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Structural Basis for Iron-mediated Sulfur Transfer in Archaeal and Yeast Thiazole Synthases†

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Abstract

Thiamin diphosphate is an essential cofactor in all forms of life and plays a key role in amino acid and carbohydrate metabolism. Its biosynthesis involves separate syntheses of the pyrimidine and thiazole moieties, which are then coupled to form thiamin monophosphate. A final phosphorylation produces the active form of the cofactor. In most bacteria, six gene products are required for biosynthesis of the thiamin thiazole. In yeast and fungi only one gene product, Thi4, is required for thiazole biosynthesis. *Methanococcus jannaschii* expresses a putative Thi4 ortholog that was previously reported to be a ribulose 1, 5-bisphosphate synthase [Finn, M. W. and Tabita, F. R. (2004) *J. Bacteriol.* 186, 6360–6366]. Our structural studies show that the Thi4 orthologs from *M. jannaschii* and *Methanococcus igneus* are structurally similar to Thi4 from *Saccharomyces cerevisiae*. In addition, all active site residues are conserved except for a key cysteine residue, which in *S. cerevisiae* is the source of the thiazole sulfur atom. Our recent biochemical studies showed that the archaeal Thi4 orthologs use nicotinamide adenine dinucleotide, glycine and free sulfide to form the thiamin thiazole in an iron-dependent reaction [Eser, B., Zhang, X., Chanani, P. K., Ealick, S.E., and Begley, T.P. (2015) submitted]. Here we report X-ray crystal structures of Thi4 from *M. jannaschii* complexed with ADP-ribulose, the C205S variant of Thi4 from *S. cerevisiae* with a bound glycine imine intermediate, and Thi4 from *M. igneus* with bound glycine imine intermediate and iron. These studies reveal the structural basis for the iron-dependent mechanism of sulfur transfer in archaeal and yeast thiazole synthases.

Thiamin pyrophosphate (ThDP 4) plays a key role in branched amino acid biosynthesis and carbohydrate metabolism, and is an essential cofactor in all living systems. Its biosynthesis

†The coordinates for ScThi4C205S/glycine imine, MjThi4/ADPr1, and MiThi4/Fe/glycine imine have been deposited in the Protein Data Bank under accession codes 4Y4L, 4Y4M, and 4Y4N, respectively.

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SUPPORTING INFORMATION

Supplementary Figure 1 is the ribbon diagram of the overall structure of ScThi4C205S.

Supplementary Figure 2 is the ribbon diagram of the overall structure of MiThi4.

Supplementary Figure 3 is the multiple sequence alignment result of archaeal Thi4 homologs with eukaryotic Thi4 homologs.

Supplementary Figures 4 and 5 show the activity of the ScThi4C205S variant.

Supplementary Figure 6 shows the activity of the ScThi4D207A variant.

Supplementary Figure 7 shows the activity of the ScThi4H237N variant.

This material is available free of charge via the Internet at <http://pubs.acs.org>.

requires the separate formation of the pyrimidine and thiazole moieties, which are then coupled to form thiamin monophosphate (ThMP **3**) which is then phosphorylated to form the cofactor (Scheme 1). The mechanism of thiazole formation in bacteria has been elucidated and involves a complex oxidative condensation in which five different enzymes and a sulfide carrier protein catalyze reactions involving three substrates (1-deoxy-D-xylulose-5-phosphate, cysteine, and glycine or tyrosine).^{1–3} In yeast, fungi, and plants, only one gene product, Thi4, is required for thiazole biosynthesis, and nicotinamide adenine dinucleotide (NAD) **5**, glycine **8** and cysteine are the substrates.^{4–6} Our previous results showed that *Saccharomyces cerevisiae* Thi4 (ScThi4) is a suicide thiazole synthase that uses a conserved Cys205 residue as the sulfur source, and that the sulfur transfer reaction is Fe(II) dependent.⁷ We have recently shown that the C205A variant of the yeast enzyme can utilize sulfide as the sulfur source.⁸ A mechanistic proposal for the Thi4 catalyzed formation of adenosine diphosphate thiazole (ADT **17**) is shown in Figure 1.⁷

Putative orthologs of Thi4 are also found in archaea. The Thi4 ortholog in *Methanococcus jannaschii* (MjThi4, also known as Mj0601) was first characterized as an NAD-dependent ribulose 1, 5-bisphosphate (RuBP) synthase, which converts ribose 1,5-bisphosphate to RuBP.⁹ *M. jannaschii* is a thermophilic methanogenic archae with 85 °C as the optimum living temperature¹⁰ and is found in anaerobic environments with high levels of sulfide.¹¹ We recently showed that MjThi4, which lacks the equivalent of ScThi4 Cys205, synthesizes the thiamin thiazole moiety (ADT **17**) using NAD, glycine, and sulfide in an iron-dependent reaction.⁸

Here, we report the structure of MjThi4 with bound ADP-ribulose (ADPr1 **7**), which copurified with the enzyme. We also report the structure of the C205S variant of ScThi4 with a bound glycine imine intermediate **12**, and the structure of *Methanococcus igneus* Thi4 (MiThi4) with **12** and Fe(II). These structures suggest a mechanism for iron-dependent sulfur transfer in the formation of the thiamin thiazole. Finally, we were unable to demonstrate RuBP synthase activity using purified MjThi4, suggesting that in archaea the only function for the Thi4 orthologs is biosynthesis of the thiamin thiazole.

MATERIALS AND METHODS

Cloning, Overexpression and Purification

The overexpression plasmid for the *M. jannaschii* Thi4 gene was constructed at the Cornell Protein Production Facility. Plasmid DNA was purified with a Fermentas GeneJet miniprep kit and DNA fragments were purified from agarose gel with a Zymoclean Gel DNA Recovery kit from Zymo Research. *Escherichia coli* strain MachI (Invitrogen) was used as a recipient for transformations during plasmid construction and for plasmid propagation and storage. Phusion DNA polymerase (New England Biolabs) was used for PCR following the manufacturer's instructions. PfuTurbo DNA polymerase (Stratagene) was used for mutagenesis. All restriction enzymes were purchased from New England Biolabs. The plasmid THT, which was purchased from Novagen, is a derivative of pET-28 and incorporates an N-terminal polyhistidine tag cleavable by TEV protease and a short sequence to improve solubility.

