl group of thiamin is transferred to any one of a variety of organic nucleophiles, e.g., aniline, quinoline, pyridine, and cysteine. A related activity, thiaminase II, catalyzes the transfer of the pyrimidinylmethyl specifically to water. Scheme I shows the reaction catalyzed by thiaminase I between thiamin and aniline.

Thiaminase I occurs in such diverse organisms as ferns, fish, mollusks, crustacea, and microorganisms (Fujita, 1954; Murata, 1965, 1982). Although the biological function of the enzyme is unknown, it is of some veterinary importance, as it has been demonstrated that animals fed a diet containing excessive quantities of the enzyme develop the neurological symptoms of thiamin deficiency (Green et al., 1941; Woolley, 1941). Production of thiaminase by ruminal microorganisms has been suggested to be the cause of certain thiamin deficiencies in livestock (Edwin & Jackson, 1970; Edwin et al.,

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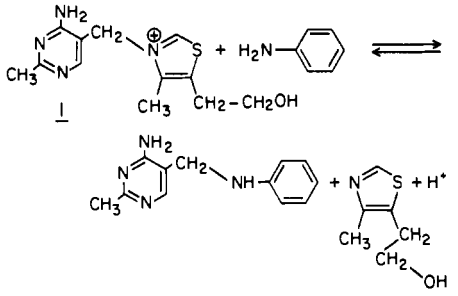
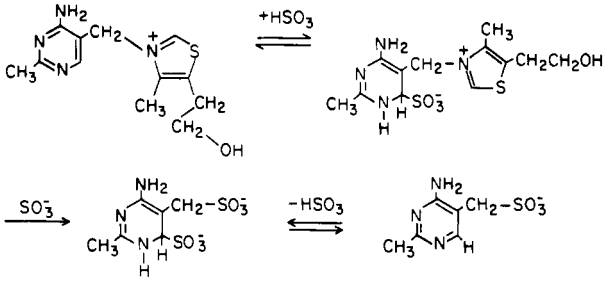
Scheme I



Scheme II



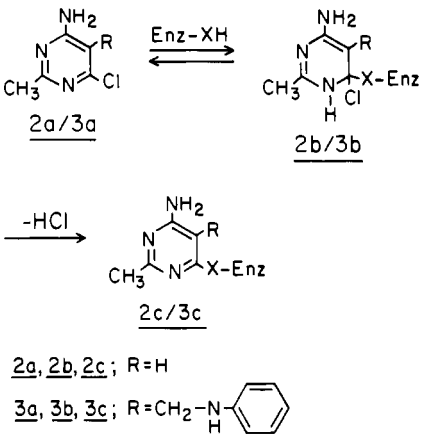
1978). One such organism, *Bacillus thiaminolyticus*, has also been isolated from the human large intestine (Matsukawa & Misawa, 1949), but the relation of this organism to any human thiamin deficiency or other pathology has not been firmly established (Duffy et al., 1981).

Despite interest in this enzyme over a period of 40 years, little is known about its mechanism. Kinetic investigations on thiaminase I from fish, mollusks, and *B. thiaminolyticus* support a double-displacement mechanism involving a pyrimidinyl-enzyme intermediate (Mazrimas et al., 1963; Lienhard, 1970; Puzach et al., 1984). The structure of this intermediate, however, is unknown.

A simple organic chemical model for the base exchange catalyzed by thiaminase I is available in the cleavage of thiamin catalyzed by bisulfite (HSO_3^-) ion (Leichter & Joslyn, 1969). This reaction has been more thoroughly investigated, and a mechanism has been proposed by Zoltewicz and Kauffman (Scheme II). The mechanism postulates nucleophilic addition of bisulfite across the 1,6 double bond of the pyrimidine ring **1**, creating a 1,6-dihydropyrimidine intermediate. Substitution at the benzylic methylene is envisioned to be the result of elimination of the thiazole to form an α,β-unsaturated imine, followed by readdition of sulfite or another competing nucleophile. Reversal of the formation of the 1,6-dihydropyrimidine completes the process (Zoltewicz & Kauffman, 1977; Doerge & Ingraham, 1980). It was suggested that this mechanism might apply to thiaminase I as well, with an as yet unidentified enzyme-bound nucleophile substituting for the bisulfite anion.

