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Mechanistic Studies on Thiaminase I: Identification of the Product of Thiamin Degradation in the Absence of the Nucleophilic Cosubstrate

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Thiaminase I catalyzes the decomposition of thiamin under basic conditions in the absence of the nucleophilic cosubstrate. The product of this reaction was identified as the adduct resulting from the displacement of the thiazole moiety of thiamin by the ring opened form of thiamin.

INTRODUCTION

Thiaminase I catalyzes the cleavage of thiamin by a wide variety of nucleophiles (Fig. 1) (*1*). The physiological function of this surprising enzymatic activity, which is widely distributed in nature, is currently unknown (*2*).

During our attempts to carry out presteady state kinetics studies on thiaminase I, we observed turnover of the enzyme in the absence of the nucleophilic cosubstrate (*3*). Here, we report the structure of the reaction product formed under these conditions.

EXPERIMENTAL PROCEDURES

General methods and materials. Column chromatography was performed with EM science silica gel 60 (230–400 mesh). Analytical TLC was performed on silica gel 60 F_{254}-precoated glass plates from EM Science and visualized by UV light. IR spectra were recorded on a Mattson Galaxy series FT-IR instrument. High resolution FAB mass spectra were run at the University of Illinois on a 70-SE-4F instrument with a resolving power of 10,000.

Thiaminase I from *Bacillus thiaminolyticus* was overexpressed in *Escherichia coli* and purified as previously described (*1a*).

Isolation of the thiaminase I catalyzed thiamin degradation product. Thiaminase I (20 μl, 10.8 mg/ml) was added to a stirred solution of thiamin · HCl (20 mg, 0.06 mmol) in aqueous NaOH (0.1 M, 1 ml). The reaction mixture (initial pH = 9.1) was stirred at room temperature for 2 h. TLC analysis (EtOAc:MeOH:AcOH:NH_4 OH = 50:48.5:0.75:0.75) demonstrated the clean formation of 4-methyl-5-(2-hydroxyethyl)-thiazole (R_f = 0.8) and

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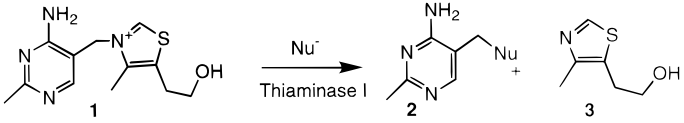


FIG. 1. The thiaminase I catalyzed degradation of thiamin.

a new compound (R_f = 0.31). In a control experiment run under identical conditions, except for the absence of thiaminase, neither product was formed. The reaction mixture was deproteinized by ultrafiltration (Centriprep-10 at 2000 RPM for 30 min) and lyophilized. The resulting solid was purified by flash column chromatography (silica gel, EtOAc/MeOH/AcOH/NH_4OH, 50:48.5:0.75:0.75) and triturated with CH_2Cl_2 to give the product as a white solid (2 mg, 17%). HRMS (FAB in magic bullet) m/z $[MH]^+$ calculated for ($C_{18}H_{26}N_7O_2S$) = 404.1869, observed = 404.1869. IR (CH_2Cl_2) 3329, 3198, 2960, 2918, 2846, 1643, 1589, 1559, 1470, 1428, 1375, 1047 cm^{-1}. The NMR parameters are listed in Table 1.

RESULTS AND DISCUSSION

The molecular formula ($C_{18}H_{25}N_7O_2S$) of the product of the thiaminase I catalyzed thiamin degradation, obtained by HRFABMS, suggested that it was composed of thiamin ($C_{12}H_{17}N_4OS$), the pyrimidine moiety of thiamin ($C_6H_8N_3$), and an additional oxygen atom. The extra oxygen atom and the presence of an amide carbonyl stretch at 1643cm^{-1} suggested that the ring opened form of thiamin (**5,** Fig. 3) functioned as the nucleophile in this reaction. The observation of correlations between H6 and H9 in the ROSEY spectrum established that the sulfur and not the pyrimidine nitrogen functioned as the nucleophile and suggested that the structure of the reaction product was compound **4** (Fig. 2). This was fully consistent with a detailed analysis of the NMR spectra (^1H, ^{13}C, HMQC, HMBC, ROSEY; Table 1). The product consisted of a mixture of isomeric amides. NOE measurements demonstrated that the trans amide was the major product. A similar mixture of isomeric amides had been previously observed for the ring-opened form of thiamin (*4*).

A mechanistic proposal for the formation of **4,** based on the established mechanism of thiaminase I and on the high tolerance of the enzyme to variations in the structure of the nucleophile, is outlined in Fig. 3 (*1*). The facile base-catalyzed ring opening of the thiazolium moiety of thiamin generates the nucleophile **5** (*4*). Reaction of this with the enzyme bound pyrimidine **7,** followed by expulsion of the active site nucleophile, would give the observed reaction product.

