From suicide enzyme to catalyst: the iron-dependent sulfide transfer in *Methanococcus jannaschii* thiamin thiazole biosynthesis

Bekir E. Eser[#], Xuan Zhang[§], Prem K. Chanani[†], Tadgh P. Begley[†], Steven E. Ealick[§]

[†]Department of Chemistry, Texas A&M University, College Station, TX, 77842, USA

[§]Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY, 14853, USA

[#]Zirve University, Department of Medical Biochemistry, Emine-Bahaeddin Nakıboglu School of Medicine, Gaziantep 27260, Turkey

Supporting Information

ABBREVIATIONS

WT, wild-type; LB, Luria-Bertani; isopropyl 1-β-D-galactopyranoside; Tris, tris(hydroxymethyl) aminomethane; FeAS, ferrous ammonium sulfate; ADP-ribose, adenosine diphosphate ribose; NAD, nicotinamide adenine dinucleotide; GuHCl, guanidinium chloride; HPLC, high-performance liquid chromatography; ADP-ribulose, adenosine diphosphate ribulose; ADT, adenosine diphosphate thiazole; ESI-MS, electrospray ionization mass spectrometry.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. All WT MjThi4 and variant proteins were overexpressed in BL21DE3 (T1R) cells. Cell growth was performed either in LB media or in M9 media. In the case of M9 media, the medium was supplemented with 0.2% glucose, 1 mM $MgSO_4$ and 0.1 mM $CaCl_2$. 1 L cultures were inoculated by cells grown overnight and allowed to reach an O.D. 600 nm of ~0.4 at 37 °C. Then, the temperature was reduced to 15 °C and cells were further grown

to an O.D. of 0.6-0.8. At this point the expression was induced with IPTG at a final concentration of 0.5 mM. After 15-20 h of incubation at 15 °C, the cells were pelleted by centrifugation and resuspended in 50 mM KPi buffer, pH 7.8, containing 150 mM NaCl, 10 % glycerol, 10 mM imidazole, lysozyme, DNAse, tris(2-carboxyethyl)phosphine and protease inhibitor cocktail (Sigma). Resuspended cells were sonicated followed by centrifugation at 15000 g for 2 h. Supernatant containing the soluble proteins was loaded on a Ni-affinity column (Histrap-GE Healthcare) and eluted with 400 mM imidazole. Fractions containing the pure proteins were pooled and buffer exchanged into the final assay buffer using a Pd-10 desalting column.

MjThi4 Activity Assays. Specific conditions for each assay are given in the figure legends. In summary, all assay components were taken inside a glovebox and allowed to sit for enough time to allow complete deaeration. Ferrous ammonium sulfate (FeAS) was taken as powder to the glovebox and dissolved in anaerobic deionized water. Sodium sulfide was purified from commercially available sodium sulfide by treatment with acid to generate H_2S which was then trapped with aqueous NaOH. The exact concentration of the sulfide solution was determined using the 5,5′-dithiobis-(2-nitrobenzoic acid) or methylene blue assays. Sulfide solutions were stored as highly basic solutions (pH>12) to prevent escape of H_2S and under anaerobic conditions to prevent oxidation. As purified apo-enzyme was first treated with FeAS and incubated for ~10 min before addition of substrates to assure binding of iron to the enzyme and to prevent precipitation of iron with sulfide. In general, assays were initiated, inside a glovebox, by addition of either ADP-ribose or NAD and quenched with an equal volume of 8 M GuHCl. Samples were centrifuge filtered before loading on a HPLC column to separate the small molecule pool from the protein. HPLC analysis was performed using a Supelcosil LC-18

reversed-phase column (150 mm x 4.6 mm, 3 μm pore size). The following linear gradient, at a flow rate of 1 mL/min, was used. Solvent A is water; solvent B is 100 mM K_2HPO_4, 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.

Spectrophotometric Determination of the 380 nm Absorbing Intermediate. For the spectrophotometric monitoring of the 380 nm chromophore, the assay components were added to an anaerobic cuvette inside the glovebox. Apo-enzyme was first incubated, for at least 10 min, with 1.5-2 fold excess FeAS. A concentration-dilution cycle was then performed, using Amicon centrifuge filters (0.5 mL), to remove excess iron. The resulting enzyme preparation was mixed with ADP-ribose and incubated at 65 °C for at least 30 min to maximize formation of the [MjThi4:Fe(II):ADP-ribulose] complex. The reaction mixture was then transferred to an anaerobic cuvette. Glycine was placed on the neck of the anaerobic cuvette as a droplet. In cases where sulfide was also reacted, a small drop of sodium sulfide solution was also placed on the neck of the cuvette, above the glycine droplet. The anaerobic cuvette was then sealed with a stopper and transferred to a diode-array spectrophotometer (outside the glovebox). After the acquisition was started, the contents of the cuvette were first mixed with the glycine droplet and changes in the spectrum were recorded immediately. After formation of the 380 nm absorbing intermediate was complete, the reaction mixture were mixed with the sulfide droplet and changes in spectra were monitored as before.