The *Thi4* gene was amplified using standard PCR conditions with desired primers. The amplified product was purified and digested with *NdeI* and *XhoI*, and inserted into similarly digested pTHT vector to yield MjThi4/pTHT and MiThi4/pTHT. Mutagenesis was performed following a standard PCR-mediated site directed protocol using primers with the desired mutation. The wild type ScThi4/p28 and MjThi4/pTHT clones were used as the template to generate the ScThi4C205S/p28 and MjThi4H164C/THT variants, respectively.

The *Thi4* plasmids were transformed into *E. coli* BL21(DE3) (Novagen) cells and grown with shaking overnight at 37 °C in a 20 mL starter culture of Luria-Bertani (LB) media containing 30 µg/mL kanamycin. The overnight starter culture was then transferred into 1.5 L of either LB medium or M9 minimal medium and incubated at 37 °C with shaking until reaching an OD₆₀₀ of ~0.6. In the case of M9 minimal medium, the medium was supplemented with 0.2% glucose, 1 mM MgSO₄ and 0.1 mM CaCl₂. The culture was then induced with 0.5 mM isopropyl 1-β-D-galactopyranoside (IPTG) and incubation was further carried out for 18 – 24 h at 15 °C. Cells were harvested by centrifugation at 1000 g for 30 min at 4 °C. The cell pellet was collected and frozen at –20 °C for storage.

The N-terminal hexahistidine-tagged MjThi4 was first purified by Ni-nitrilo acetic acid (Ni-NTA) affinity chromatography. Cell pellets from 2 L of culture were resuspended in a lysis buffer containing 20 mM Tris, 500 mM NaCl, 10 mM imidazole, pH 8.0, and lysed by sonication. The lysate was centrifuged at 4500 g for 30 min at 4 °C and the resulting supernatants were subjected to a column containing 2 mL Ni-NTA resin (Qiagen) preequilibrated with the lysis buffer. The column was washed with 10 column volumes of buffer containing 20 mM Tris, 500 mM NaCl, 30 mM imidazole, pH 8.0, and bound protein was recovered with 20 mM Tris, 500 mM NaCl, 250 mM imidazole, pH 8.0. The eluted samples were further purified using a Superdex G200 column (GE Healthcare) with 10 mM Tris, 50 mM NaCl, pH 8.0, and the peaks corresponding to MjThi4 were pooled, concentrated to 15 mg/mL using an Amicon concentrator (10 kDa MWCO filter, Millipore), flash frozen, and stored at –80 °C. MiThi4 was purified using the same procedure.

N-terminal hexahistidine-tagged ScThi4C205S was expressed in M9 minimal media and purified in a glove box under anaerobic conditions. Cell pellets from 2 L of culture were lysed by sonication on ice in 20 mM Tris, 500 mM NaCl, 10 mM imidazole, pH 8.0. The resulting supernatant was subjected to a Ni-NTA column and the column was washed with 10 column volumes of buffer containing 20 mM Tris, 500 mM NaCl, 50 mM imidazole, pH 8.0. Bound protein was recovered by elution with 20 mM Tris, 500 mM NaCl, 250 mM imidazole, pH 8.0. The eluted samples were buffer exchanged into 10 mM Tris, 50 mM NaCl, pH 8.0 using an Econo-Pac 10DG desalting column (Bio-Rad). The purified protein was concentrated to 10 mg/mL using an Amicon Ultra-0.5 concentrator (10 kDa MWCO filter, Millipore) and incubated at room temperature for 1 h with 2 mM NAD, 2 mM glycine, 0.5 mM ferrous ammonium sulfate (FeAS), and 2 mM dithiothreitol.

Crystallization of MjThi4

Crystals of MjThi4 were grown using the vapor diffusion hanging drop method. Subsequent analysis showed that MjThi4 selectively crystallized as the MjThi4/ADPrI 7 complex. Equal volumes of protein and reservoir solutions were mixed and equilibrated at 22 °C against a

total volume of 400 μ L well solution. The initial crystallization condition was determined using the commercially available Wizard I sparse matrix screen (Emerald Biosystems). The optimized condition for MjThi4/ADPrI 7 was 1.05 M potassium/sodium tartrate, 0.1 M *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES)/sodium hydroxide, pH 9.5 and 0.2 M lithium sulfate. Rod shaped crystals grew in about six days to their maximum size of 0.5–0.6 mm $\times$ 0.1–0.2 mm. The crystals were cryoprotected for data collection in a solution composed of the mother liquor supplemented with 2.5% (v/v) ethylene glycol.

Crystallization of ScThi4C205S/Glycine Imine

Crystals of ScThi4C205S were grown under anaerobic conditions using the vapor diffusion hanging drop method. Equal volumes of protein and reservoir solutions were mixed and equilibrated at 22 °C against a total volume of 400 μ L well solution. The initial crystallization condition was determined using the commercially available Wizard IV sparse matrix screen (Emerald Biosystems). The optimized conditions were 25% (w/v) PEG1500, 0.0125 M succinic acid, 0.044 M sodium dihydrogen phosphate, and 0.044 M glycine, pH 8.5. ScThi4C205S crystals grew in about 10 days to their maximum size of ~0.1 mm $\times$ 0.1 mm $\times$ 0.4 mm. Crystals were cryoprotected for data collection in a solution composed of the mother liquor supplemented with 15% (v/v) glycerol.

