Saccharomyces cerevisiae THI4p is a suicidal thiamin thiazole synthase

Abhishek Chatterjee[2,3], **N. Dinuka Abeydeera**[1], **Shridhar Bale**[2,3], **Pei-Jing Pai**[1], **Pieter C. Dorrestein**[4], **David H. Russell**[1], **Steven E. Ealick**[*,2], and **Tadhg P. Begley**[*,1]

[1]Department of Chemistry, Texas A&M University College Station, TX 77843

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

[4]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California at San Diego, La Jolla, California 92093

Abstract

Thiamin pyrophosphate 1 (Figure 1A) is an essential cofactor in all living systems[1]. Its biosynthesis involves the separate syntheses of the pyrimidine 2 and thiazole 3 precursors, which are then coupled[2]. Two biosynthetic routes to the thiamin thiazole have been identified. In prokaryotes, five enzymes act on three substrates to produce the thiazole via a complex oxidative condensation reaction, the mechanistic details of which are now well established[2–6]. In contrast, only one gene-product is involved in thiazole biosynthesis in eukaryotes (THI4p in *Saccharomyces cerevisiae*)[7]. Identification of three adenylated metabolites (structures 5, 12 and 17 in Figure 1B), co-purifying with THI4p, provided three molecular snapshots of the reaction pathway catalyzed by this protein. In addition, two partially active mutants were identified (C204A and H200N), which catalyzed the conversion of NAD (nicotinamide adenine dinucleotide) 6 and glycine 9 to an advanced intermediate 12[8]. A mechanism for thiazole formation, consistent with these observations, is outlined in Figure 1B.[8–11] However, the source of the thiazole sulfur remained elusive, precluding us from deciphering the subsequent steps leading to the adenylated thiazole 5. Here we report the preparation of fully active recombinant wild type THI4p, the identification of an iron-dependent sulfide transfer reaction from the protein to a reaction intermediate and the demonstration that THI4p is a suicidal enzyme undergoing only a single turnover.

*To whom correspondence should be addressed: Department of Chemistry, Texas A&M University, College Station, TX 77843. begley@chem.tamu.edu or Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853. see3@cornell.edu.
[3]Current address: The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037

High resolution ESI-FTMS (Electrospray Ionization Fourier Transformed Mass Spectrometry) analysis of wild type THI4p (wtTHI4p), recombinantly expressed in *E. coli*, revealed a mass that was 34±1 Da less than the calculated mass of the protein. The active site mutants of THI4p[8], which did not copurify with any bound metabolites and did not show any activity, were unmodified suggesting that the 34 Da mass loss was in some way related to the catalytic activity of the protein. To localize the site of this modification, chymotrypsin digestion of modified wtTHI4p was carried out, followed by MALDI (Matrix assisted laser desorption ionization) and ESI MS analysis of the peptide fragments. Prior to the digestion, free thiol residues of the protein were alkylated with iodoacetamide to protect them from oxidation. As a control, we performed the same procedure on an inactive and unmodified THI4p mutant (R301Q)[8] in parallel. Upon comparing the results for the wtTHI4p and the mutant THI4p, two modified peptide fragments, spanning the same region of the protein sequence, were identified (Figure 2A and Supplementary figure 2). Fragmentation analysis localized the modification to a pair of adjacent cysteine residues (Cys204, Cys205, Figure 2B, highlighted in red). Both of these residues failed to alkylate during the iodoacetamide treatment of the peptide fragments. In contrast, peptide fragments originating from THI4p (R301Q) under the same conditions were completely alkylated (Figure 2A). These observations may be explained by the transfer of H_2S from Cys204 or Cys205 of wtTHI4p, to a thiazole intermediate, generating a dehydroalanine residue (M-34 Da), which is subsequently trapped by the adjacent cysteine-thiol producing a seven membered cyclic thioether (Figure 2B and Supplementary figure 2).

Based on these observations, the crystal structures of THI4p[9] and THI1[12] (THI4p ortholog from *Arabidopsis thaliana*) were reanalyzed for evidence of an active site dehydroalanine (DHA) or cyclic thioether. The $2F_o$-F_c and F_o-F_c electron density maps clearly demonstrate a lack of electron density for the sulfur atom of Cys205, consistent with a dehydroalanine residue (Figure 2C, 2D). The loop containing Ala199 – Asp207, disordered in our previous structure, was completed and shown to extend into the active site of a fourfold-related monomer. The interpretation of the high resolution structure of THI1 (PDB ID 1RP0) is also consistent with an active site dehydroalanine. The $2F_o$-F_c and F_o-F_c electron density maps for this protein confirm that electron density for the sulfur atom of Cys172 is missing and demonstrate that the C_α is planar (Supplementary figure 3). The identification of adjacent cysteine/dehydroalanine residues in THI4p suggests that the formation of the cyclic thioether occurs under the denaturing conditions used for the preparation of the sample for MS analysis.