If such an addition–elimination mechanism is indeed applicable to thiaminase, 6-chloropyrimidines **2a**/**3a** (Scheme III) will react with the putative catalytic nucleophile to form an analogous intermediate **2b**/**3b** which could then revert to **2a**/**3a** or eliminate chloride irreversibly to give the stable enzyme adduct **2c**/**3c**. Thus, we predict that 4-amino-6-chloro-2-methylpyrimidine (**2a**) and 4-amino-5-(anilinomethyl)-6-chloro-2-methylpyrimidine (**3a**) would be mechanism-based irreversible inhibitors of thiaminase I. Here we report evidence that confirms this prediction.

Scheme III



Experimental Procedures

Materials. All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification except as noted. Buffers were prepared from house-distilled water passed through a demineralization unit (Continental Demineralizer). Prepared thin-layer chromatography (TLC) plates were purchased from Analtech (Newark, DE; silica gel GHLF, 2-mm layer).

General Methods. Spectrophotometric determinations were performed on a Varian Cary 219 spectrophotometer at ambient temperature. Proton magnetic resonance (^{1}H NMR) spectra were determined at 90 MHz (JEOL FX-90Q), and chemical shifts are expressed as δ values (in ppm) relative to an internal standard of Me_4Si. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories (Knoxville, TN).

Enzyme Purification. B. thiaminolyticus strain Matsukawa et Misawa (American Type Culture Collection 13203) was cultured according to Douthit and Airth (1966). Thiaminase I was purified according to the procedure of Wittliff and Airth (1968). In this paper, "crude" enzyme refers to the water-redissolved pellet from the 0–75% $(NH_4)_2SO_4$ precipitation step, having a specific activity of about 500 milliunits/mg; "purified" enzyme refers to that material conducted through the purification procedure, with a specific activity of about 12000 milliunits/mg. Where it was necessary to free enzyme from buffer, dilution and ultrafiltration (through Amicon YM10 membranes) were used in lieu of dialysis, which in our hands usually led to enzyme inactivation. Ultrafiltration and dilution back to original volume typically left an enzyme solution with at least 97% of its original activity. In our hands the published purification protocol did not yield homogeneous material, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis [10% gels, run by using a stacking gel and stained with Coomassie Blue, following the published procedure (Laemmli, 1970)], though enrichment in a 45-kilodalton peptide had clearly been achieved. [Wittliff and Airth (1968) report a molecular weight for thiaminase of 44 kilodaltons.] Purity was assessed by active-site count (see below) and was no better than 75%.

4-Amino-6-chloro-2-methylpyrimidine. **2a** was synthesized as described by Henze et al. (1952), with modification. The immediate precursor, 4,6-dichloro-2-methylpyrimidine, was heated overnight at 100 °C in 15% ethanolic NH_3. The resulting mixture was partitioned between water and ether, and the organic layer was dried to give **2a**, mp 181–186 °C [lit. mp 190–191 °C (Henze et al., 1952)]. Thin-layer chromatography (silica gel developed with ethyl acetate) showed that the starting material (R_f 0.9) had been converted to a single product, R_f 0.58. To verify the identity of this material, it was hydrogenated according to the procedure of Whittaker (1953)

to give 4-amino-2-methylpyrimidine and identified by ^1H NMR comparison with authentic material (David & Estramareix, 1960).

Tritium-Labeled 4-Amino-6-chloro-2-methylpyrimidine (**2a**). Tritiation was performed by use of acid-catalyzed exchange under conditions described by Hutchinson (1971). By use of this procedure, label is incorporated into the C-2 methyl of the pyrimidine. Use of either HCl or CF_3CO_2H catalyst results, in our hands, in only extremely low labeling, typically 15 cpm/nmol.

4-Amino-5-(anilinomethyl)-6-chloro-2-methylpyrimidine (**3a**). 5-(Bromomethyl)-4,6-dichloro-2-methylpyrimidine (0.26 g; Hasegawa, 1953) was suspended in aniline (180 μL) and heated for 15 min at 100 °C. Anilinium hydrobromide was removed by precipitation with CH_2Cl_2, the supernatant was evaporated, and the residue was heated in 15% ethanolic NH_3 at 100 °C overnight to displace a single chloride. Solvent was evaporated and **3a** was purified by preparative TLC (R_f 0.72 on 2-mm silica gel developed with ethyl acetate), followed by recrystallization from methanol: mp 201–203 °C; ^1H NMR (90 MHz, CD_3OD) δ 2.36 (3 H, s, methyl), 4.27 (2 H, s, methylene), 6.5–7.2 (5 H, m, aryls). Anal. Calcd for $C_{12}H_{13}N_4Cl$: C, 58.06; H, 5.24. Found: C, 57.52; H, 5.24.