Crystallization of MiThi4/Fe/Glycine Imine

MiThi4 (300 μ L, 500 μ M) was incubated anaerobically with 1.5 equivalents of FeAS at 22 °C for 30 min. The mixture was then incubated with 1.5 equivalents of adenosine diphosphate ribose (ADPr) at 80 °C for 30 min, and finally 1.5 equivalents of glycine were added to the reaction system and incubated at 80 °C for a further 30 min to form MiThi4/Fe/glycine imine 12. The protein complex was concentrated to 15 mg/mL and removed from the glove box for further crystallization experiments. The initial crystallization conditions for MiThi4/Fe/glycine imine 12 were determined using the commercially available Wizard II sparse matrix screen (Emerald Biosystems). The optimized condition was 1.0 M sodium citrate, 0.1 M CHES/sodium hydroxide, pH 9.5. Bipyrimidal-shaped crystals grew in about 4 days to a maximum dimension of 0.4–0.5 mm. Crystals were cryoprotected for data collection in a solution composed of the mother liquor supplemented with 2.5% (v/v) ethylene glycol.

X-ray Data Collection and Processing

X-ray diffraction data for MjThi4/ADPrI 7, ScThi4C205S/glycine imine 12 and MiThi4/Fe/glycine imine 12 were collected at the Advanced Photon Source Northeastern Collaborative Access Team (NE-CAT) beamline 24-ID-C using an ADSC Q315 CCD detector with a crystal to detector distance of 475 mm for MjThi4/ADPrI 7, 260 mm for ScThi4C205S/glycine imine 12, and 320 mm for MiThi4/Fe/glycine imine 12. The wavelength was 0.9791 Å and the data collection temperature was 100 K. Individual frames were collected using 1 s for each 1.0° oscillation over a range of 180° for MjThi4/ADPrI, 120° for ScThi4C205S/glycine imine 12, and 150° for MiThi4/Fe/glycine imine 12. X-ray diffraction data were indexed, integrated, scaled, and merged using the program HKL2000.¹² Data collection and processing statistics are shown in Table 1.

Structure Determination and Refinement

The structure of MjThi4/ADPr 7 was determined by molecular replacement using Phaser¹³ as implemented in the PHENIX¹⁴ program package. An octamer of *Arabidopsis thaliana* thiamin thiazole synthase (AtThi1) (PDB entry 1RP0)¹⁵, which shares a 32% sequence identity with MjThi4, was used as the template to generate a search model using Chainsaw¹⁶. The initial refinement resulted in an R-factor of 33.1% and R-free of 39.7%. Side chains were added and the model was manually adjusted using COOT¹⁷ and refined using Refmac 5.0.¹⁸ After several cycles of refinement and model building, ADPr 7 was built into the active site based on the $F_o - F_c$ electron density, and then water molecules were added in the model. Geometry was verified using COOT¹⁷ and PROCHECK.¹⁹ The final refinement statistics are summarized in Table 1.

The structure of ScThi4C205S/glycine imine **12** was determined by molecular replacement using Phaser¹³ as implemented in the PHENIX¹⁴ program package. A dimer of wild-type ScThi4 (PDB entry 3FPZ)⁷ was used as the search model. The initial refinement resulted in an R-factor of 25.7% and R-free of 21.3%. The model was manually adjusted using COOT¹⁷ and refined using Refmac 5.0.¹⁸ After several cycles of refinement and model building, the glycine imine intermediate **12** was built into the active site based on the $F_o - F_c$ and composite omit maps, and then water molecules were added to the model. Geometry was verified using COOT¹⁷ and PROCHECK.¹⁹ The final refinement statistics are summarized in Table 1.

The structure of MiThi4/Fe/glycine imine **12** was determined by molecular replacement using Phaser¹³ as implemented in the PHENIX¹⁴ program package. An octamer of wild-type AtThi1 (PDB entry 1RP0),¹⁵ which shares 29% sequence identity with MiThi4, was modified using Chainsaw¹⁶ to generate a search model. The initial molecular replacement solution was refined to an R-factor of 40.8% and R-free of 46.9%. All side chains were added and the model was manually adjusted using COOT.¹⁷ After several cycles of refinement using COOT¹⁷, Fe, the glycine imine intermediate **12**, and water molecules were added to the model. The final model was refined to an R-factor of 19.9% and R-free of 21.6%. Refinement statistics are summarized in Table 1.

MjThi4 Activity Assays

All assays were performed in a glove box either at 25 °C or 75 °C with components prepared with degassed, deionized water. Sodium sulfide was prepared by passing H₂S through an aqueous solution of NaOH. The final concentration of the sulfide solution (5–20 mM Na₂S) was determined by both the 5,5'-dithiobis-(2-nitrobenzoic acid) and methylene blue methods.^{20, 21} The sulfide solutions were stored at highly basic solutions (pH>12) to prevent escape of H₂S and under anaerobic conditions to prevent oxidation. Stock solutions of ADPr **7**, NAD **5**, and glycine **8** were prepared at concentrations of 1 mM, 10 mM, and 100 mM, respectively. Buffer A (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.1, 0.1 M KCl, and 10% glycerol) was added to make the final volume of each mixture 100 µL. Purified MjThi4 (20–100 µM) was first incubated with FeAS (400 µM to 1 mM) for approximately 10 min to assure binding of iron to the enzyme and to prevent precipitation of iron with sulfide. The conversion of NAD **5** to ADT **17** by wild-type MjThi4

was carried out in the presence 100 μ M MjThi4, 300 μ M NAD **5**, 1 mM glycine **8**, 400 μ M FeAS and 800 μ M Na₂S at 25 °C, and quenched with 8 M guanidinium chloride. The activity assay for the H164C variant was performed in the presence or absence of 400 μ M Na₂S with 190 μ M variant enzyme, 500 μ M ADPr **7**, 1 mM glycine **8**, 500 μ M FeAS, and 1 mM dithionite at 75 °C. After incubation of the assay ingredients for 1 h for the sample with Na₂S or for overnight for the sample without Na₂S, the samples were quenched with equal volumes of 8 M guanidinium hydrochloride. The ScThi4 variants (C205S, D207A, and H237N) were similarly assayed. All the reaction components were allowed to sit for 2 h in the glovebox for complete deoxygenation. Purified enzyme was first incubated with freshly prepared FeAS (final concentration 400 μ M) for 10 min followed by the addition of sulfide (final concentration 600 μ M), glycine (final concentration 1 mM) and NAD or ADPr (final concentration 500 μ M). Reactions were incubated at room temperature for 6 h followed by quenching with 8 M guanidinium hydrochloride. The reaction mixture was deproteinized by passing through a 10 kD cut off filter and then analyzed by HPLC using a Supelcosil LC-18 reversed-phase column (150 mm $\times$ 4.6 mm, 3 μ m ID). The following linear gradient, at a flow rate of 1 mL/min, was used. Solvent A is water; solvent B is 100 mM K₂HPO₄, pH 6.6; and solvent C is methanol. 0 min: 100% B; 3 min: 10% A, 90% B; 10 min: 25% A, 60% B, 15% C; 14 min: 25% A, 60% B, 15%; 19 min: 30% A, 40% B, 30% C; 21 min: 100%B; 30 min 100% B.