Kinetics of the Steps after Sulfur Transfer. In order to determine the rates of ADT tautomer **9** and ADT **10** formation, reactions were carried out in a glovebox as described above. After quenching with 8 M GuHCl, the reaction mixture was analyzed by HPLC as described above.

For the spectrophotometric analysis of the sulfide addition, to the 380 nm intermediate, a drop of sodium sulfide was put on the neck of the anaerobic cuvette. After the acquisition was started, the contents of the cuvette were mixed with the sulfide droplet and immediately the changes in the spectrum were recorded.

Analysis of the C205A Variant of ScTHI4p. All the components were stored inside the glovebox for two hours for deoxygenation. Purified enzyme was first incubated with freshly prepared FeAS (final concentration 400 μM) for 10 min followed by the addition of sulfide (final concentration 600 μM), glycine (final concentration 1 mM) and NAD or ADP-ribose (final concentration 500 μM). Reactions were incubated at room temperature for 6 h followed by quenching with 8M GuHCl. The reaction mixture was analyzed by HPLC as described above.

SUPPLEMENTARY FIGURES

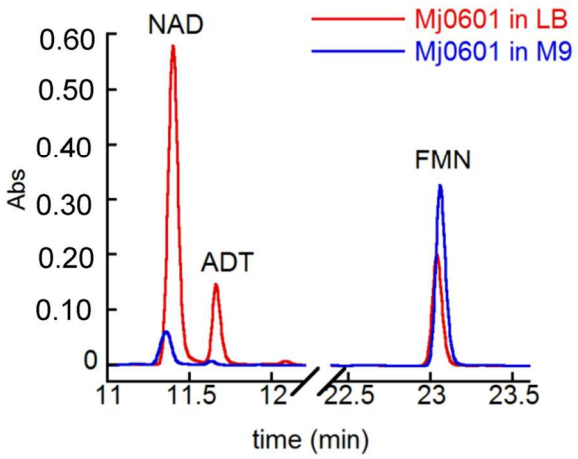

Figure S1. HPLC chromatogram of the major metabolites that co-purify with MjThi4 expressed in LB (red) and M9 (blue) medium. Purified protein samples were denatured with 4 M GuHCl (final), centrifuge filtered, and analyzed by HPLC. HPLC analysis was performed using a Supelcosil LC-18 reversed-phase column (150 mm x 4.6 mm, 3 μm pore size). The following linear gradient at a flow rate of 1 mL/min was used. Solvent A is water; solvent B is 100 mM K_2HPO_4, 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.

