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Expression, Purification, and Activity of ActhiS, a Thiazole Biosynthesis Enzyme from *Acremonium chrysogenum*

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Abstract—Thiamine pyrophosphate is an essential coenzyme in all organisms. Its biosynthesis involves independent syntheses of the precursors, pyrimidine and thiazole, which are then coupled. In our previous study with overexpressed and silent mutants of ActhiS (thiazole biosynthesis enzyme from *Acremonium chrysogenum*), we found that the enzyme level correlated with intracellular thiamine content in *A. chrysogenum*. However, the exact structure and function of ActhiS remain unclear. In this study, the enzyme-bound ligand was characterized as the ADP adduct of 5-(2-hydroxyethyl)-4-methylthiazole-2-carboxylic acid (ADT) using HPLC and ^{1}H NMR. The ligand-free ActhiS expressed in M9 minimal medium catalyzed conversion of NAD^+ and glycine to ADT in the presence of iron. Furthermore, the C217 residue was identified as the sulfur donor for the thiazole moiety. These observations confirm that ActhiS is a thiazole biosynthesis enzyme in *A. chrysogenum*, and it serves as a sulfur source for the thiazole moiety.

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Keywords: *Acremonium chrysogenum*, ActhiS, ADT, thiazole biosynthesis

Thiamine (vitamin B1), a water-soluble vitamin, is essential for all living organisms as it plays an important role in carbohydrate and amino acid metabolism [1]. It was discovered in 1911 by Casimir Funk, isolated from yeast in 1932, and synthesized in 1935. Thiamine is the universal name for several compounds including free thiamine, monophosphorylated (ThMP), diphosphorylated (ThDP), and triphosphorylated thiamine (ThTP) [2-4]. Among them, ThDP is identified as an enzymatic cofactor for over 20 well-characterized enzymes, such as pyruvate decarboxylase (PDC), pyruvate dehydrogenase (PDH), etc. [5].

The biosynthesis of thiamine consists of independent formation of the thiazole and pyrimidine precursors, which are subsequently coupled [6, 7]. However, only prokaryotes (bacteria and archaea) and a few eukaryotes, such as fungi and plants, can synthesize thiamine. Humans and other animals must obtain thiamine from food [6, 8, 9]. The biosynthesis process of thiazole in bacteria has been studied comprehensively, in which five different enzymes are needed to catalyze thiazole formation [10, 11]. In archaea, the pathway for *de novo* biosynthesis of thiamine is a hybrid of the prokaryotic and eukaryotic pathways. In other words, bacterial related enzymes are used for synthesis of the pyrimidine moiety and yeast related enzymes are used for formation of the thiazole derivative of thiamin [12].

The molecular mechanism of thiazole ring formation in eukaryotes has also been revealed. The process involves several thiazole biosynthesis enzymes, such as THI4p from *Saccharomyces cerevisiae* [13-18], THI1 from *Arabidopsis thaliana* [19, 20], ThiA from *Aspergillus oryzae* [21, 22], and CyPBP37 from *Neurospora crassa* [23, 24]. Labeling studies in THI4p have shown that the thiazole moiety of thiamine is synthesized from five-carbon carbohydrates, glycine, and cysteine [17, 18]. Subsequent study demonstrated that THI4p expression is repressed by thiamine and disruption of the gene generates strains that are auxotrophic for thiamine, but can grow in the presence of hydroxyethyl thiazole, one of the two precursor compounds of thiamine [16].

In recent years, it has been shown that THI4p catalyzes the conversion of NAD^+ and glycine to the ADP

Abbreviations: ActhiS, thiazole biosynthesis enzyme from *Acremonium chrysogenum*; ADT, adenylated thiazole (ADP adduct of 5-(2-hydroxyethyl)-4-methylthiazole-2-carboxylic acid); PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase; ThDP, thiamine diphosphorylated; ThMP, thiamine monophosphorylated.

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adduct of 5-(2-hydroxyethyl)-4-methylthiazole-2-carboxylic acid (ADT) in a single turnover reaction, and Cys205 of THI4p provides the sulfur to the thiazole (Fig. 1) [13-15]. This reaction is Fe^{2+}-dependent and generates inactive enzyme with the formation of dehydroalanine through loss of sulfur of Cys205, making THI4p a single turnover suicide enzyme. More recently, it was reported that MjThi4, an ortholog of THI4p from *Methanococcus jannaschii*, catalyzed the formation of ADT using exogenous sulfide. Later, it was found that the C205A variant of THI4p could catalyze the formation of ADT in the presence of sulfide [25, 26].

In our previous study, we compared the proteomics of two different cephalosporin C producers using two-dimensional (2-D) gel electrophoresis. One potential sulfur carrier, protein ActhiS, was identified and may be related to thiamine metabolism. The DNA sequence (*Acthi*) of ActhiS was obtained from RNA-seq and then deposited into a public database (GenBank: KF010923). An ActhiS overexpression mutant and an ActhiS silencing mutant indicated enhanced thiamine biosynthesis and reduced thiamine biosynthesis, respectively [27, 28]. Thus, we postulated that ActhiS may play an important role in thiamine biosynthesis, and it might be the thiazole biosynthesis enzyme in *Acremonium chrysogenum*. Here, we report the characterization of the ActhiS-bound metabolite that consists of ADT, and the preparation of active recombinant ActhiS that catalyzes the enzymatic reaction in the presence of Fe^{2+}.