Assay for the Putative RuBP Activity of MjThi4 by Derivatization of Downstream Reaction Products⁹

The derivatization reaction uses 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) as a coupling reagent and pyridine as a catalyst.²² Formation of RuBP **18** (Figure 2A) was tested by derivatization of 3-phosphoglycerate (3-PGA **19**), which would form upon reaction of RuBP with ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO).⁹The substrate proposed for RuBP **18** formation is ribose 1, 5-bisphosphate,⁹which is not available commercially. Ribose 1, 5-bisphosphate was generated from 5-phospho- α -D-ribose 1-diphosphate (PRPP) by incubating with Mg²⁺ at ~80 °C for a few hours at neutral pH.⁹To test for MjThi4 catalyzed RuBP **18** formation, apo MjThi4 (~500 μ M final) was incubated, in a glove box, with ribose 1, 5-bisphosphate (~5 mM final) and NAD **5** (final ~500 μ M) in 50 mM HEPES (pH 7.5). After a few hours, RuBisCo (~0.2 μ M final) and NaHCO₃ (20 mM final) were added and the resulting mixture was anaerobically incubated overnight at room temperature. A control reaction, containing FeAS (final ~ 500 μ M) in addition to the above components, was also run. The reaction mixture was then treated with calf intestinal phosphatase (CIP) for 1 h at 37 °C. For the derivatization (Figure 2)²², 200 μ L of the dephosphorylated reaction mixture were mixed with 2,4-DNPH (80 μ L of 40 mM solution in 1:1 0.2 M HCl/ethanol) and EDC (100 μ L of 125 mM solution in 1.5% pyridine, 50% ethanol, 48.5% water) and incubated at 80 °C for 1 h. The samples were then centrifuged to remove the precipitate and analyzed by HPLC using Supelcosil LC-18 reversed-phase column (150 mm $\times$ 4.6 mm, 3 μ m ID). Two mobile phases were used for HPLC analysis; (A) acidified water with HCl at pH 4.5, (B) acetonitrile. Elution was performed isocratically in 90% (A) and 10% (B) at a flow rate of 1 mL/min. The 2,4-DNP hydrazide **22** of 3-PGA could be detected with a sensitivity of 30 – 50 μ M.

Assay for RuBP Formation by ^{31}P NMR

MjThi4 (500 μM) was loaded with ~50% iron (to simultaneously assay the apo and the metalloenzyme) and reacted overnight in a glove box with ~5 mM PRPP and ~25 mM MgCl_2 at 50 $^\circ\text{C}$ in the presence and absence of NAD **5** (0.5 mM final). The samples, in 1 mL of $\text{D}_2\text{O}:\text{H}_2\text{O}$ (1:10), were transferred to 5 mm NMR tubes and ^{31}P NMR spectra were acquired on a Bruker Avance III 400 Mhz spectrometer at 161.98 MHz. A 45 $^\circ$ read pulse was used for 100 scans with Waltz-16 proton decoupling. For comparison, the NMR spectrum of a RuBP **18** standard was also taken.

Figure Preparation

All figures were prepared using PyMOL²³ or ChemDraw (Cambridge Biosoft), and compiled in Photoshop (Adobe).

RESULTS

Overall Structure of MjThi4 and MiThi4

MjThi4 and MiThi4 show approximately 30% sequence identity to ScThi4⁷ and AtThi1¹⁵ and the four structures are very similar (Figure 3). All Thi4 orthologs are homooctamers. Each protomer consists of 12 β -strands organized as one six-stranded and two three stranded β -sheets flanked by nine α -helices. The Thi4 octamer has 422 symmetry and can be thought of as a tetramer of closely packed dimers. The buried dimer interface has an area of ~1500 $\text{\AA}^2$. The octamer contains a channel along the fourfold axis with a maximum opening of about 30 $\text{\AA}$.

ADPrI Binding Site of MjThi4

The active site of MjThi4 is located near the central channel and involves two adjacent protomers. Most of the residues come from one protomer, and a loop containing Gly162'-Asp166' from a fourfold related protomer completes the active site. Analysis of MjThi4 samples expressed in LB media showed high levels of NAD **5**, ADT **17**, and flavin mononucleotide and smaller amounts of ADPr **6** and ADPrI **7** eluting with the protein (data not shown). Based on the $F_o - F_c$ electron density in the active site, the intermediate ADPrI **7** was included in the model (Figure 4A). The structure of MiThi4 also showed ADPrI **7** unless further treated with glycine and iron. The interactions of ADPrI **7** with the surrounding residues are illustrated in Figure 4B. The N1, N3, N6, and N7 atoms of the adenine base hydrogen bond to the Val138 amide nitrogen atom, the Arg67 amide nitrogen atom, the Val138 carbonyl oxygen atom and the Ser184 side chain hydroxyl group, respectively. The side chain carboxylate group of Glu66 hydrogen bonds to the O2' and O3' hydroxyl groups of the ADPrI. The ADPrI α -phosphate hydrogen bonds to the Gly74 amide nitrogen atom and the His164' side chain from the fourfold related protomer. The β -phosphate hydrogen bonds to the Ser47 side chain and amide group, the Met230 amide group and two water molecules. The ribulose moiety hydrogen bonds to the carbonyl groups of Gly74 and Gly242, and both carboxylate oxygen atoms of Asp166' from the adjacent protomer.