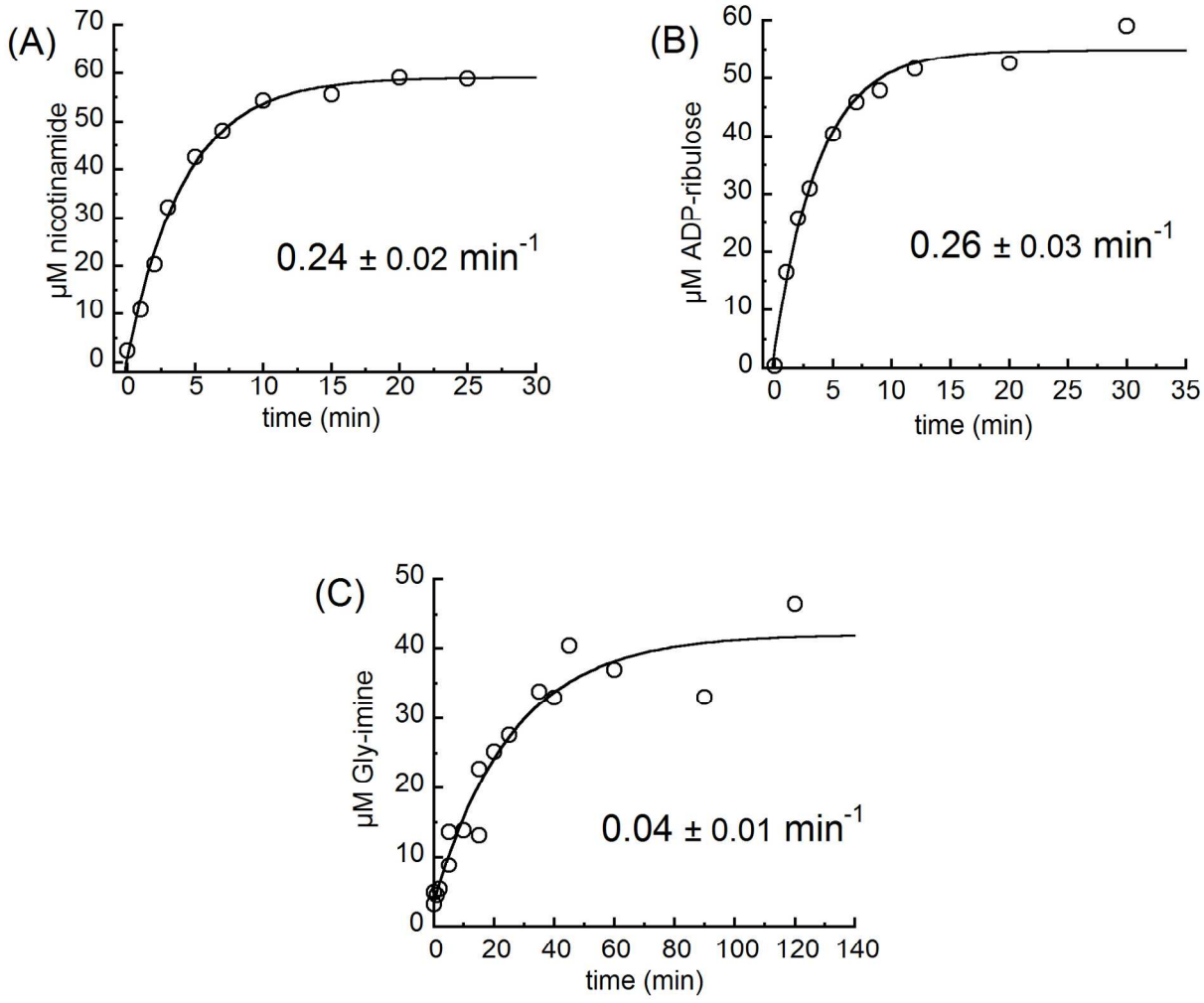

Figure S2. (A) Kinetics of the formation of nicotinamide upon reaction of 150 µM apo-MjThi4 with 140 µM NAD. (B) Kinetics of the formation of ADP-ribulose upon reaction of 150 µM apo-MjThi4 with 140 µM ADP-ribose. (C) Kinetics of the formation of glycine imine intermediate with apo-MjThi4. Conditions were 150 µM MjThi4, 400 µM ADP-ribose, 120 µM glycine at 65 °C. All data were fitted to a single-exponential function for comparison of the rates. Circles show the data and solid lines are the corresponding fits with rate constants given on each graph.