MATERIALS AND METHODS

Overexpression and purification of ActhiS. Standard protocols were used for DNA manipulation [29]. The *Acthi* gene (GenBank accession number KF010923) was amplified with the Acthi-R1 and Acthi-R2 primers (see table). To overexpress ActhiS in *E. coli*, the amplified DNA fragment containing the coding region of ActhiS was digested with *Eco*RI and *Hin*dIII, then subcloned into pET-28a(+) vector to generate the overexpression plasmid pET28a-Acthi [28]. *Escherichia coli* BL21(DE3) bearing the pET28a-Acthi overexpression plasmid was cultured in LB medium containing kanamycin (50 μg/ml) at 37°C until the OD_{600} reached 0.6. The expression of the recombinant protein was induced by 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 25°C for 12 h. The cells were then harvested by centrifugation, and the cell pellets were stored at −20°C until needed.

To purify recombinant ActhiS, the cell pellets from 2 liters of culture were resuspended in 50 ml of 20 mM Tris buffer (pH 7.9) containing 300 mM NaCl. The mixture was lysed using a Nano Homogenize Machine (ATS Engineering Inc. AH100B), and the insoluble cell lysate was excluded by centrifugation at 8000 rpm for 10 min at 4°C. The supernatant containing soluble ActhiS was purified on Ni-NTA resin according to manufacturer's instructions (Novagen, USA). After elution, the protein was desalted using an Amicon Ultra 10-kDa centrifugal filter device (Millipore, USA) pre-equilibrated with 50-mM potassium phosphate buffer (pH 7.9). The concentration of total protein was determined using the Bradford Protein Assay Kit (Sangon Biotech, China) with bovine serum albumin (BSA) as the standard. The purified protein was stored at −80°C for later experiments.

Primers used in the study

Primer name	Primer sequence (5′-3′)
Acthi-R1	GCGGAATTCATGTCGCCTCCCGCTGCTAC
Acthi-R2	CCGTCTAGATCAGACCGCATTCTCCTTCT
R3	TCCATGGACCCCAACACCATCAACG
R4	GGACTGGTCGTCATGGTGCATGGTGACG

SDS-PAGE. The purity and molecular mass of ActhiS were evaluated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [30]. The molecular weight of ActhiS was determined by comparison of its electrophoretic mobility with molecular weight standard markers.

Analyzing bound ligands. The purified ActhiS was denatured at 100°C for 3 min, and the precipitated protein was removed by centrifugation. The supernatant was filtered through an Amicon Ultra 10-kDa centrifugal filter device, and 500 μl of the filtrate was analyzed by reverse phase HPLC (C18 column, XSELECT™ HSS T3, 5 μm, 4.6 × 150 mm).

The following mobile phase was used at 1 ml/min flow rate (solvent A is methanol, solvent B is water, solvent C is 100 mM potassium phosphate, pH 6.6); 0 min – 100% C; 5 min – 10% B/90% C; 8 min – 15% A/25% B/60% C; 14 min – 15% A/25% B/60% C; 19 min – 30% A/30% B/40% C; 21 min – 100% C; 30 min – 100% C [13]. The absorbance at 261 nm was monitored. The UV-visible spectra of the resolved components were analyzed by an inline diode array UV detector. The UV-visible spectra of the bound small molecules from ActhiS were analyzed within the 200-700 nm range using a UV-visible spectrophotometer (BioMate 3S; Thermo).

Purification of the product. The purified ActhiS obtained from 10 liters of culture was concentrated to 100 ml by ultrafiltration using a 10-kDa membrane (Millipore) and aliquoted into 10-ml fractions. Each fraction, frozen at −80°C and lyophilized, was supplemented with 1 ml of distilled water and denatured at 100°C for 3 min. The precipitated protein was removed by centrifugation, and the supernatant was filtered through an Amicon Ultra 10-kDa centrifugal filter device. All the filtrates were analyzed by reverse phase HPLC as