Glycine Imine Intermediate Binding Site in ScThi4C205S/Glycine Imine

Electron density for the ScThi4C205S variant treated with NAD and glycine showed the glycine imine intermediate **12** in the active site (Figure 5A and S1). Interactions between the intermediate and the active site residues are shown in Figure 5B. The N1 and N6 atoms of the adenine base hydrogen bond to the Val170 amide and carbonyl groups, analogously to MjThi4/ADPr1 **7**, and the Ser98 amide donates a hydrogen bond to N3. The side chain carboxylate group of Glu97 hydrogen bonds to the O2' and O3' hydroxyl groups of the ADPr1 **7** ribose, analogously to MjThi4/ADPr1 **7**, and also hydrogen bonds to the Ser98 and a water. The ADP α -phosphate group hydrogen bonds to the Gly105 amide nitrogen atom and two water molecules. The ADP β -phosphate hydrogen bonds to the Met291 amide group and two water molecules. The hydroxyl groups of the ribulose fragment only interact with water molecules. The nitrogen atom of the glycine fragment forms two strong hydrogen bonds with Asp207' of the adjacent protomer.

Fe and Glycine Imine Intermediate Binding Sites in MiThi4/Fe/Glycine Imine

MiThi4 and MjThi4 share 78% sequence identity and the ADP portion of the binding site in MiThi4/Fe/glycine imine **12** is essentially identical to that of MjThi4/ADPr1 **7** (Figures 2, 6A and S2). Likewise, the glycine imine **12** conformation is very similar to that observed for the ScThi4C205S complex; however, a small movement takes place as required to form the octahedral iron binding site. The ribulose and glycine fragments of the glycine imine **12** occupy three positions of the octahedral Fe(II) binding site through coordinate covalent bonds with a ribulose oxygen atom and the glycyl nitrogen and carboxylate atoms (Figure 6B). The other three sites are occupied by His176, Asp161' from the adjacent protomer and a water molecule. The Fe(II) to oxygen or nitrogen distances range from 2.0 Å to 2.2 Å. Analogous to ScThi4C205S, the glycyl carboxylate group of the glycine imine **12** forms a strong salt bridge with Arg236 through two strong hydrogen bonds (Figure 6C).

Activity of Thi4 Variants

The activity assays of MjThi4 and H164C MjThi4 were performed under anaerobic conditions and HPLC was used to detect the reaction products. Wild-type MjThi4 catalyzes thiazole biosynthesis with the substrates NAD **5**, glycine **8** and sodium sulfide in the presence of FeAS (Figure 7A). The enzyme can only use free sulfide as the sulfur source for ADT **17** formation, and the H164C variant still requires free sulfide for the reaction (Figure 7B). The ScThi4C205S variant is catalytic in the presence of exogenous sulfide, the ScThi4D207A variant is inactive, and the ScThi4H237N variant only catalyzes the conversion of ADPr **6** to ADPr1 **7**.

Determination of RuBP Activity of MjThi4 by Derivatization of 3-PGA with 2, 4-DNPH

Analysis of standards showed that concentrations of RuBP **18** as low as 30–50 μ M can be detected using the RuBisCO/2, 4-DNPH assay (Figure 2A). Using this assay, we demonstrated that MjThi4 does not catalyze the formation of RuBP **18** (Figure 2C). Neither NAD **5** nor Fe(II) had any effect on RuBP **18** formation (Figure 2C).⁹

³¹P-NMR for Detection of RuBP Formation Activity of MjThi4

The ³¹P-NMR spectra for reaction samples with and without NAD **5** did not exhibit any peak that corresponds to the ³¹P-NMR spectrum of the RuBP **18** standard (Figure 8) indicating no detectable RuBP **18** synthase activity.

DISCUSSION

Structural Comparison of MjThi4 to ScThi4 and AtThi1

A structural similarity search for MjThi4 was performed using the DALI²⁴ online server. ScThi4, AtThi1, MjThi4, and MiThi4 are homooctamers, and the protomers are structurally similar (Figure 9). Unsurprisingly, the most similar structures are Thi4 orthologs. The structure of AtThi1 (PDB ID 1RP0), which was used as the search model during molecular replacement, showed the highest similarity with a Z score of 34.6. The structure of ScThi4 (PDB ID 3FPZ) shows a Z score of 32.4. MjThi4 shares a 29% sequence identity with ScThi4 and superimposes to ScThi4 with an rmsd for 158 C_α atoms of 1.0 Å, while MjThi4 and AtThi1 share 32% sequence identity and superimpose with an rmsd for 180 C_α atoms of 0.9 Å. The key difference between MjThi4 and ScThi4 or AtThi1 is the length of the active site loops β10 - α6 and α5 - β9. The β10 - α6 loop and α5 - β9 loop in MjThi4 are 3–5 residues shorter than the corresponding loops in ScThi4 and AtThi4, which makes the active site of MjThi4 more open to the solvent (Figure 9B).

In addition to Thi4 orthologs, similar structures include NAD(FAD)-utilizing dehydrogenases (e.g., PDB ID 2IOZ with an rmsd of 2.6 and a Z score of 20.3). The NAD(FAD) binding domains are structurally similar to the NAD binding domain of Thi4²⁵; however, NAD serves as a cofactor for the dehydrogenases and as a substrate for Thi4.