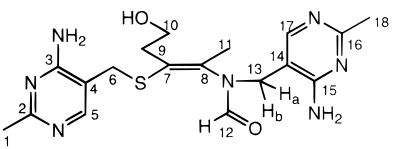
Thiaminase I rapidly looses activity when treated with thiamin in the absence of the nucleophilic cosubstrate (*5*). Addition of **4** to the reaction mixture does not result in inhibition and the mechanism of this inhibition reaction is currently unknown. One possibility, based on the facile addition of nucleophiles to N1 methyl thiamin (*6*), is that N1 of thiamin or of **5** reacts with **7** and that the resulting pyrimidinium cation alkylates the enzyme.

TABLE 1

^{1}H and ^{13}C NMR Chemical Shifts[a] and HMQC and HMBC and ROSEY Correlations[b] of the Thiamin Degradation Product

Position	^{1}H NMR δ	^{13}C NMR δ	HMQC	HMBC	ROSEY
1	2.36 or 2.38(s)	23.8 or 24.0	C1	C2	H5
2		166.1			
3		161.8			
4		111.5			
5	7.70(major, s)	154.2		C2, C3, C6	H1, H6, H9
	7.81(minor, s)				
6	3.51(br s)	29.3	C6		H5, H9
7		132.6(major)			
		132.2(minor)			
8		138.8(major)			
		136.1(minor)			
9	2.65(*t*, *J* = 6.5 Hz)	33.9	C9	C7, C8, C10	H5, H6, H10
10	3.69(*t*, *J* = 6.5 Hz)	60.0	C10	C7, C9	H9, H17
11	1.97(major, s)	18.2	C11	C7, C8	H12, H17
	1.79(minor, s)			C7, C8	
12	7.30(s)	165.2	C12	C13	H11
13a	4.50(*d*, *J* = 15.2 Hz)	40.2			H17(minor)
13b	4.35(*d*, *J* = 15.2 Hz)				H17(minor)
14		109.5			
15		163.2			
16		167.1			
17	7.73(major, s)	151.8		C13, C15	H10, H11, H18
	8.15(minor, s)				H13a(minor), H13b(minor)
18	2.36 or 2.38(s)	23.8 or 24.0	C18	C16	H17

[a] Recorded in D_2O at 500 MHz (^{1}H) and 125 MHz (^{13}C), in ppm.
[b] HMQC optimized for $^{1}J_{C\text{-}H}$ = 140 Hz. ROSEY mixing time t_m 700 ms.



4 (Major isomer)

FIG. 2. The structure of the thiamin degradation product formed in the absence of the nucleophilic cosubstrate.

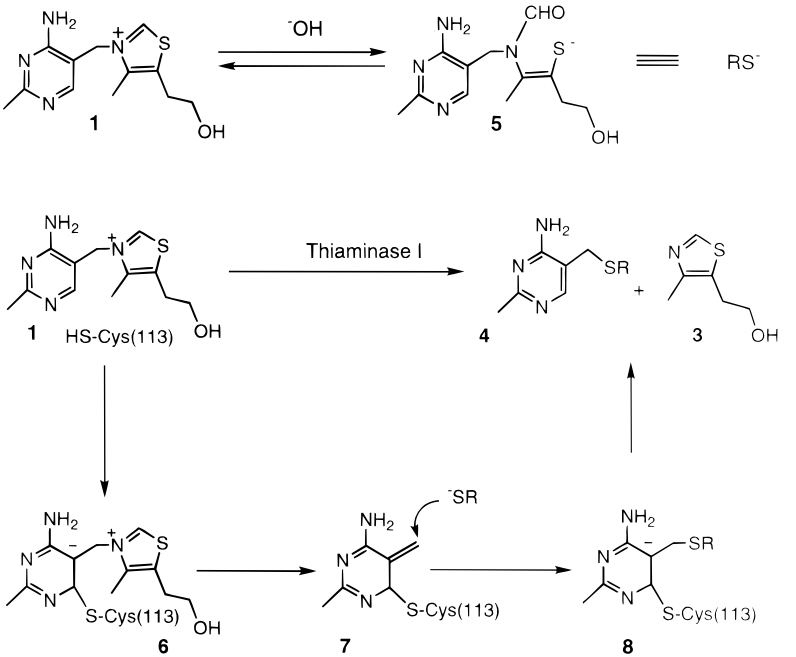


FIG. 3. Mechanistic proposal for the formation of the thiamin degradation product (*4*).

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