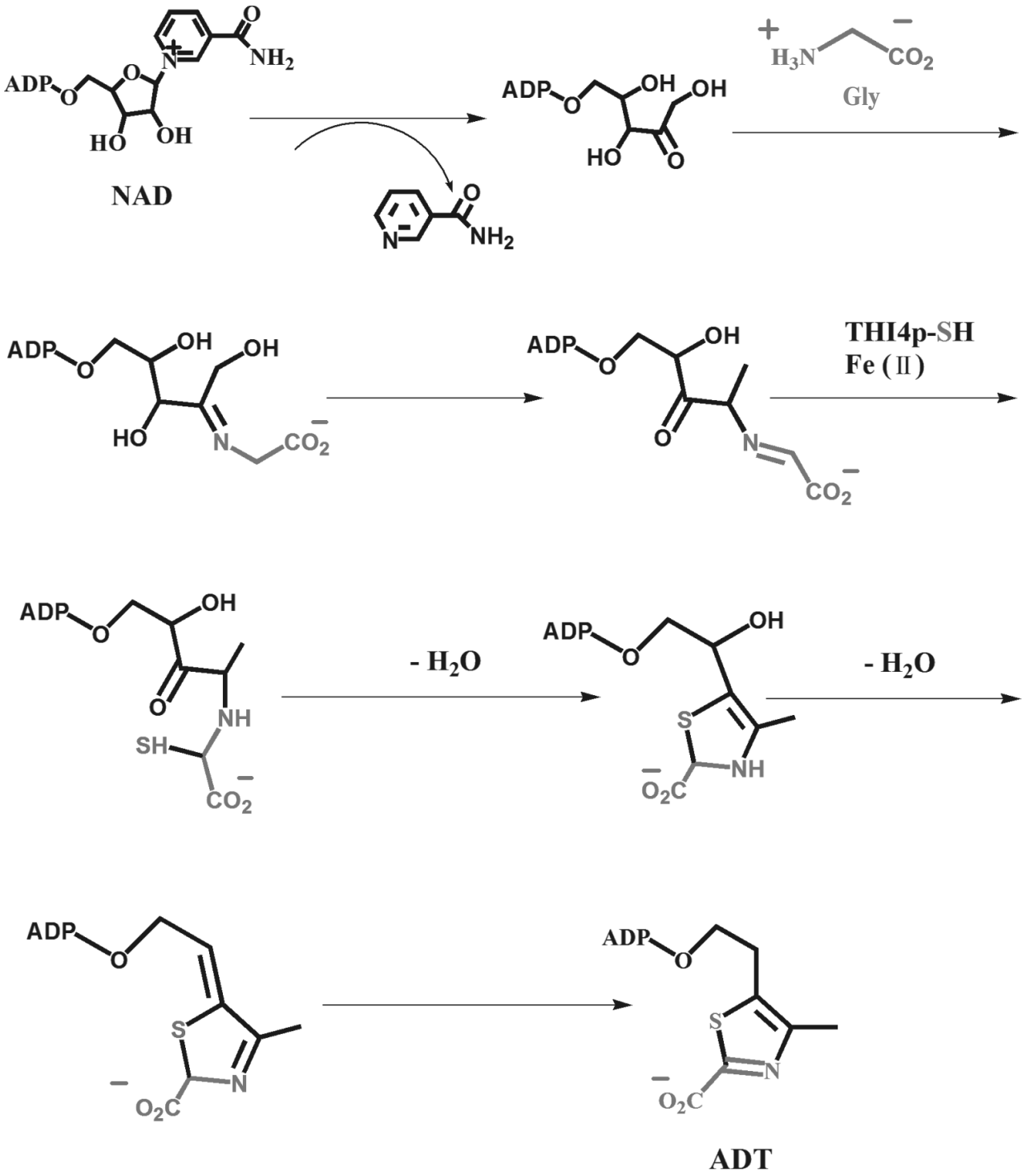

Fig. 1. Proposed scheme of biosynthesis of thiazole in *Saccharomyces cerevisiae*. THI4p catalyzes the conversion of NAD^+ and glycine to ADT in a single turnover reaction, and Cys205 of THI4p provides the sulfur to the thiazole.

described above. One major peak (retention time 9.59 min) was collected in tubes, frozen at −80°C, and lyophilized later.

NMR and LC-MS analysis of the purified product. The lyophilized product was dissolved in 500 µl D_2O and subjected to ^1H NMR analysis (Bruker Avance III 400 MHz). A sample of adenosine diphosphate (ADP), believed to be a precursor of ADT, was also assayed to identify the ligands from the protein.

The lyophilized product was redissolved in 50 µl of methanol, and 5 µl of the solvent was injected into the LC-MS system. The fractions were separated on a

reverse-phase Luna C18 (2.0 × 150 mm, ID: 5 μm) column by a linear gradient between eluent A (1 mM ammonium acetate in methanol containing 0.2% formic acid) and eluent B (2 mM ammonium acetate in water containing 0.2% formic acid).

***In vitro* enzymatic reaction catalyzed by ActhiS.** NAD^+ (final concentration 2 mM), glycine (final concentration 2 mM), and freshly prepared $FeSO_4$ (final concentration 0.5 mM) were combined with the purified recombinant protein (500 μM) expressed in M9 minimal medium to initiate the reaction. The control reaction was set up by omitting $FeSO_4$. The reactions were incubated at 25°C for 6 h, heat quenched, and analyzed by HPLC as described above.

Point mutation of ActhiS. Point mutagenesis was performed by a standard protocol using a KOD-Plus-Mutagenesis Kit (Toyobo Co., Ltd, Japan). For C217S, primers R3 and R4 (see table) were designed.

RESULTS

Overexpression and purification of ActhiS. ActhiS purified from *E. coli* was analyzed by SDS-PAGE. As shown in Fig. 2, we obtained ActhiS with purity higher than 90% and molecular mass of about 36 kDa.