Thiaminase I Assay. Activity was measured spectrophotometrically, at room temperature (25 °C), in 0.175 M sodium phosphate, pH 5.8, with thiamin (100 μM) as primary substrate and with quinoline (1 mM, distilled over zinc) as acceptor base. Thiamin was added to start the reaction, in a final volume of 1 mL, and the absorbance increase at 319 nm was monitored (Lienhard, 1970). Activity (milliunits) was found by multiplying ΔA_{319}/min by 123. For determination of specific activities, protein was measured with Folin phenol reagent (Fisher) according to the directions of Dawson et al. (1969), with bovine serum albumin (Sigma) as standard.

Chloride Assay. A modification of the method of Mentasti and Pelizzetti (1975) was used. A 400-μL sample was mixed with 300 μL of 160 μM $Tl(ClO_4)_3$ (Aesar; Johnson Matthey Inc., Seabrook, NH) in 0.66 M $HClO_4$, allowed to stand exactly 250 s, and then mixed with 50 μL of 500 μM 4,4′-biphenol in methanol; the absorbance increase over 80 s at 399 nm was noted and compared with those of NH_4Cl (Fisher) standards. The assay was sensitive to 3 μM chloride. Enzyme solutions that were to be assayed for chloride were first subjected to several dilution/ultrafiltration cycles, to bring base line chloride levels well below the range of sensitivity for this assay. Dilutions were made with water deionized through a Millipore Milli-Q apparatus; dilution was ultimately 2.2 million-fold. Purified enzyme so treated was incubated with 0.35 mg/mL 4-amino-6-chloro-2-methylpyrimidine over 3 days (i.e., until all enzyme activity was lost) and then ultrafiltered to remove protein; the filtrate was then assayed for chloride and compared against standards spiked with an equal concentration of this enzyme inhibitor.

Active-Site Count. The radiometric procedure (Agee & Airth, 1973) was not used due to interfering impurities in commercially available (Amersham) [^{14}C]thiamin. A fluorometric method, which operates on the same principle, was used instead. In this method, purified enzyme (estimated to be no greater than 13 μM in active sites) and 1-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-8-hydroxyquinolinium chloride (20 μM) were incubated in a total volume of 100 μL at room temperature and then diluted with 900 μL of *p*-dioxane/tris(hydroxymethyl)aminomethane (Tris)/$Mg(NO_3)_2 \cdot 6H_2O$ (80% v/v, 0.1 M, and 0.01 M, respectively). Released 8-quinolinol was then measured fluorometrically (excitation 360 nm, emission 530 nm) (Watanabe et al., 1963) against 8-quinolinol (Mallinckrodt) standards similarly diluted. Release was found to reach a plateau by 10 min after mixing of enzyme and substrates and to remain at this level until at least 30 min after mixing; these kinetics of release are similar to those reported for the radiometric procedure (Agee & Airth, 1973). The maximum amount of 8-quinolinol released in the absence of an acceptor base corresponds to the number of active sites. This fluorogenic substrate was made enzymatically, as follows: sodium phosphate, thiamin, and 8-quinolinol were combined to give the concentrations 0.175 M, 0.1 mM, and 1 mM, respectively, in 20 mL of water. 8-Quinolinol was introduced as 2 mL of a 10 mM stock solution in 20% (v/v) methanol. Finally, 1 mL of crude thiaminase I was added, and the mixture (final pH 5.8) was allowed to stand for 4 h at room temperature. This was ultrafiltered to remove protein and extracted continuously with $CHCl_3$ overnight to remove thiamin, thiazole, and excess 8-quinolinol. To verify that the reaction had gone to completion, yielding a 100 μM solution of fluorogenic substrate, this solution was decomposed enzymatically in the presence of 1 mM L-cysteine (which can function as an acceptor base), and released 8-quinolinol was measured fluorometrically as described. As expected, 100 μM was released. No attempt was made to remove sodium phosphate from this preparation.

