An assay for thiaminase I in complex biological samples

Jeremiah W. Hanes a, Clifford E. Kraft b, Tadhg P. Begley a, *

a Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853, USA
b Department of Natural Resources, Cornell University, Ithaca, NY 14853, USA

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Abstract

An alternative method for measuring thiaminase I activity in complex samples is described. This assay is based on the selective consumption of the highly chromophoric 4-nitrothiophenolate by thiaminase I, resulting in a large decrease in absorbance at 411 nm. This new assay is simple and sensitive, and it requires only readily available chemicals and a visible region spectrophotometer. In addition, the assay is optimized for high-throughput analysis in a 96-well format with complex biological samples.

Keywords: Thiaminase I; Thiaminase II; TenA; Vitamin B 1; Thiamin; Early mortality syndrome; Salmon; Trout; Beriberi; Forage fish; Great Lakes; New York Finger Lakes

Thiaminases degrade thiamin (vitamin B 1) and were initially observed in foods more than 6 decades ago. This activity has been detected in a wide variety of sources, including bacteria, marine organisms, and plants [1–3]. Animals that consume thiaminase-containing foods can experience thiamin deficiency-related illnesses [4–7]. Currently, problems exist regarding early mortality syndrome (EMS),1 which occurs in the offspring of large predatory fish (coho salmon, chinook salmon, steelhead trout, brown trout, lake trout, and walleye) of the Laurentian Great Lakes and the New York Finger Lakes [8]. A similar ailment in Atlantic salmon, called M74, occurs in the Baltic Sea. These maladies have been associated with low egg thiamin concentrations and are manifested clinically as a loss of equilibrium, a spiral swimming pattern, lethargy, hyperexcitability, hemorrhage, and death between hatch and first feeding [9]. This thiamin deficiency occurs as a result of predatory fish consuming forage fish, such as nonnative alewife, that often contain high levels of thiaminase [8,10–21].

Two types of thiaminase activity have been identified: type I catalyzes the degradation of thiamin by replacing the thiazole moiety with a variety of nucleophiles, whereas type II is specific for the use of water as the cosubstrate (Scheme 1). Both types operate by a ping-pong kinetic mechanism, but type I is thought to be solely responsible for the cited thiamin deficiencies. Thiaminase I from Bacillus thiaminolyticus has been cloned and overexpressed, and its mechanism has been studied in detail [22–26]. The enzyme functions as a 42-kDa monomer and has been characterized structurally [27,28]. In contrast to thiaminase II, which is likely to play a role in thiamin salvage, the physiological role of thiaminase I is still unknown after decades of study.

A 30-year-old quantitative radiochemical assay for thiaminase I activity in complex samples [29,30] was recently optimized due to the need to further understand the serious environmental issues surrounding thiamin degradation [21]. This assay uses 14C-labeled thiamin and, therefore, requires a sophisticated laboratory environment for its implementation. In the current article, we describe an...
alternative method for measuring thiaminase activity in complex samples. This new assay is based on the selective consumption of the highly chromophoric 4-nitrothiophenolate (Scheme 2) by thiaminase I, which is able to use a variety of nucleophiles as cosubstrates. This assay is sensitive and uses readily available chemicals and a visible region spectrophotometer. In addition, the assay can be easily performed in a high-throughput fashion in either 96- or 384-well plates.

Materials and methods

Recombinant thiaminase I

*B. thiaminolyticus* thiaminase I was overexpressed and purified as described previously [22]. After purification, the enzyme was buffer-exchanged into 50 mM phosphate (pH 7.2 at room temperature [RT]), 100 mM NaCl, and 2 mM dithiothreitol (DTT) in 20% glycerol using a 10 DG column purchased from Bio-Rad (Hercules, CA, USA). The protein was flash-frozen in liquid nitrogen and stored at −80°C until use.

Bacillus subtilis thiaminase II (TenA) was overexpressed and purified as described previously [31].