Comparison of the MjThi4 active site with those of ScThi4 or AtThi1 reveals a highly conserved arrangement around the ribulose moiety of ADPrI **7** (Figure 9C). Active site residues Asp166' and His164' (MjThi4 numbering) are from loop α5 - β9 of the adjacent monomer, His181 and Glu182 are from loop β10 - α6. Three highly conserved residues, Asp166', Arg240 and Gly242, interact with the thiazole moiety in the ScThi4 and AtThi1 structures (Figure 9C). Residues His189 and Asp190 in AtThi1 are involved in the coordination of a zinc ion in the active site¹³. These two residues are conserved in ScThi4, while in MjThi4 Asp190 is replaced by Glu182 (Figure 9C). All of these active site residues near the thiazole moiety in ScThi4 and AtThi1 are highly conserved or partially conserved in MjThi4, except for His164, which corresponds to Cys205 of ScThi4. ScThi4 was identified as a suicide enzyme and Cys205, which is conserved in all eukaryotic Thi4 orthologs, was shown to be the sulfur donor in a suicide reaction for thiazole synthesis⁵. The multiple sequence alignment result of eukaryotic Thi4 and archaea Thi4 homologs shows that both MjThi4 and MiThi4 lack this cysteine residue (Figure S3), and our studies show that thiazole synthesis in MjThi4 and MiThi4 utilizes sulfide as the sulfur source.

Structural Comparison of ADPrI, Glycine Imine, Fe/Glycine Imine and ADT Binding Sites

Together with the previously reported structure of ScThi4/ADT (PDB ID 3FPZ)⁷ and AtThi1/ADT (PDB ID 1RP0)¹⁵, complexes of four Thi4 orthologs are now available.

Different orthologs were used because the crystallization behavior varied, and not all intermediates could be trapped for a single ortholog. While multiple intermediates were detected for purified MjThi4 and MiThi4, both selectively crystallized with bound ADPr **7**. Previous studies showed the presence of the glycine imine **12**, the final intermediate **16**, and the product ADT **17**, in purified ScThi4; however, ScThi4 selectively crystallized with ADT **17**. We were only able to trap the glycine imine **12** intermediate using the ScThi4C205S variant, and only MiThi4 crystallized with bound glycine imine and iron.

Superposition of the active sites of MjThi4/ADPrI, ScThi4C205S/glycine imine, MiThi4/Fe/glycine imine **12** and ScThi4 (PDB ID 3FPZ) provide snapshots along the Thi4 reaction coordinate (Figure 10). The residues involved in binding the ADP moiety are highly conserved and the ADP moieties from the four structures superimpose closely (Figures 3, 4 and 5). Arg301 (ScThi4 numbering) anchors both intermediates and the product through hydrogen bonds with the carboxylate tail. This arginine residue is conserved in all Thi4 orthologs. The glycine imine intermediate **12** undergoes a conformational change upon Fe(II) binding, resulting in the formation of **23**. Two residues, Asp161 and His176 (MiThi4 numbering), which chelate Fe(II) are conserved in MjThi4, MiThi4, ScThi4, and other orthologs indicating a similar Fe(II) binding site for ScThi4. The iron is released from the enzyme after the conversion of the glycine imine **12** to a structure that is no longer able to function as a tridentate ligand (Figure 11).

Analysis of ScThi4 Active Site Variants

C205S, D207A, and H237N. Cys205 has previously been identified as the sulfide donor in the ScThi4 catalyzed reaction and the C205A variant can catalyze the formation of ADT **17** in the presence of sulfide. In contrast, the “dead enzyme” with dehydroalanine at position 205 is unable to utilize sulfide for thiazole formation. We therefore tested the C205S variant to further explore the constraints on the use of exogenous sulfide and were surprised to find that this variant could also use sulfide (Figures S4 and S5). This suggests that planarization of the alpha carbon at position 205 locks the enzyme into an inactive conformation.

Asp207 in ScThi4 corresponds to the iron ligating Asp161 of MiThi4 and is likely to have multiple functions. The D207A variant is inactive and unable to catalyze the conversion of NAD **5** to ADPr **6** or the conversion of ADPr to ADPrI **7** (Figure S6). This suggests that Asp207 plays a role in the initial *N*-glycosyl bond cleavage of NAD and in the tautomerization of **6** to **7** (see Figure 5B). H237 in ScThi4 corresponds to the iron ligating His176 of MiThi4 and is also likely to have multiple functions. The H237N variant catalyzes the conversion of ADPr **6** to ADPrI **7** but not the conversion of ADPr **6** to the glycine imine **12**, suggesting that in addition to serving as a ligand for the iron, His237 plays an essential role in the conversion of **7** to **12** (Figure S7).

Sulfur Source for MjThi4

Cysteine, thioacetate, thiosulfate, sulfide, and glutathione were previously tested as possible sulfur sources in the MjThi4 activity assays, but only sulfide supported ADT **17** formation.⁸ Because His164 corresponds structurally to Cys205 in ScThi4, it was mutated to cysteine to determine if a cysteine residue in this position could serve as the sulfur source

for thiazole formation. Neither free cysteine nor cysteine at position 164 in the protein functioned as the sulfur source. *Methanococci* inhabit strictly anaerobic environments with high levels of sulfide⁹ and require high sulfide concentrations in the medium for cultivation (~4.5 mM).²⁶ In addition, recent studies on iron-sulfur cluster, thiouridine and methionine biosynthesis in *Methanococcus maripaludis* suggests that the sulfur in these molecules was predominantly derived from exogenous sulfide.^{27,28} Therefore, we conclude that free sulfide is the likely sulfur donor for the *in vivo* biosynthesis of thiazole in *M. jannaschii*.

The Role of Metal in Thi4 Catalysis

Our previous biochemical studies showed that the biosynthesis of the thiamin thiazole in yeast is Fe(II)-dependent.⁷ We recently showed that ADT **17** formation catalyzed by MjThi4 is also dependent on metal; however, in addition to Fe(II) and Fe(III), many divalent metals including Mn(II), Co(II), Zn(II), Ni(II), Mg(II), and Ca(II) also support activity of MjThi4 at varying levels.⁸ We further showed that upon reaction of MjThi4 with Fe(II), ADPr **6** and glycine **8**, a chromophore at 380 nm was observed.⁸ The crystal structure of this chromophoric intermediate showed that the iron is ligated by the carboxylate of the glycine imine intermediate **12**, Asp161, His176, and one water molecule (Figure 6). The formation of the 380 nm absorbing species was only observed in the presence of Fe(II), suggesting a possible charge-transfer interaction between Fe(II) and the glycine imine intermediate. After the addition of free sulfide, the ADT-tautomer **16** was observed and the 380 nm peak decayed simultaneously, indicating that the loss of the 380 nm signal is due to the formation of dihydrothiazole **16**.