Analysis of Kinetic Data. The best fits for the straight lines and hyperbola in Figures 1–3 were obtained through the damped Gauss–Newton method, using a computer program supplied by Dr. F. J. Kezdy (Department of Biochemistry, University of Chicago), adapted from the procedure described by Yamaika et al. (1981). The value of K_I was taken as the inhibitor concentration at half-maximal inactivation rate. The value of k_{cat} was determined by using the formula of Jung and Metcalf (1975).

RESULTS

Incubation of thiaminase I with 4-amino-6-chloro-2-methylpyrimidine (**2a**) results in a time-dependent loss of enzyme activity as shown in Figure 1. The semilogarithmic plot of the extent of inactivation vs. time is linear as expected for a pseudo-first-order process involving an inactivation by a simple reaction of the inhibitor with the active site. The pseudo-first-order rate constants k_{obsd} for the inactivation were directly proportional to inhibitor concentration (Figure 1, insert) up to 5.6 mM. The low rate of inactivation by **2a**, as well as its failure to evidence saturation, is most probably due to its poor resemblance to thiamin (**1**). The corresponding nonhalogenated compound, 4-amino-2-methylpyrimidine, does not competitively inhibit thiaminase I at concentrations up to 500 μM even when the assay is conducted at thiamin concentrations near its K_m (21 μM; J. A. Hutter, unpublished experiments). The nonhalogenated pyrimidine further does not cause any time-dependent inactivation, even after overnight incubation.

To demonstrate that inactivation by **2a** occurs by reaction at the active site, thiaminase was inactivated in the presence of varying concentrations of its substrates. The time-dependent loss of activity under these conditions is shown in Figure 2. Quinoline, an acceptor base, was supplied along with thiamin, since treatment with thiamin alone is known to inactivate the enzyme (Agee & Airth, 1973). It is evident that inactivation by **2a** proceeds more slowly as substrate concentrations increase, as predicted for an active-site-directed process.

Inactivation was found to go to completion, given sufficiently long incubation times, and to be irreversible. Repeated dilutions with water (ultimately 5000-fold) and ultrafiltrations

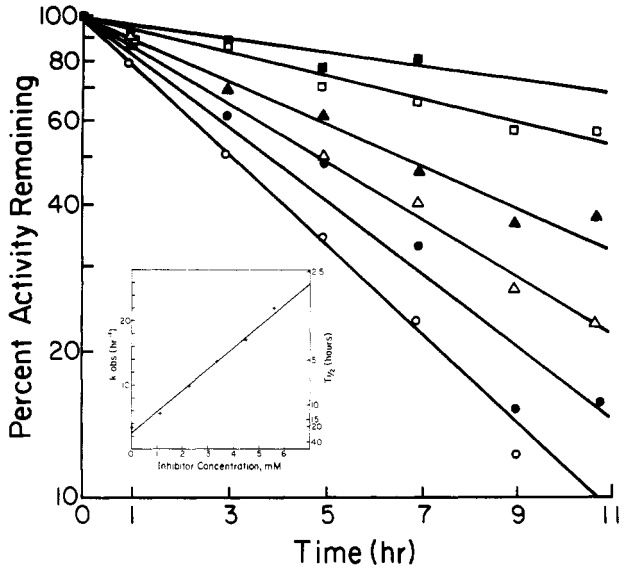


FIGURE 1: Time-dependent inactivation of partially purified thiaminase I by 4-amino-6-chloro-2-methylpyrimidine (**2a**) at (■) 0, (□) 1.12, (▲) 2.24, (△) 3.36, (●) 4.48, or (○) 5.60 mM. Incubations were conducted at 25 °C in 0.175 M sodium phosphate buffer, pH 5.8. Inhibitor **2a** was supplied from a 50 mM methanolic stock solution. Assays were performed spectrophotometrically with quinoline as the acceptor base. Aliquots from the incubation mixtures were removed at the time indicated and diluted 10-fold into the assay mixture. (Insert) Rate of thiaminase inactivation (expressed as k_{obsd}, the pseudo-first-order rate constant for inactivations determined above) vs. concentration of inhibitor **2a**. Rates of inactivation at higher concentrations of **2a** could not be measured due to the insolubility of **2a** in water.