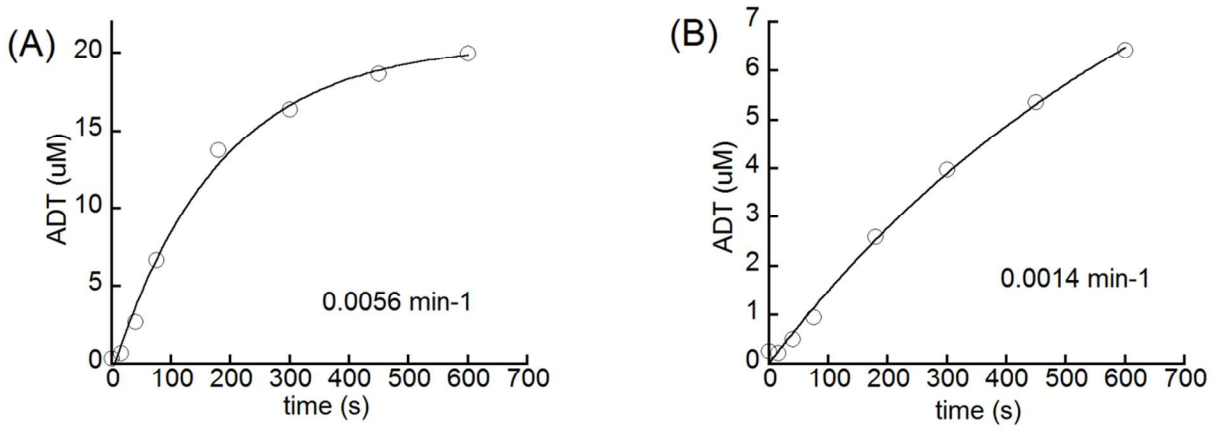

Figure S3. Comparison of the rates of ADT **10** formation by ferrous reconstituted MjThi4 (A) vs. ferric reconstituted MjThi4 (B). Data was fitted to a single-exponential function for comparison of the rates. Circles show the data and solid lines are the corresponding fits with rate constants given on each graph. 155 μM apo-MjThi4 was incubated with 400 μM of either ferrous or ferric iron inside the glovebox followed by a concentration dilution cycle to remove excess metal. Reconstituted enzymes were mixed with 620 μM ADP-ribose and 830 μM glycine and were incubated for at least 2 h for glycine imine complex formation at 65 °C. Afterwards reactions were initiated by addition of 200 μM sulfide, quenched with an equal volume of 8 M GuHCl and analyzed by HPLC for ADT formation as described above. All concentrations given are final.

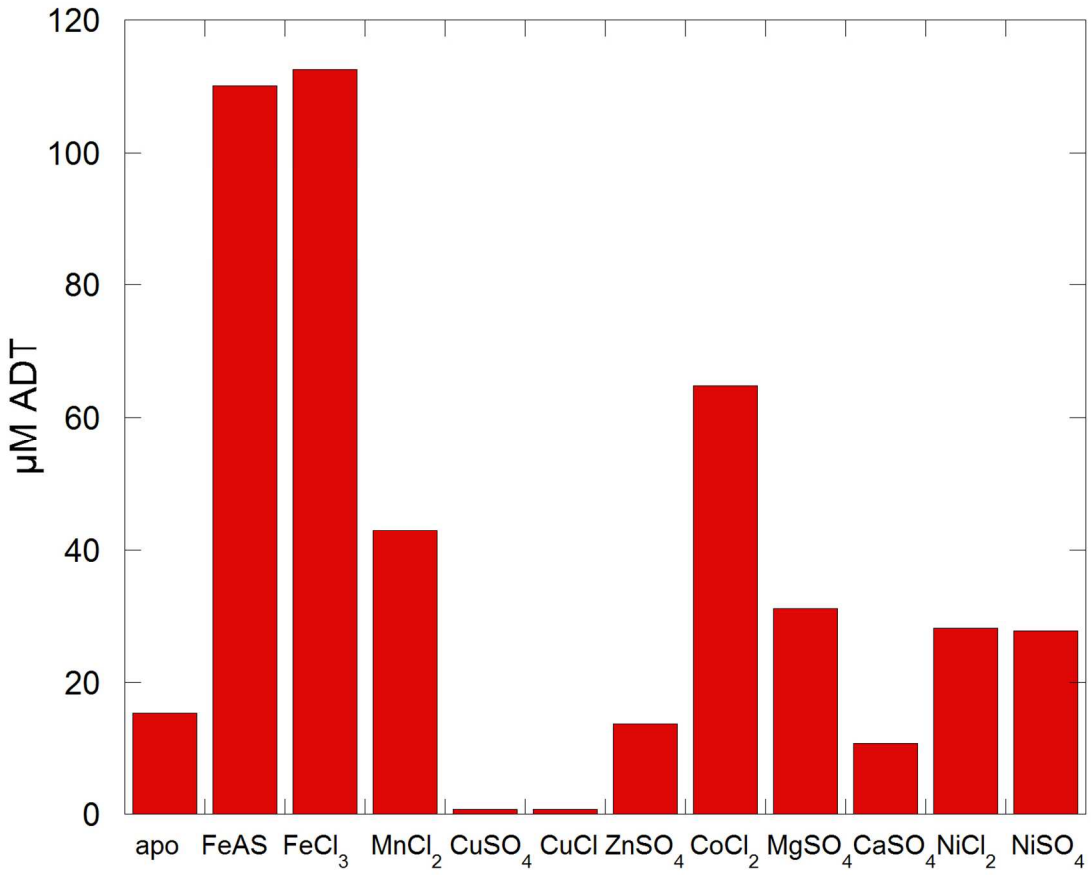

Figure S4. Comparison of the activities of MjThi4 proteins reconstituted with various metals. 50 µM apo-MjThi4 was incubated with 150 µM of each metal inside glovebox. Reconstituted enzymes were mixed with 500 µM ADP-ribose and 500 µM glycine and were incubated for at least 2 h for glycine imine complex formation. Afterwards reactions were initiated by addition of 800 µM sulfide and reaction mixtures were allowed to proceed for 3.5 h inside a glovebox before being quenched with an equal volume of 8 M GuHCl. Analysis of ADT formation was carried out by HPLC as described above. All concentrations given are final and all assays were carried out under identical conditions.