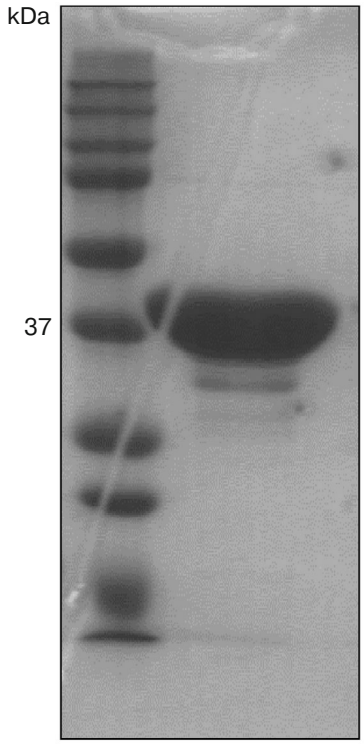

Fig. 2. Overexpression and purification of ActhiS. SDS-PAGE indicated that the molecular mass of ActhiS was about 36 kDa.

Sequence alignment. The sequence of ActhiS was BLAST searched in the current available protein databases to find homologous proteins. The results revealed that the ActhiS sequence has the highest homology with CyPBP37 protein (*N. crassa*), THI4p (*S. cerevisiae*), and THI1 (*A. thaliana*), which are the only known enzymes to participate in thiazole biosynthesis in eukaryotes. The sequence identities with these three published protein were 83.81, 61.31, and 58.84%, respectively. The sequence of MjThi4 was also added to the sequence alignment. The identity between ActhiS and archaeal MjThi4 was only 26.5%. Besides, the result showed that MjThi4 lacks the cysteine residue in position 164 (corresponds to C217 in ActhiS and C205 in THI4p), which was replaced by histidine (Fig. 3).

***In vitro* activity assay of ActhiS.** To study the activity of ActhiS, a full reaction with NAD^+, Gly, ActhiS (isolated from LB medium), and freshly prepared $FeSO_4$ was performed as reported by Chatterjee et al. [13]. Comparison of the full reaction (Fig. 4, curve *1*) and control (Fig. 4, curve *3*) showed the formation of a new compound (retention time 9.59 min). However, the HPLC analysis of denatured ActhiS alone (Fig. 4, curve *2*) displayed the same peak (Fig. 4, inset). Thus, we speculated that ActhiS overexpressed in LB medium was inactive due to binding of the product.

THI4p could use itself as a sulfur donor for thiazole formation during the process of protein overexpression, leading to the inactivation of the enzyme. After the reaction was completed, the reaction product ADT could bind to the active site of the THI4p, which was revealed by the structure of THI4p [15, 31]. Since LB medium contains the substrates and the iron needed for the enzymatic reaction, we speculated that ActhiS overexpressed in LB medium has already bound to the enzymatic reaction product and is inactive, which can explain the full reaction result above.

Next, ActhiS overexpressed in LB was heat-denatured to study the bound ligands. HPLC analysis revealed that four bound molecules were present (Fig. 5a, curve *1*). Meanwhile, the non-denatured ActhiS showed nothing released (Fig. 5a, curve *2*). Besides, these four bound molecules had a maximum absorption at 260 nm (Fig. 5b), suggesting that the bound molecules might be composed of adenine dinucleotide motif. This result indicated that ActhiS, which can release the bound molecules, has already completed the reaction and become inactive during the process of overexpression. We tried to obtain active ActhiS without bound molecules using minimal medium for overexpression.

M9 minimal medium was used to express ActhiS to obtain active protein. HPLC analysis of the bound metabolites from expressed protein after denaturing demonstrated apparent decrease in peak A (retention time 9.59 min) (Fig. 5c, curve *4*). After 200 μM Fe^{2+} was added, the HPLC analysis result (Fig. 5c, curve *3*) was similar to the result of protein overexpressed in LB. This suggested that the catalytic reaction of ActhiS is associat-