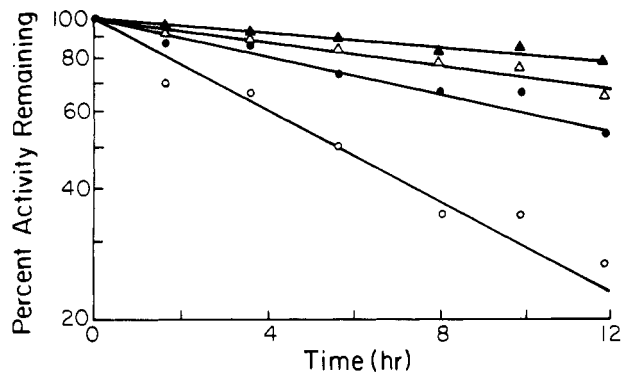


FIGURE 2: Time-dependent inactivation of partially purified thiaminase by 4-amino-6-chloro-2-methylpyrimidine (**2a**) in the presence of substrate. Incubations were conducted in the presence of 3.36 mM inhibitor and with varying concentrations of thiamin: (○) 0, (●) 8, (△) 16, or (▲) 24 mM. Quinoline was present at 10 times the listed thiamin concentrations.

failed to restore activity in inactivated preparations. This is consistent with the formation of a covalent bond between inhibitor and enzyme.

Inactivation was accompanied by the release of approximately 1 chloride ion per active site. In the experiment described under Chloride Assay under Experimental Procedures, chloride release by purified enzyme incubated with **2a** came to 5 μM. The concentration of enzyme specifically inactivated by **2a** was calculated to be 5.4 μM, after introducing a 60% correction for inactivation occurring over the course of the incubation that was not attributable to the action of **2a**, thus yielding an observed stoichiometry of 0.92 chloride ion released per equivalent of active site inactivated. Thus, it appears **2a** is modifying only one position on the enzyme, almost certainly the active site. Noncovalent binding elsewhere on the enzyme cannot yet be ruled out, however. We have not been able to

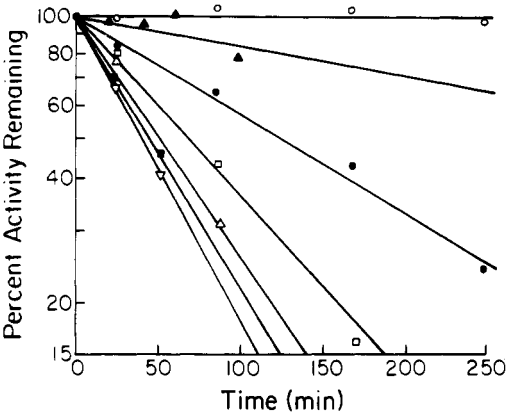


FIGURE 3: Time-dependent inactivation of partially purified thiaminase I by 4-amino-5-(anilinomethyl)-6-chloro-2-methylpyrimidine (**3a**) at (○) 0, (●) 25, (□) 50, (△) 75, (■) 100, or (▽) 150 μM. The symbol ▲ denotes an incubation conducted with 75 μM **3a** in the presence of 100 μM thiamin and 1 mM quinoline. The inactivation was otherwise conducted as described in Figure 1.

make a direct determination of enzyme–inhibitor stoichiometry, because **2a** labeled at sufficiently high specific radioactivity is not available.

It is unlikely that **2a** works as a nonspecific electrophile, inactivating thiaminase I by nonspecific modification of nucleophilic amino acid groups on the enzyme molecule. The inhibitor **2a** is in fact a fairly stable compound: it will not react noticeably with ordinary nucleophiles like CH_3O^-, $C_2H_5S^-$, or CH_3NH_2 unless heated at concentrations above 0.1 M overnight in sealed vessels at temperatures above 100 °C. It does not form a stable adduct with bovine serum albumin (BSA), even after incubation for 2 days at room temperature with protein at 50 mg/mL (this was verified with [^{3}H]-**2a**, whose low specific radioactivity could be compensated for by using very high BSA concentration). It further will not inhibit α-chymotrypsin at concentrations and incubation times sufficient to inactivate thiaminase I.