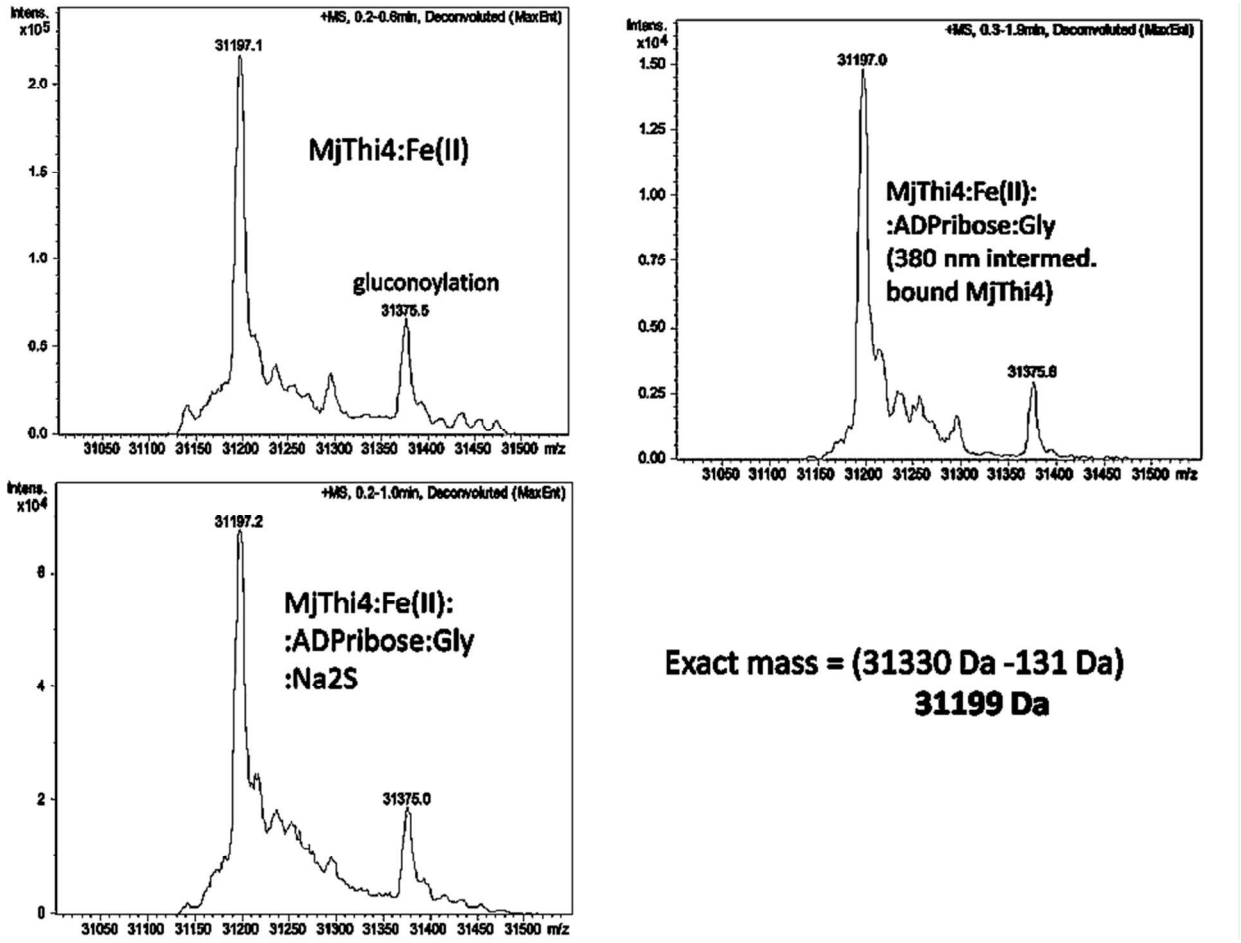

Figure S5. ESI-MS analysis of MjThi4 samples under various reaction conditions showing that there is no covalent modification of the protein. Reaction mixtures were desalted before being loaded for analysis. Agilent 1260 HPLC instrument was coupled to a MicroTOF-Q II mass spectrometer for LC-MS analysis in the positive mode. Exact mass is shown after subtracting the mass (131 Da) for the loss of N-terminal methionine.