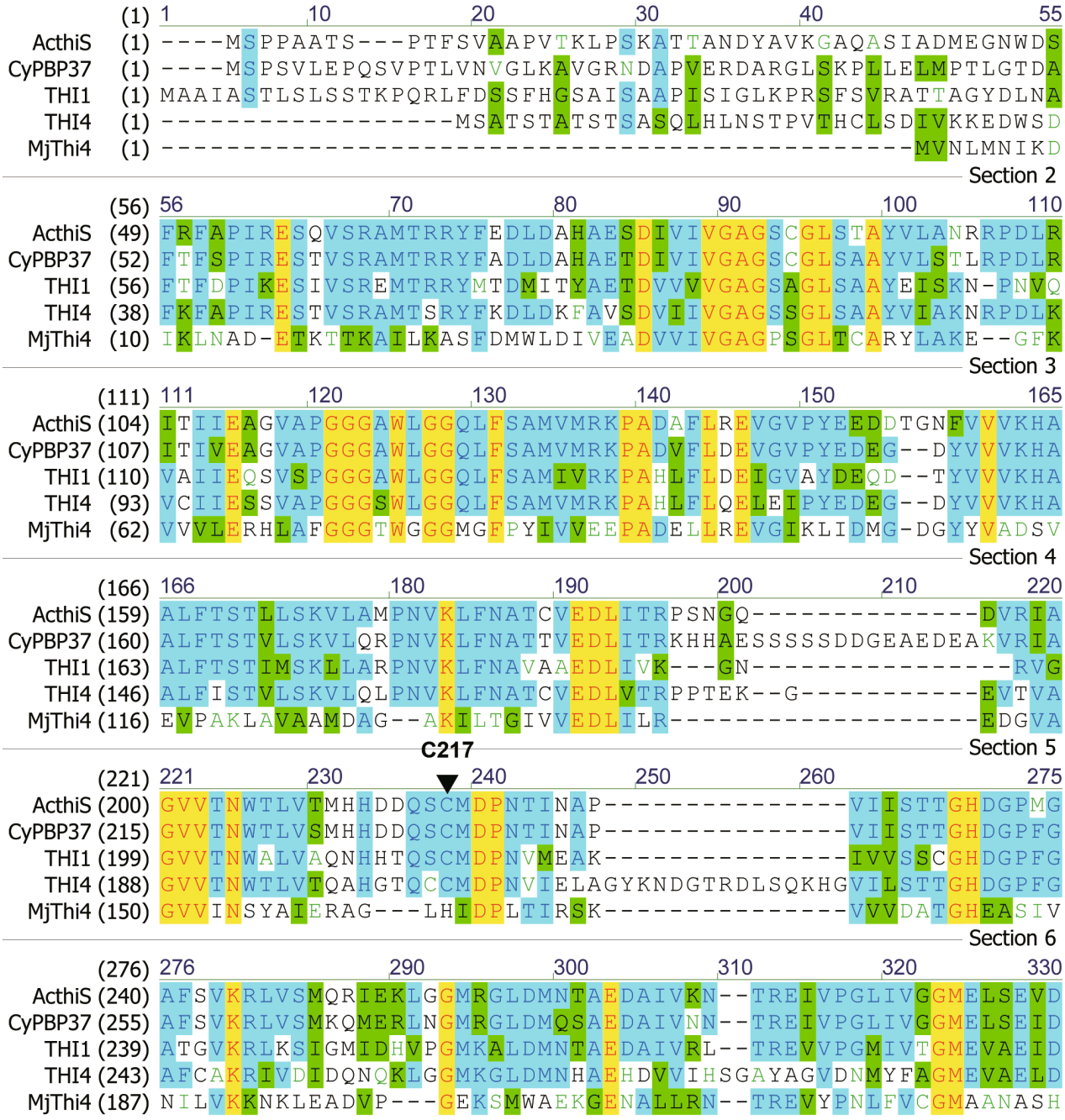

Fig. 3. Sequence alignment. Alignment of amino acid sequences of ActhiS with those of CyPBP37 protein (*N. crassa*), THI4p (*S. cerevisiae*), THI1 (*A. thaliana*), and MjThi4 (*M. jannaschii*). Identical residues are highlighted in yellow, blocks of similar are highlighted in green, and conservative residues are highlighted in blue, which was generated in the Vector NTI program. The conserved cysteine is indicated by an arrow.

ed with Fe^{2+}, and peak A is most likely to be the product related to the reaction that is mediated by Fe^{2+}.

Characterization of the bound ligand. The component with retention time of 9.59 min (peak A on Fig. 5) was purified by preparative HPLC. Figure 6A shows a selected region from the ^1H NMR for the ligands obtained from the ActhiS along with the spectrum for ADP, a molecule considered to be part of the NAD^+ structure. The spectrum of ADP was used to help assign the peaks in the NMR spectra of the purified product (retention time 9.59 min) obtained from ActhiS. The peaks arising from the pentose proton (Fig. 6A, peaks c-f) and from two adenine protons (Fig. 6A, peaks a and b) of purified peak A could be assigned and confirmed by using chemical shifts previously reported [13, 14]. The spectra also showed the presence of ADP in the sample. We conclude that NAD^+

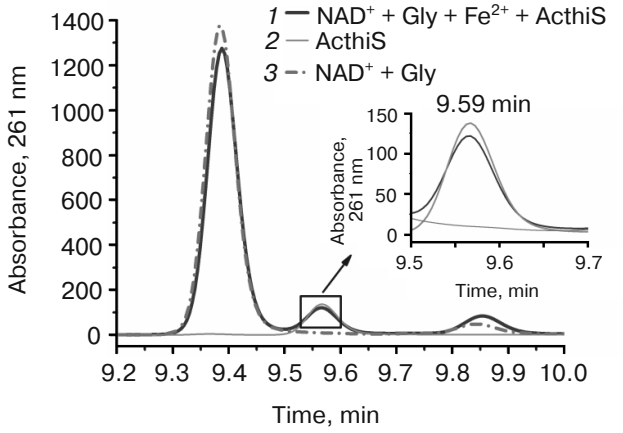

Fig. 4. HPLC analysis of ActhiS (overexpressed in LB medium) catalyzing the full reaction and relevant control reactions. Curves: *1*) full reaction; *2*) control reaction, where denatured ActhiS was alone; *3*) control reaction (NAD^+ + Gly). The compound (retention time 9.59 min) is shown in the inset.

is one of the substrates for ActhiS, and the purified bound ligand is ADT.