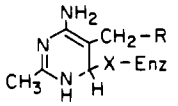
The extremely low rate of inactivation of thiaminase I by **2a** compared to the normal rate of catalytic turnover caused some concern that the mechanism of inactivation might differ significantly from the mechanism of catalysis. A 6-chloropyrimidine that more closely resembles a thiaminase I substrate (or product) was thus sought, with the expectation that it would bind more tightly to the active site and so inactivate the enzyme more quickly. 4-Amino-5-(anilinomethyl)-6-chloro-2-methylpyrimidine (**3a**) was selected for study since it is easy to synthesize and since it is the 6-chloro analogue of "anilinothiamin" [4-amino-5-(anilinomethyl)-2-methylpyrimidine], the product of the reaction between thiamin (**1**) and aniline in the presence of thiaminase (Scheme I).

Incubation of thiaminase I with **3a** results in a rapid first-order loss of activity (Figure 3). Inactivation was complete and irreversible and was slowed significantly in the presence of thiamin and quinoline (Figure 3). As predicted, the rate of inactivation of thiaminase by **3a** gives evidence for saturation at low inhibitor concentrations. Plots of the rate of inactivation vs. inhibitor concentration are hyperbolic, and a K_I of 96 ± 20 μM may be calculated. This value is quite close to the K_m for thiamin of 21 μM that was determined in this laboratory. The k_{cat} for inhibition by **3a** was determined to be 0.31 ± 0.004 min^{-1}.

Discussion

We have demonstrated that 6-chloropyrimidines **2a** and **3a** are irreversible inhibitors of the thiaminase I from *B. thiaminolyticus*. The kinetics of inactivation are demonstrated to

be first order with respect to the concentration of the inhibitor. Inactivation is slowed in the presence of substrates, and where the stoichiometry could be measured, reaction with 1 equiv. of inhibitor was found to inactivate one active site. Further, 6-chloropyrimidines such as **2a** were demonstrated to be relatively unreactive electrophiles, which apparently require activation by the enzyme active site prior to enzyme inactivation. Taken together, these observations imply that **2a** and **3a** are acting as mechanism-based irreversible inhibitors of thiaminase I (Abeles & Maycock, 1976). The mechanism of inhibition that we consider most likely is depicted in Scheme III and involves the formation of a 1,6-dihydropyrimidinyl-enzyme intermediate, followed by the irreversible elimination of HCl to trap the active-site nucleophile as a stable 6-substituted pyrimidine. If this mechanism of inactivation can be confirmed, the inactivation would imply that a covalent 1,6-dihydropyrimidinyl-enzyme intermediate **4** is on the normal



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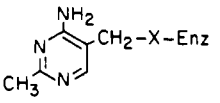

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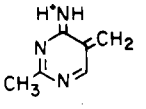

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pathway for thiamin cleavage. Kinetic investigations on the thiaminase I from mollusks (Puzach et al., 1984) and from *B. thiaminolyticus* (Lienhard, 1970) support such a double-displacement mechanism involving a pyrimidinyl-enzyme intermediate. No evidence as to the structure of this intermediate has heretofore been presented. A covalent intermediate, linked by a bond between some enzymatic nucleophile and the 5-pyrimidinylmethylene such as **5**, and a noncovalent enzyme-stabilized *o*-quinone methide imine such as **6** have been proposed as possible intermediate structures (Lienhard, 1970). Subsequently, the 1,6-dihydropyrimidinyl-enzyme adduct **4** was proposed on the basis of the analogy between the reaction catalyzed by thiaminase I and the bisulfite-catalyzed cleavage of thiamin (Zoltewicz & Kauffman, 1977). Mechanism-based irreversible inhibition of the enzyme by 6-chloropyrimidines **2a** and **3a** would not be predicted if either **5** or **6** correctly represented the structure of the enzyme-bound intermediate, but it is a direct consequence of pyrimidinyl-enzyme **4**. The observation that **2a** and **3a** inhibit thiaminase thus may represent the successful trapping of this intermediate and the first experimental support of the proposal that the mechanism advanced for the bisulfite-catalyzed cleavage of thiamin applies to the enzymatic case as well. Future work will be directed toward substantiating this mechanism of inactivation, toward the identification of the pyrimidinyl–amino acid adduct, and toward the extension of the mechanism to other related enzymatic reactions.

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