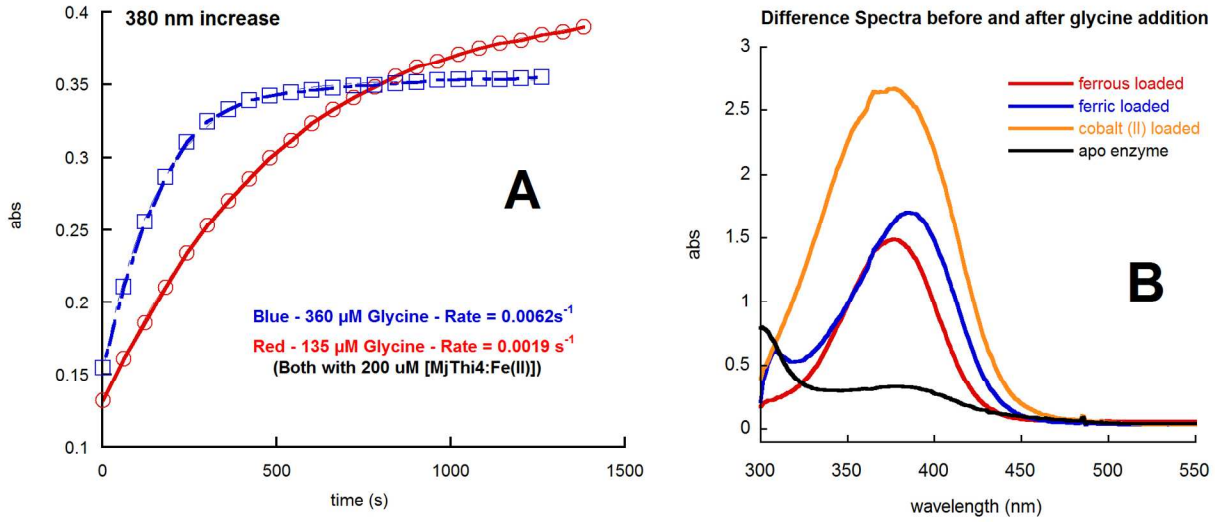

Figure S6. (A) Kinetics of 380 nm chromophore formation upon addition of two different glycine concentrations to the [MjThi4:Fe(II):ADP-ribulose] complex. (B) Difference spectra of the 380 nm chromophore with apo- and metal reconstituted enzymes (spectrum before glycine addition was subtracted from spectrum after glycine addition).

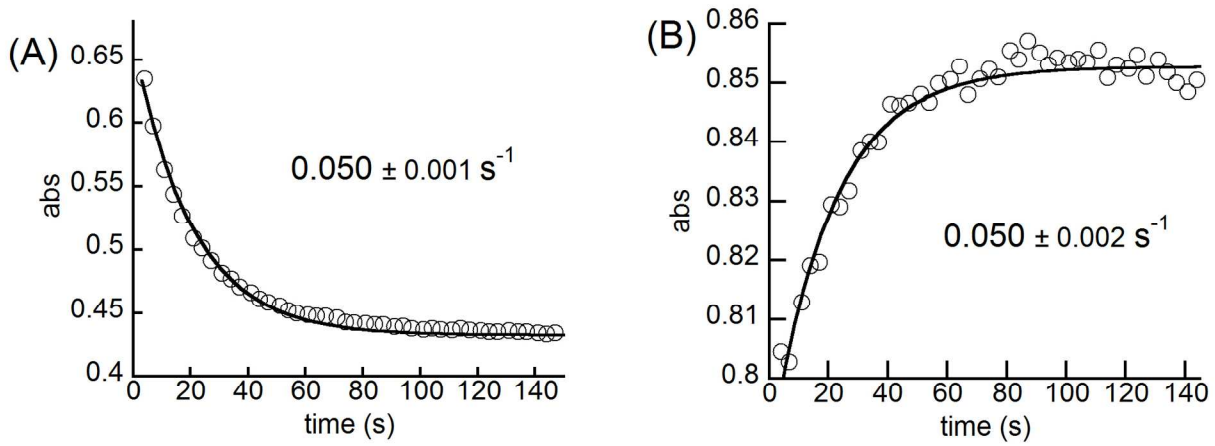

Figure S7. (A) Decrease in the 380 nm chromophore upon addition of sodium sulfide to the reaction mixture. (B) The simultaneous increase at 305 nm due to formation of the thiazole tautomer upon sulfur transfer. Both data were fitted to a single-exponential function. Circles show the data and solid lines are the corresponding fits with rate constants given on each graph.