Positive-mode LC-MS analysis identified the molecular mass of peak A to be 596 Da (Fig. 6B). This result agrees with that of the ^1H NMR analysis. We concluded that peak A is ADT produced by the reaction catalyzed by ActhiS. Thus, we determined that ActhiS is the thiazole biosynthesis enzyme in *A. chrysogenum*.

***In vitro* reconstitution of ActhiS-catalyzed reaction.** To accomplish reconstitution of the ActhiS-catalyzed reaction, NAD^+, Gly, and freshly prepared $FeSO_4$ were mixed with the purified ActhiS (overexpressed in M9 minimal medium) to initiate the reaction. A control reaction was set up without Fe^{2+}. After 6 h, the reaction mixture was heat quenched and analyzed by HPLC as previously described. Figure 7 showed that the formation of ADT *in vitro* in the presence of Fe^{2+} is successful. This further showed that the reaction is Fe^{2+}-dependent.

Activity assay of mutant C217S. Cys205 in THI4p is strictly conserved in eukaryotic thiazole synthases, and it donates the sulfur atom to thiazole [15]. Multiple sequence alignment between ActhiS and orthologs indicated that C217 of ActhiS was most likely the donor of the sulfur atom. Thus, we mutated Cys217 of ActhiS to Ser to study the role of C217. HPLC analysis revealed that there was no peak of ADT released after denaturing mutant C217S (expressed in LB medium) (Fig. 8a). This could be either due to a weaker affinity of the mutant towards its reaction products, or due to a catalytical impairment of the mutant that makes it unable to produce the later intermediates. Thus, full reconstitution of the C217S catalyzed reaction was carried out. NAD^+, glycine, and freshly prepared $FeSO_4$ were mixed with the purified C217S (expressed in M9 medium) to initiate the reaction. After 6-h reaction, HPLC analysis still showed no production of ADT (Fig. 8b). This result demonstrated that the second suggestion (mutant C217S is unable to catalyze the biosynthesis of ADT) is valid rather than the first (weaker mutant affinity towards its reaction products). Thus, this refers to the change of the sulfur-donating residue, which indicates that the C217 is the sulfur-donating residue.

We then tested whether C217S can use exogenous sulfur sources (cysteine, sodium thiosulfate, sodium thioglycolate, sodium sulfide) to catalyze the reaction of ADT formation in the presence of Fe^{2+}, as MjThi4 and the C205A variant of THI4p do [25, 26]. HPLC analysis showed that only sodium sulfide led to the formation of ADT (Fig. 8c). This indicated that ActhiS uses the C217 as the sulfur-donating residue. However, when Cys217 of ActhiS was mutated, the enzyme could also use exogenous sodium sulfide as the sulfur donor.

DISCUSSION

Our laboratory has been working on genetic engineering of *A. chrysogenum* for many years [32]. In a previ-

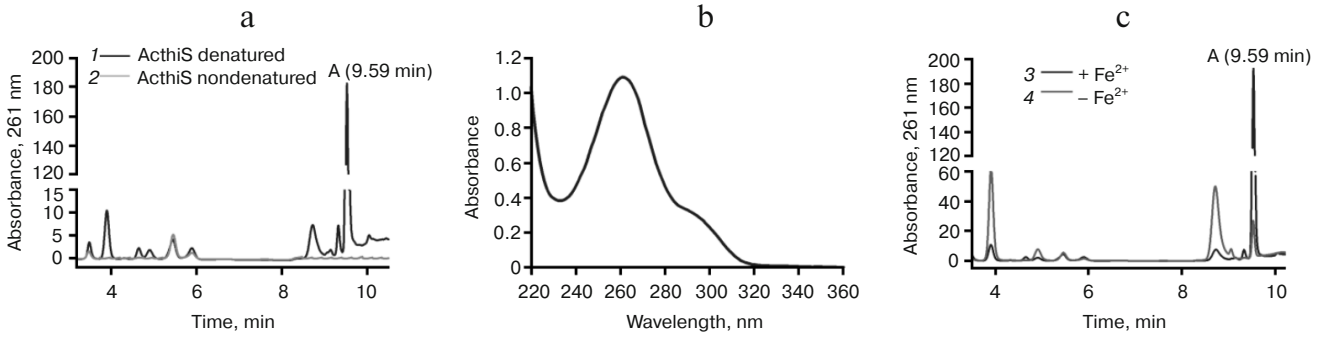

Fig. 5. Identification of ActhiS-bound molecules. a) HPLC analysis of the ActhiS (overexpressed in LB medium)-bound ligands. Curves *1* and *2* refer to denatured AchiS and non-denatured ActhiS, respectively. b) UV-vis spectroscopy of the bound ligands. c) Analysis of the ActhiS (overexpressed in M9 minimal medium ± 200 μM Fe^{2+})-bound ligands. Curve *3* refers to 200 μM Fe^{2+} added to M9 minimal medium, and curve *4* refers to M9 minimal medium without 200 μM Fe^{2+}.