Chemicals and reagents

Unless otherwise specified, all buffers, salts, and chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA) and were of the highest purity offered. The 4-nitrothiophenol used in this study was purchased from Sigma–Aldrich and was of technical grade (>80% pure). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was obtained from Soltec Ventures (Beverly, MA, USA).

Equipment

A Hitachi (Berkshire, UK) U-2010 UV/visible spectrophotometer was used to characterize the spectral changes during the enzymatic reaction. A model SF-2004 stopped-flow apparatus (KinTek, Austin, TX, USA) was used to measure the steady-state kinetic parameters of the recombinant enzyme. Complex biological samples were pulverized in a SPEX SamplePrep Freezer Mill (model 6850, SPEX CertiPrep, Metuchen, NJ, USA). Absorbance measurements on complex samples were performed in 96-well plates (Greiner plate model 655801) purchased from Omega Scientific (Tarzana, CA, USA) in a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT, USA).

Steady-state measurement on recombinant enzyme

On the day of any experiment in which 4-nitrothiophenol was used, a fresh stock solution was made by dissolving it in dimethyl sulfoxide (DMSO) to a final concentration of 20 to 200 mM. The reaction conditions under which the steady-state parameters of the recombinant enzyme were measured were as follows: 20 nM thiaminase I (as judged by Bradford assay), 50 mM phosphate (Na+, pH 7.2, at RT), 100 mM NaCl, 2 mM TCEP, and variable substrate concentrations as described in the main text. The decrease in absorbance at 411 nm was recorded during the initial linear phase of the reaction using a stopped-flow apparatus. Each rate is the result of an average of at least five separate mixing events. The absorbance signal was converted to concentration using an extinction coefficient of 13,650 M⁻¹ cm⁻¹, which was estimated from the absorbance of a known concentration of 4-nitrothiophenolate at 411 nm under the same buffer conditions.

Evaluation of 4-nitrothiophenolate as a cosubstrate for thiaminase II

*B. subtilis* thiaminase II was assayed for activity using 4-nitrothiophenolate as a potential cosubstrate under the following conditions: 1 μM thiaminase II (as judged by Bradford assay), 50 mM phosphate (Na+, pH 7.2, at RT), 100 mM NaCl, 2 mM TCEP, 400 μM thiamin, and 80 μM 4-nitrothiophenolate. The reaction was initiated by the addition of enzyme and was monitored for 3 h at RT.

Preparation of complex samples

Whole gizzard shad and alewife from aquatic ecosystems (northcentral United States) of high thiaminase I levels (gizzard shad from “pond 6S”), moderate thiaminase I levels (alewife from Cayuga Lake), presumably low thiaminase I levels (alewife from Cayuta Lake), and unknown thiaminase I levels (alewife from Canadarago Lake) were stored at −80°C until pulverization. Prior to and leading up to pulverization, the samples were kept in a cooler filled approximately halfway with finely ground dry ice. Samples (12–18 g fish) were cut into approximately 3-g segments.
with diagonal cutting pliers until the entire fish was inside the polycarbonate sample container. The tube was closed and inserted into the freezer mill and submerged in liquid nitrogen. The grinding was performed in cycles according to the following protocol: 1- to 10-min pre-cool followed by 2- to 3-min grinding cycles with 2-min interruptions in between each grinding cycle to allow for recooling of the samples. When grinding was completed, the samples were quickly taken out of the tube and transferred into 50-ml plastic screwcap tubes and stored at −80 °C until further use. The resulting biological material was a fine gray powder of uniform consistency.