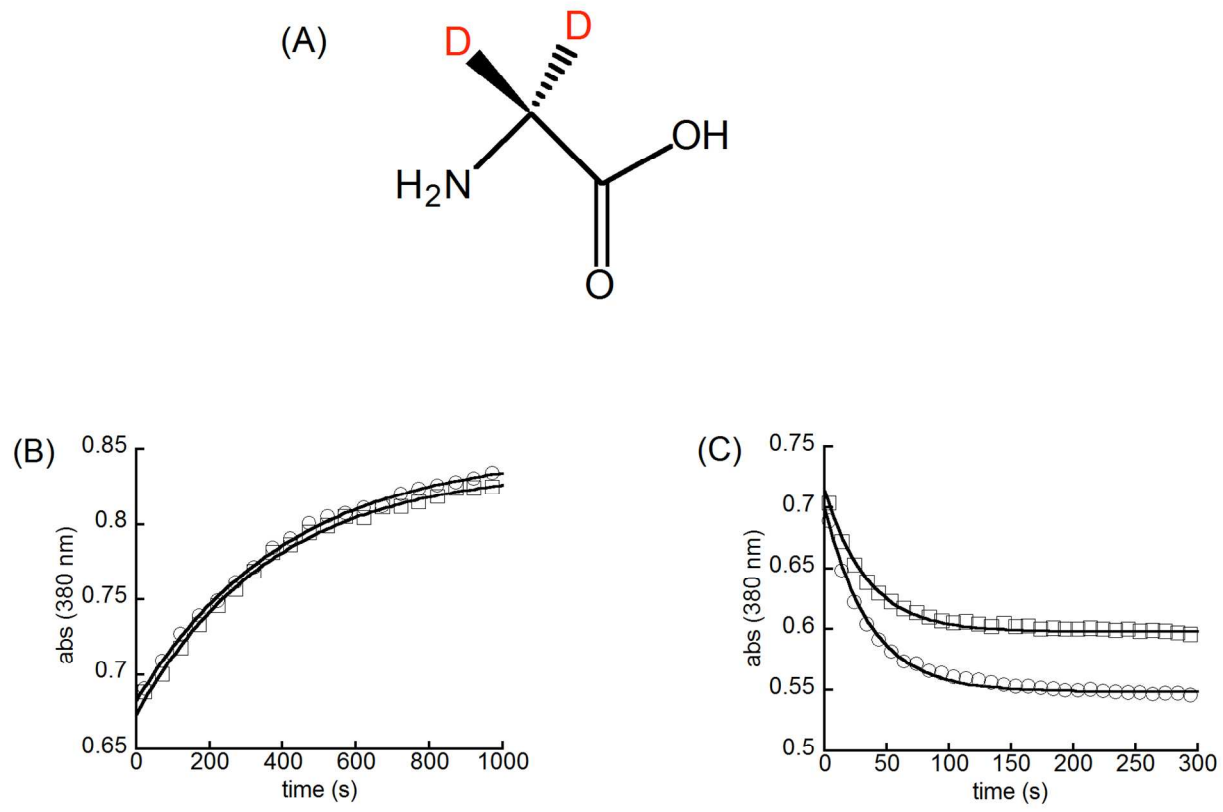

Figure S8. (A) Structure of 2,2-dideuterated glycine used as a substrate in isotope effect studies. (B) Time-dependent formation of 380 nm chromophore using proteated glycine (squares) and dideuterated glycine (circles). Solid lines are the fits by a single-exponential with rate constants of 0.0027 s^{-1} and 0.0024 s^{-1} for proteated and dideuterated substrates, respectively. (C) Time-dependent decay of 380 nm chromophore using proteated glycine (squares) and dideuterated glycine (circles). Solid lines are the fits by a single-exponential with rate constants of 0.029 s^{-1} and 0.028 s^{-1} for proteated and dideuterated substrates, respectively. Conditions were as follows: 195 μM MjThi4:Fe(II), 200 μM ADP-ribose, 400 μM proteated or dideuterated glycine, 200 μM Na_2S at 65 °C.