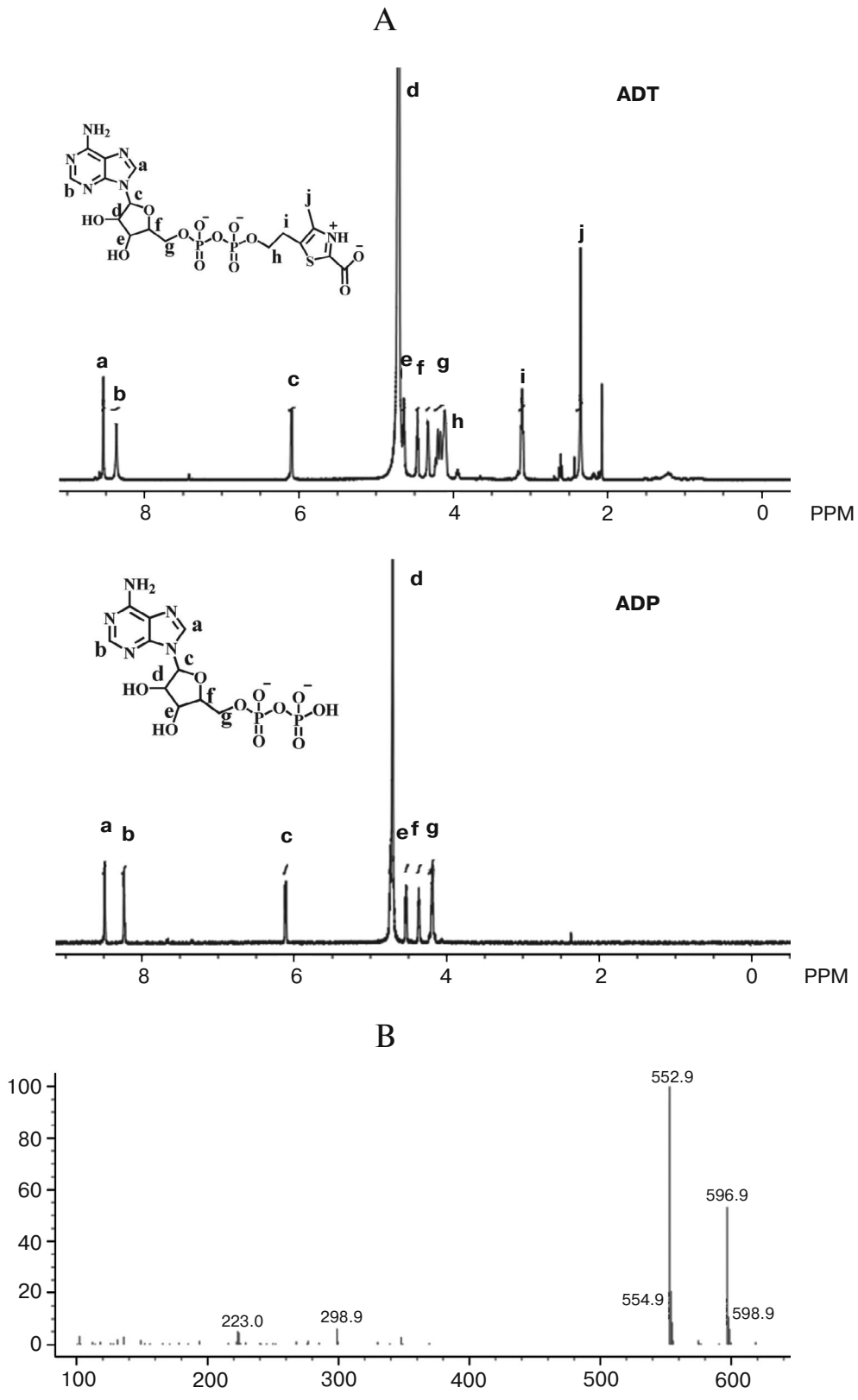

Fig. 6. [1]H NMR and LC-MS positive-mode spectra of the purified peak of bound ligand from ActhiS and of ADP. A) Peaks arising from the pentose proton (c-f) and from two adenine protons (a, b) of purified peak A. ADP peaks were present in both samples. All the peaks were noted on the structure of ADT and ADP. B) Molecular mass of peak A is 596 Da, which is consistent with the molecular mass of ADT.

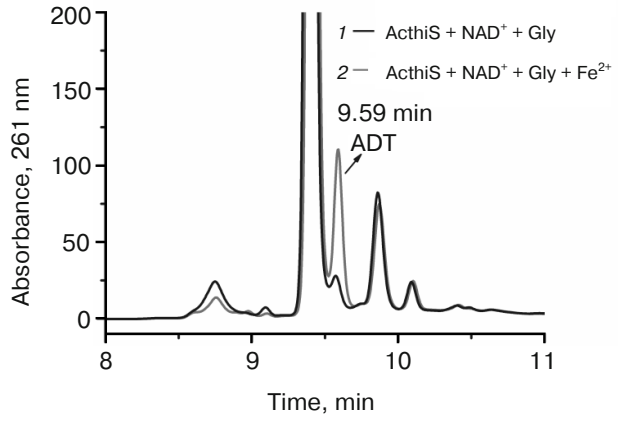

Fig. 7. *In vitro* reconstitution of ActhiS-catalyzed reaction. Curves: *1*) control reaction (ActhiS + NAD^+ + Gly) (black line); *2*) full reaction (ActhiS + NAD^+ + Gly + Fe^{2+}) (gray line).