Measurement of thiaminase I activity in complex samples

On the day of an experiment, approximately 0.25 to 0.5 g of finely ground biological material was taken out and placed into a preweighed 15-ml screwcap tube. A volume of 5 ml buffer per gram of biological material was added to each sample tube and placed on wet ice. The buffer was prechilled and consisted of 50 mM phosphate (pH 7.2 at RT), 100 mM NaCl, and 10 mM TCEP. Three rounds of vortexing (~10 s each at maximum speed) were performed in intervals separated by 1-min incubations on ice. After all samples were vortexed, they were transferred into 1.5-ml Eppendorf tubes and centrifuged at 17,200 relative centrifugal force (rcf) at 4 °C for 20 min. At this time, the supernatant was assayed for thiaminase I activity directly or diluted using the same buffer and assayed as described in the main text. The assay cocktail for the complex samples consisted of the following (final concentrations during reaction): 50 mM phosphate, 100 mM NaCl, 10 mM TCEP, 400 μM thiamin, and 80 μM 4-nitrothiophenol (pH 7.2 at RT). To start the reaction, 50 μl of sample supernatant (or dilution of the sample supernatant) was added to 250 μl of assay cocktail (in a 96-well plate), and the absorbance at 411 nm was recorded as a function of time using the microplate reader as described above. To convert the absorbance measurement to a 4-nitrothiophenolate as a function of concentration, the absorbance value was multiplied by 91.7, which normalized the initial “y value” to approximately 80 μM. The number 91.7 was determined by measuring the absorbance of samples (at 411 nm) containing all of the assay cocktail components minus the 50 μl of complex biological mixture but plus 50 μl of buffer in its place (the complex samples tend to slightly increase the absorbance at 411 nm). Furthermore, because the decrease in absorbance at 411 nm in complex samples lacking thiamin was determined to not be a consequence of thiaminase I activity, wells containing everything except thiamin were run for each dilution of the complex mixture so that the raw data could be corrected by calculating the difference in the raw data. It was observed that the background decrease in absorbance at 411 nm varies with dilution of the sample; therefore, it is particularly important to run a “− thiamin” sample under identical conditions to allow for reliable data correction.

Data analysis

All data fitting by linear and nonlinear regression was performed with the computer program GraFit 5 (Erithacus Software, Surrey, UK) by the least squares method. All other data normalization and manipulation was done in Microsoft Excel (Redmond, WA, USA). Steady-state parameters were obtained by fitting the observed rate as a function of concentration by nonlinear regression to the following Michaelis–Menten equation:

$$k_{obs} = \frac{k_{cat} \cdot [S]}{K_m + [S]}.$$  

Results

4-Nitrothiophenolate as a substrate for thiaminase I

Because thiaminase I is able to use a variety of nucleophiles as cosubstrates, we tested the enzyme with the highly chromophoric 4-nitrothiophenolate with the expectation that there would be a large change in the extinction coefficient on formation of product (Scheme 2). The λmax of the 4-nitrothiophenolate anion, under the buffer conditions used during this study, was 411 nm with an extinction coefficient of approximately 13,650 M⁻¹ cm⁻¹. Fig. 1A shows UV/visible scans taken as a function of time under the following conditions: 300 pM thiaminase I, 800 μM thiamin, 100 μM 4-nitrothiophenolate, 2 mM TCEP, and 100 mM NaCl buffered in 50 mM phosphate (pH 7.2 at RT). There is a large change in absorbance that occurs as the reaction proceeds; the absorbance initially present in the visible region of the spectrum drops to less than 15% of the original value (estimated as the sum of absorbance >400 nm). Furthermore, the product absorbs predominantly in the UV region. There is an isosbestic point centered at 362 nm. The absorbance change is sufficiently large that the reaction can easily be followed in real time by eye. Fig. 1B shows that the total drop in absorbance is approximately 1.2 units at 411 nm under the reaction conditions.

Kinetic characterization of product formation

The steady-state kinetic parameters were obtained by measuring the initial linear decrease in absorbance at 411 nm as a function of time in a stopped-flow apparatus (see Materials and methods). Fig. 2A shows the change in the steady-state rate of consumption of 4-nitrothiophenolate as a function of concentration. For this experiment, the thiamin concentration was held constant at 400 μM. The data were fit by nonlinear regression as described in Materials and methods. Fig. 2A shows the change of product formation as a function of thiamin concentration. The steady-state rate of reaction was fit to the Michaelis–Menten equation:

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]}.$$  

The steady-state kinetic parameters were obtained by measuring the initial linear decrease in absorbance at 411 nm as a function of time in a stopped-flow apparatus (see Materials and methods). Fig. 2A shows the change in the steady-state rate of consumption of 4-nitrothiophenolate as a function of concentration. For this experiment, the thiamin concentration was held constant at 400 μM. The data were fit by nonlinear regression as described in Materials and methods. The 4-nitrothiophenolate anion is a surprisingly good substrate for thiaminase I, with a $k_{cat}$ of 297 ± 5 s⁻¹ and a $K_m$ of 36.4 ± 1.4 μM to yield a specificity constant ($k_{cat}/K_m$) of 8.38 ± 1.6 μM⁻¹ s⁻¹. Another experiment was performed to obtain the steady-state
kinetic parameters for thiamin in the presence of a fixed concentration of 4-nitrothiophenolate (80 μM), and the results are shown in Fig. 2B. A $k_{cat}$ of $260 \pm 10 \text{s}^{-1}$ and a $K_m$ of $21.1 \pm 1.3 \text{μM}$ were determined for thiamin to define a specificity constant of $12.4 \pm 1.0 \text{μM}^{-1} \text{s}^{-1}$. The $k_{cat}$ for thiamin is lower than the one obtained for the nitrothiophenolate because the experiment in which the thiamin concentration was varied was done with a slightly less than saturating concentration of 4-nitrothiophenolate. These data show that thiaminase I can use 4-nitrothiophenolate as a substrate efficiently and rapidly.

4-Nitrothiophenolate as a substrate for native thiaminase I in a complex biological sample

To test whether the 4-nitrothiophenolate would function as a substrate for thiaminase I in a complex biological mixture derived from native sources, we obtained freshwater fish samples that were known to contain measurable amounts of thiaminase I (measurable by the radiochemical assay). The samples were prepared in a manner similar to that of previous methods [21]. The final assay cocktail contained nearly saturating concentrations of both substrates (80 μM 4-nitrothiophenolate and 400 μM thiamin). Critical to success of the assay was the inclusion of a nonnucleophilic reducing agent (10 mM TCEP) that prevents the nonenzymatic oxidation of the 4-nitrothiophenolate. Thiaminase I is also sensitive to oxidation, presumably because of its essential active site cysteine residue [22], and TCEP extends its catalytic lifetime (unpublished results).

Fig. 1. Time-dependent changes in the UV/visible spectrum of the thiaminase reaction mixture. (A) An assay reaction was performed under steady-state conditions, and the changes in the UV/visible spectrum were monitored as a function of time over a period of 30 min. The arrows indicate the directionality of the absorbance changes as the reaction proceeded. The reaction was performed with 300 pM thiaminase I, 800 μM thiamin, and 100 μM 4-nitrothiophenolate. (B) The absorbance value at 411 nm is plotted as a function of time, showing a total change of approximately 1.2 units.

Fig. 2. Steady-state kinetic parameters. (A) The initial rate of 4-nitrothiophenolate consumption is plotted as a function of 4-nitrothiophenolate concentration. Error bars are shown but are obstructed by the data points. The experiment was performed with a constant concentration of thiamin equal to 400 μM. A fit of the data by nonlinear regression to the Michaelis–Menten equation provided a $k_{cat}$ of $297 \pm 5 \text{s}^{-1}$, a $K_m$ of $36.4 \pm 1.4 \text{μM}$, and a specificity constant ($k_{cat}/K_m$) of $8.38 \pm 1.6 \text{μM}^{-1} \text{s}^{-1}$ for 4-nitrothiophenolate. (B) The initial rate of 4-nitrothiophenolate consumption is plotted as a function of thiamin concentration. A $k_{cat}$ of $260 \pm 10 \text{s}^{-1}$, a $K_m$ of $21.1 \pm 1.3 \text{μM}$, and a specificity constant of $12.4 \pm 1.0 \text{μM}^{-1} \text{s}^{-1}$ were defined for thiamin. The experiment was run in the presence of a constant concentration of 4-nitrothiophenolate equal to 80 μM.