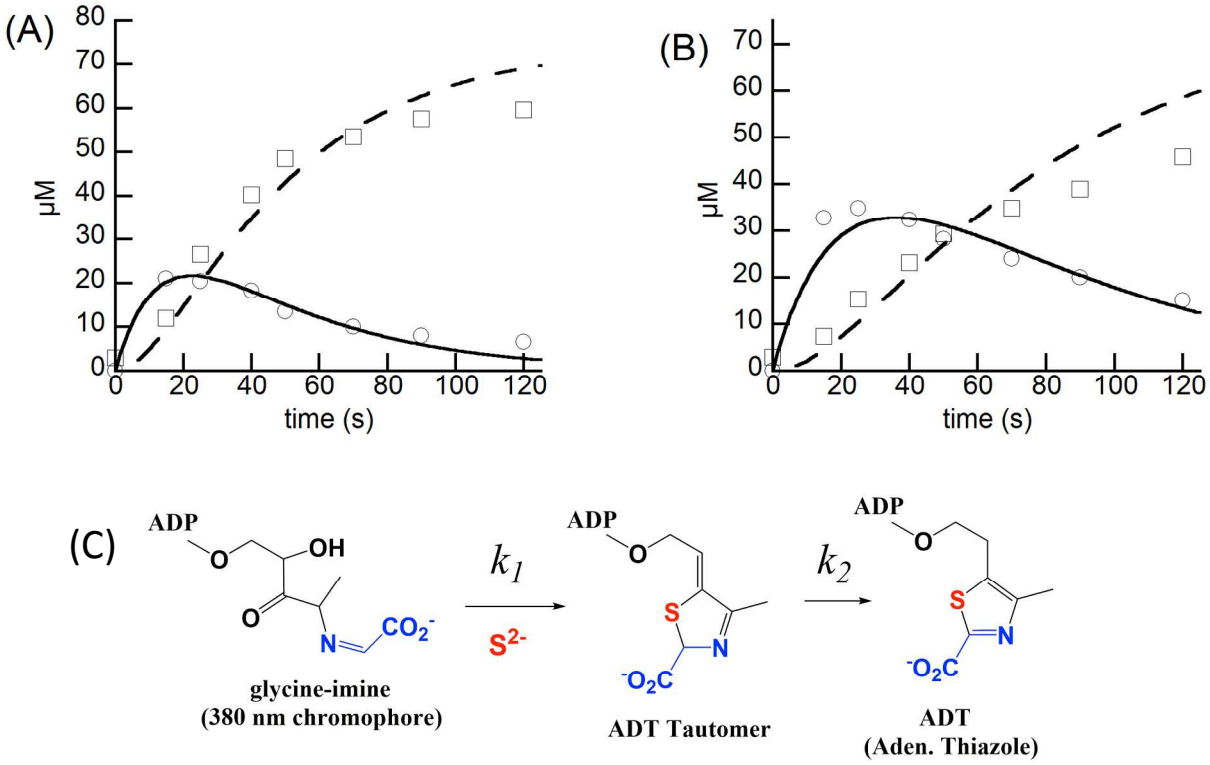

Figure S9. Data and simulations of the kinetics of ADT tautomer **9** and ADT **10** formation upon mixing (at 55 °C) of 200 μM Na_2S with about 100 μM of the 380 nm chromophore (glycine imine complex) generated by using either proteated glycine (A) or 2,2 - dideuterated glycine (B). Time-dependent changes in the concentration of ADT tautomer (circles) and ADT (squares) were simulated to a two-step mechanism (panel C) using KinTek software (KinTek Corporation, USA) with rate constants of $k_1 = 0.185\ mM^{-1}s^{-1}$ and $k_2 = 0.05\ s^{-1}$ for (A) and rate constants of $k_1 = 0.185\ mM^{-1}s^{-1}$ and $k_2 = 0.02\ s^{-1}$ for (B).

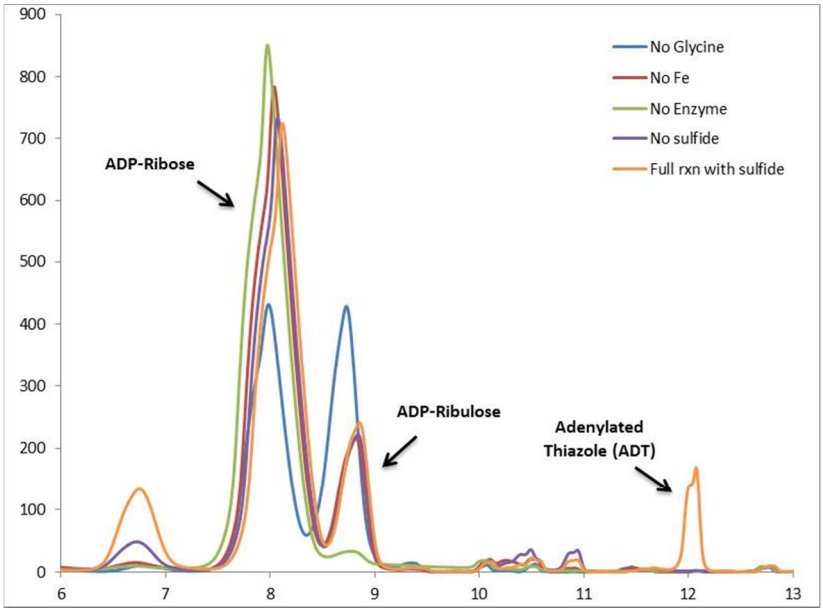

Figure S10. HPLC analysis of the production of ADT by the C205A variant of ScTHI4p. The C205dehydroalanine form of the enzyme is inactive under these conditions.