Mechanistic Implications

MjThi4 and ScThi4 both use NAD **5** and glycine **8** to form the glycine imine intermediate **12** and the residues involved in glycine imine binding and conversion to the thiazole **17** are highly conserved. We therefore suggest that both enzymes use a similar mechanism for thiazole formation and that the structure of the MiThi4/glycine imine intermediate **12** with bound iron (Figure 6) provides important insight into the previously poorly understood role of iron in the ScThi4 mediated sulfur transfer chemistry. Based on this structure, we propose that sulfide from the reaction medium displaces the ketone oxygen from the iron, thus allowing the carbonyl carbon to reorient to satisfy the stereoelectronic requirements for sulfide addition to the carbonyl carbon (**23** to **24**, Figure 11) in the active site of MjThi4. While the reorientation requires nearly a 180° flip of the carbonyl group, the conformational change is consistent with the crystal structures of **23** and the ADT product complex. For the ScThi4, sulfide, generated from Cys205 by an elimination reaction, forms **24**. Cys205 is in a flexible loop but in the C205S structure, His200, which hydrogen bonded to Glu171, could serve as a base to deprotonate Cys205. The reaction then proceeds by a common pathway involving addition of the iron-bound sulfide to the carbonyl of **24** followed by loss of water to give **26**. No protein side chain is positioned to serve as a base; however, the β -phosphate of the ADP could serve this function. As the thiol of **26** cannot reach the glycine Ca, we propose that Fe(II) dissociation followed by bond rotation gives **27**. Thioenolization, followed by addition of the resulting thiol to the imine gives **15**. Water elimination and tautomerization completes the thiazole formation.

While our studies elucidate the sulfur transfer chemistry involved in thiamin thiazole biosynthesis, they also show that there is no mechanistic requirement for enzyme autoinactivation in the sulfur transfer. Why then does yeast use an active site cysteine rather than exogenous sulfide for thiazole biosynthesis? We do not yet know the answer to this interesting question. One possibility is that thiazole formation triggers a posttranslational modification and that “inactive” Thi4 has additional functions in yeast.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ThMP	thiamin monophosphate
ThDP	thiamin pyrophosphate
LB	Luria-Bertani
Tris	tris(hydroxymethyl)aminomethane
IPTG	isopropyl 1-β-D-galactopyranoside
ADT	adenosine diphosphate thiazole
Ni-NTA	Ni-nitrilo acetic acid
NAD	nicotinamide adenine dinucleotide
RuBP	ribulose 1, 5-bisphosphate
ADPr	adenosine diphosphate ribose
ADPrI	adenosine diphosphate ribulose
rmsd	root mean squared deviation
NE-CAT	Northeastern Collaborative Access Team
CHES	N-cyclohexyl-2-aminoethanesulfonic acid
FeAS	ferrous ammonium sulfate
2,4-DNPH	2,4-dinitrophenylhydrazine
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride

3-PGA	3-phosphoglycerate
PRPP	5-phospho- α -D-ribose 1-diphosphate
CIP	calf intestinal phosphatase
RuBisCO	ribulose 1,5-bisphosphate carboxylase/oxygenase

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Figure 1.

Mechanistic proposal for the formation of ADT **17** from NAD **5**, glycine **8** and a sulfur source based on previous research. These studies left unresolved the geometry and tautomeric state of the glycine imine **12** as well as the initial site of sulfide addition (carbonyl vs imine; see Figure 11).

Figure 2.

Assay of MjThi4 for RuBP synthase activity. (A) The RuBisCO/DNPH assay for the detection of RuBP. (B) HPLC analysis of the derivatization reaction. Various concentrations of 3-PGA were first treated with CIP and then derivatized with 2,4-DNPH as described in the experimental section. (C) HPLC chromatograms of the full reactions in the absence (pink trace) and presence of NAD (green trace) and control reactions without PRPP (blue trace) and without RuBisCO (black trace). Only the RuBP standard shows the expected product peak (red trace). Similar results were obtained using MjThi4/Fe(II).

Figure 3.

Crystal structure of Thi4 from *M. jannaschii*. (A) The ribbon diagram of the overall structure of MjThi4 with the secondary structural elements colored blue for α -helices, green for β -strands and yellow for loops. The α -helices and β -strands are labeled in black. The ligand ADPr is shown as sticks and colored in magenta. (B) The dimeric structure of MjThi4. Two monomers are related by a 2-fold axis and colored by chain with blue and pink, respectively. The α -helices $\alpha1$, $\alpha1'$, $\alpha4$ and $\alpha4'$ are highlighted in black. (C) Topology diagram of MjThi4. The green arrows represent β -strands and blue cylinders α -helices. The

first and last residue number of each secondary structural element is indicated. The quaternary structure of MjThi4 is shown with (D) a top view and (E) a side view of the octamer ring.

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A **B** **Figure 4.**

Stereoview of the MjThi4 active site. (A) The $F_o - F_c$ map of ADPrI 7 contoured at 3σ is shown in light blue. (B) Active site residues and ADPrI are depicted as sticks with the carbon atoms colored gray and purple, respectively. Residues from the adjacent monomer are shown as sticks with the carbon atoms colored yellow. Hydrogen bonds are shown as black dash.

A **B** **Figure 5.**

A stereodiagram of the ScThi4C205S active site. (A) The $F_o - F_c$ map of glycine imine intermediate **12** contoured at 3σ is shown in light blue. (B) Active site residues and the glycine imine **12** intermediate are depicted as sticks with the carbon atoms of residues colored in green and purple, respectively. Only residues making direct contacts with the ligand are shown. Residues from the adjacent monomer are shown as sticks with the carbon atoms colored yellow. Hydrogen bonds are shown as black dash. Water molecules are shown as red spheres.

Figure 6.