Examination of 4-nitrothiophenolate as a cosubstrate for thiaminase II

An experiment was performed with $B.\ subtilis$ thiaminase II to inspect the possibility that 4-nitrothiophenol can function as a cosubstrate. Thiaminase II (1 μM) was mixed with thiamin (400 μM) and 4-nitrothiophenolate (80 μM) and was allowed to incubate at room temperature for 3 h. Under these conditions, no significant depletion of 4-nitrothiophenolate was observed.
A time course for 4-nitrothiophenolate consumption in a 72-fold dilution (of the original solid sample, w/v) of the complex biological mixtures was run and is shown in Fig. 3A (labeled “+ thiamin”). The activity was measured in a 96-well plate by monitoring the changes in absorbance as a function of time using a microplate reader. As evidenced in the “– thiamin” control, this experiment clearly shows that it is important to include such a measurement performed under identical conditions to ensure that there is a quantifiable difference in the rate of decrease in absorbance between the two samples. A slower rate of consumption of the 4-nitrothiophenolate relative to the sample containing thiamin always was detected but is due to unknown causes. Further experiments that were performed clearly excluded the possibility that the background decrease is due to endogenous thiamin or other potential thiaminase I substrates present in the tissue samples. This was accomplished by adding purified recombinant thiaminase I to the “– thiamin” sample (1.6 μM final concentration). No change in the rate of absorbance decrease at 411 nm was observed (data not shown). It seemed logical that another possible route to the decrease in absorbance could be due to various possible reactions catalyzed by transition metals present in the complex samples. Therefore, the assay was performed again, but instead a final concentration of 10 mM ethylenediaminetetraacetic acid (EDTA) was included, with the results also being shown in Fig. 3A (labeled “+ EDTA”). There was a small, but significant, change upon the addition of EDTA to the assay cocktail. However, because the relative change was nearly identical when one compares the “+ thiamin” and “– thiamin” data, the assay was essentially unaffected overall. Unexpectedly, EDTA increased the rate of 4-nitrothiophenolate consumption in the “blank” sample where buffer was used in place of the tissue supernatant. It seems likely that the addition of EDTA to the assay cocktail may have a greater consequence if the samples are likely to contain higher concentrations of transition metals than the ones used here. Therefore, in those cases it may be prudent to characterize the effect of metal chelation.

Sensitivity of thiaminase I assay

The thiaminase I activity in four fish samples, with different amounts of native thiaminase I, is shown in Fig. 3B. The plot is presented in double log format to better illustrate the relative magnitudes of the rates of 4-nitrothiophenolate consumption as a function of sample dilution. The assays were run in duplicate for 60 min, and the decrease in absorbance for all samples was linear in this time window after correcting for the decrease seen in the “– thiamin” control wells. The linear decrease in absorbance was converted into concentration as described in Materials and methods and fit by linear regression to obtain the activity of thiaminase I in units of μM/min. The predicted relative amounts of thiaminase I present in the samples using this assay agreed well with the expected levels, and the relationship of the activities remained intact up to a dilution of approximately 1000-fold, easily sensitive enough for a reasonable dynamic range.

Discussion

To understand and control thiaminase I-catalyzed degradation of thiamin in nature, it will be necessary to know the distribution, seasonal variation, and persistence of this enzyme in a variety of environments. In particular, the elucidation of the factors influencing the amount of thiaminase in forage fish will benefit from a robust and facile assay that can be carried out with simple equipment. The assay described in this article is a step in this direction, requiring only readily available reagents and a simple UV/visible spectrometer. In addition, this assay is specific
for thiaminase I. The radioactive assay reports on an undefined combination of thiaminase I and thiaminase II activity. This difference is well worth noting because only thiaminase I has been documented to be toxic, whereas thiaminase II likely plays a role in thiamin salvage in bacteria. Eventually, this simplified thiaminase I assay may also aid in the elucidation of the true biological function of this toxic enzyme.

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References