ous work, we found that ActhiS plays a considerable role in thiamine biosynthesis according to the results of *Acthi* silencing and overexpression in *A. chrysogenum* [28]. Sequence alignment also revealed that this is a putative thiazole biosynthesis enzyme. In this study, the function of ActhiS and its role in thiazole biosynthesis are identified using a series of biochemical methods *in vitro* for the first time. We used gel electrophoresis to determine the molecular mass of ActhiS. We demonstrated that denatured ActhiS expressed in LB medium could release small molecules. The ^1H NMR results showed that the structure of one molecule (retention time 9.59 min) matched the structure of ADT reported earlier [13]. Afterwards, ligand-free ActhiS was obtained when the overexpression strain was grown in M9 minimal medium, which provided a source of native enzyme with an unoccupied active site. Transforming NAD^+ and glycine by this active form of the enzyme in the presence of Fe^{2+} generates ADT *in vitro*. Further study indicated that the sulfur of the thiazole is derived from Cys217. Taken together, the observations reported here suggest that ActhiS is an enzyme involved in thiazole biosynthesis pathway in *A. chrysogenum* and that it uses a THI4p-type mechanism for sulfur relay to the thiazole ring.

ActhiS is homologous with CyPBP37 protein (*N. crassa*), THI4p (*S. cerevisiae*), and THI1 (*A. thaliana*), which are the only known enzymes to participate the thiazole biosynthesis in eukaryotes. The result of the BLAST search is also consistent with the conclusion of the published work that plants and fungi share a common pathway of thiazole biosyntheses [33]. We also found using alignment that MjThi4 has a histidine residue in the conserved position of the active site instead of the cysteine residue (Cys217) of the ActhiS. The Cys217 residue of ActhiS, which is highly conserved among eukaryotic homologs, is required for ActhiS activity and believed to be a sulfur donor in a Fe^{2+}-mediated sulfur transfer reaction to form the thiazole ring. MjThi4 lacking the conserved cysteine utilizes sulfide as a sulfur source. When the Cys217 of ActhiS was mutated to Ser, the enzyme could also use exogenous sodium sulfide as the sulfur donor.

Why are THI4p and ActhiS suicide enzymes when there appears to be no chemical necessity to use the Cys residue as the sulfur donor? We think that the "inactive" form of THI4p and ActhiS may have additional biological activities. To support this, other features of ActhiS and its orthologs are worth mention. CyPBP37 can bind to NcCyP41 (a CyP40-type protein) in *N. crassa*, forming a complex that implies an important function in cells [23, 24]. Genetic studies indicated that the THI1 protects mitochondrial DNA from damage. Our previous study also indicated that ActhiS expression level correlated with cephalosporin C production [28]. These observations suggest that ActhiS is likely to have alternative physiological functions *in vivo*, which needs further investigations.

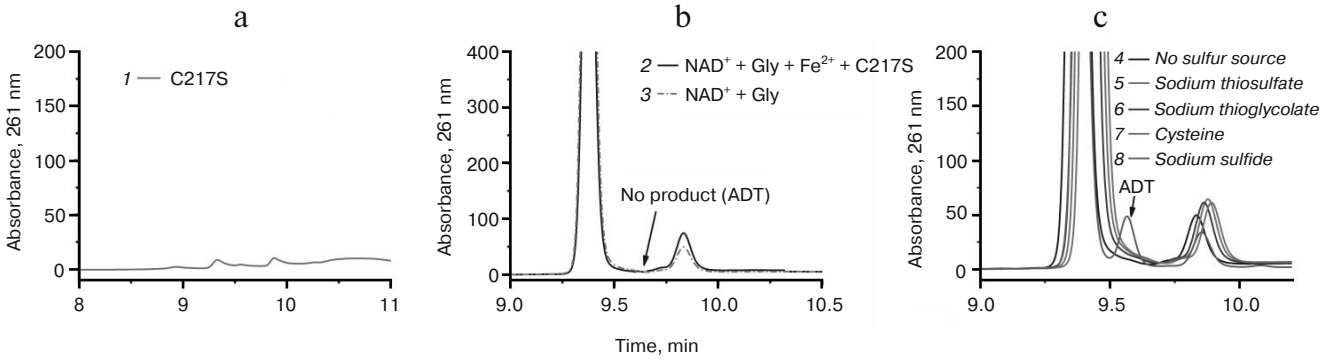

Fig. 8. Activity assay with mutant C217S. a) HPLC analysis of bound ligands of the C217S mutant (curve *1*). b) HPLC analysis of products of C217S-catalyzed full reaction and control reaction. Curves: *2*) full reaction; *3*) control reaction (NAD^+ + Gly). c) HPLC analysis of products of C217S-catalyzed reaction in the presence of different sulfur sources. Curves: *4-8*) no sulfur source and with sodium thiosulfate, sodium thioglycolate, cysteine, or sodium sulfide, respectively. Product ADT is only present in curve *8*.

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