Stereoview of the (A) $F_o - F_c$ electron density map of Fe and glycine imine intermediate **12**, (B) active site and, (C) Fe binding site of MiThi4. The $F_o - F_c$ map of glycine imine **12** contoured at 3σ is shown in light blue, the $F_o - F_c$ map of Fe and phosphorous atoms contoured at 15σ is shown in green. Active site residues and the glycine imine intermediate are depicted as sticks with the carbon atoms colored green and purple, respectively. Residue Asp161' from the adjacent monomer is shown as sticks with the carbon atoms colored

yellow. Hydrogen bonds are shown as black dashes. The six coordinate bonds are shown as black dashes and labeled with bond lengths.

Figure 7. MjThi4 activity assays. (A) HPLC chromatogram showing conversion of NAD into ADT catalyzed by wild-type MjThi4. (B) Activity of H164C variant.

Figure 8.

Assessment of RuBP formation by ^{31}P NMR. (A) $\sim 500\ \mu\text{M}$ MjThi4 was loaded with $\sim 50\%$ Fe (II) and reacted overnight in a glove box with $\sim 5\ \text{mM}$ PRPP and $\sim 25\ \text{mM}$ MgCl_2 at $50\ ^\circ\text{C}$ in the absence of NAD (B) $\sim 500\ \mu\text{M}$ MjThi4 was loaded with $\sim 50\%$ Fe (II) and reacted overnight in glove box with $\sim 5\ \text{mM}$ PRPP and $\sim 25\ \text{mM}$ MgCl_2 at $50\ ^\circ\text{C}$ in the presence of $0.5\ \text{mM}$ NAD (C) $\sim 5\ \text{mM}$ PRPP and $\sim 25\ \text{mM}$ MgCl_2 were incubated overnight inside glove box at $50\ ^\circ\text{C}$ (D) RuBP standard solution. In full reaction spectra (panels A and B), the peak at $2.7\ \text{ppm}$ (Ribose-1-P) is more intense than Ribose-5-P peak and there is an additional

peak at around 2 ppm. We attribute these changes due to side reactions that might have occurred at high temperature (50 °C) and long incubation time (overnight) of the assay.

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Figure 9.

Structural comparison of MjThi4 (blue), ScThi4 (red), and AtThi1 (green). (A) Superposition of monomeric structures. (B) Comparison of the two loops in three structures. (C) Comparison of the active residues around the ribulose moiety of **7** in MjThi4 with residues around the thiazole moiety **17** in ScThi4 and AtThi1. All the residues and ligands are shown as sticks and colored blue for MjThi4, red for ScThi4 and green for AtThi1, respectively.

Figure 10.

Structural comparison of active sites of MjThi4 (green), ScThi4C205S (red), MiThi4 (pink) and ScThi4 (PDB 3FPZ) (sky blue). The ADPr1 **7** in MjThi4, glycine imine intermediate **12** in ScThi4C205S, glycine imine intermediate **12** in MiThi4 and ADT **17** in ScThi4 (PDB 3FPZ) are shown as sticks and colored green, red, pink and sky blue, respectively. The Fe from the MiThi4 structure is shown as a sphere and colored gray. The residues from MjThi4, ScThi4C205S, MiThi4 and ScThi4 are labeled green, red, pink and sky blue, respectively.

Figure 11.
Proposed mechanism for the iron-mediated sulfide transfer chemistry involved in the biosynthesis of thiazole **17** catalyzed by the archaeal and yeast thiazole synthases.

Scheme 1.

Table 1

Summary of data collection and refinement statistics.

	MjThi4/ADPrI	ScThi4C205S/ glycine imine	MiThi4/Fe/ glycine imine
resolution (Å)	2.7	2.0	2.1
wavelength (Å)	0.97918	0.97918	0.97918
space group	<i>P</i> 3 ₂	<i>P</i> 4	<i>P</i> 3 ₁
a (Å)	180.8	127.4	101.3
b (Å)	180.8	127.4	101.3
c (Å)	73.3	72.6	204.3
protomers/asu	8	4	8
total no. of reflections	695339	930786	1010442
No. of unique reflections	72800	78765	136825
Average <i>I</i> /σ	13.3 (2.3) ^a	14.5(6.7)	19.7 (2.3)
Redundancy	3.5 (3.5)	5.0 (5.0)	3.3(3.4)
Completeness (%)	99.3 (99.7)	100.0 (100.0)	98.6 (99.8)
<i>R</i> _{sym} (%) ^b	7.2 (39.6)	7.0 (26)	4.9(43.2)
No. of protein atoms	15270	9145	15563
No. of ligand atoms	353	156	320
No. of water atoms	21	333	222
no. of reflections	68627	74673	128008
<i>R</i> -factor/ <i>R</i> _{free} (%) ^c	17.7/20.3	16.3/18.6	19.9/21.6
rms deviation from ideals			
bonds (Å)	0.0090	0.006	0.007
angles (°)	1.4	1.1	1.3
average <i>B</i> factor for protein (Å ²)	55.4	23.1	46.6
average <i>B</i> factor for water (Å ²)	39.4	24.6	39.8
average <i>B</i> factor for ligand (Å ²)	70.4	19.4	36.5
Ramachandram plot			
most favored (%)	95.1	96.3	95.5
allowed (%)	4.9	3.7	4.5
disallowed (%)	0.0	0.0	0.0

^a Values in parentheses are for the highest-resolution shell.^b $R_{\text{merge}} = \sum_i |I_i - \langle I \rangle| / \sum_i \langle I \rangle$, where $\langle I \rangle$ is the mean intensity of the *N* reflections with intensities *I_i* and common indices *h,k,l*.^c *R*-factor = $\sum_{\text{hkl}} |F_{\text{obs}} - k F_{\text{cal}}| / \sum_{\text{hkl}} |F_{\text{obs}}|$ where *F_{obs}* and *F_{cal}* are observed and calculated structure factors, respectively, calculated over all reflections used in the refinement. *R_{free}*, is similar to *R_{work}* but calculated over a subset of reflections (5%) excluded from all stages of refinement.