The activity dependent loss of H_2S from THI4p suggested that the sulfur atom of the thiazole could be derived from THI4p. The resulting modification renders the enzyme inactive and explains our previous inability to reconstitute active wtTHI4p. The problem of obtaining active wtTHI4p was solved by a surprising set of observations regarding the effect of the growth medium on the activity of isolated wtTHI4p. When the overexpression strain was grown in M9 minimal medium instead of LB medium, the purified protein was mostly free of the 34 Da mass modification (Figure 3B). HPLC analysis of this protein preparation demonstrated greatly reduced formation of the sulfur-containing metabolites **17** and **5** and the accumulation of **12** (Figure 3A, red HPLC trace). This suggests that the sulfur transfer

chemistry involved in thiazole formation is greatly retarded when wtTHI4p is isolated from cells grown in minimal medium.

Addition of 100 μM iron (II) to the growth medium was sufficient to restore the sulfide transfer activity of wtTHI4p as indicated by the formation of **17** and **5** shown in Figure 3A (black HPLC trace). In addition, the protein thus isolated showed the 34 Da mass loss, as observed with wtTHI4p overexpressed in LB medium (Figure 3B). These observations suggested that the THI4p catalyzed sulfur incorporation reactions are iron-dependent.

These observations suggested conditions for the successful reconstitution of the THI4p-catalyzed reaction. To accomplish this, the unmodified wtTHI4p, overexpressed in minimal media, was freed from bound metabolites by multiple rounds of gel-filtration. This protein preparation catalyzed the conversion of ADP-ribose (ADPr **7**) to **12** in the presence of glycine **9**, via the intermediacy of ADP-ribulose (ADPrl **8**, Figure 3C). Addition of Fe^{+2} to this reaction mixture resulted in the conversion of **12** to the final product ADP-thiazole (ADT, **5**; Figure 3C). Other divalent metal ions (Mg^{+2}, Ca^{+2}, Mn^{+2}, Co^{+2}, Ni^{+2}, Cu^{+2} and Zn^{+2}) did not activate the enzyme for the sulfide transfer chemistry. While this reaction mixture did not contain an exogenous sulfur source, nearly one full turnover could be observed (380 ± 5 μM ADT from 420 ± 21 μM THI4p). Inclusion of excess sulfide or cysteine in the reaction mixture did not enhance the turnover number or protect the protein from modification. The production of ADT **5** was oxygen sensitive and had to be performed under an anaerobic atmosphere, in the presence of a reducing agent dithiothreitol (DTT) or tris-(2-carboxyethyl)-phosphine (TCEP). Formation of ADT was accompanied by the loss of H_2S (M=34 Da) from wtTHI4p, as evidenced from ESI-FTMS analysis (Figure 3E). In control reactions, lacking either Fe^{+2} or ADPr **7**, no modification of wtTHI4p was observed. A time course for the reaction demonstrates a consistent stoichiometry of 1:1 for protein modification and thiazole production (Figure 3D and 3E).

The full reconstitution of ADT **5** formation, using purified wtTHI4p, in a reaction mixture lacking any exogenous sulfide donor, coupled with the observed loss of 34 Da from the protein and the structural characterization of a dehydroalanine residue at Cys205 provides compelling evidence that the thiazole sulfur is derived from Cys205 of wtTHI4p. Consistent with this model is the observation that Cys205 is strictly conserved in eukaryotic thiazole synthases, whereas Cys204 is often replaced with a serine residue. Also, the THI4p C205S mutant was shown to be inactive, whereas the C204S mutant retains its ability to produce ADT, suggesting Cys205 as the sulfur donating residue.

Efforts towards the *in vitro* reactivation of modified THI4p under a variety of different conditions were unsuccessful. This suggests that THI4p is a single turnover enzyme. In addition, while thiamin biosynthetic enzymes are generally present in the proteome at very low concentrations, THI4p is an exception and a high level of expression (approx. 1.5%)[13] is observed during the exponential growth phase of *Neurospora crassa*. To investigate the possibility that THI4p is a single turnover enzyme, we first characterized THI4p, expressed at native levels in a yeast strain, in which a His_6 tag had been inserted at the carboxy terminus of THI4p to facilitate its detection and purification. THI4p was isolated from this strain grown to mid-log phase in a vitamin-free defined medium using immunoaffnity

chromatography and was analyzed by gel electrophoresis and western blotting (Figure 4A and 4B). The THI4p band was excised, digested with chymotrypsin and the peptide fragments were subjected to MS analysis (MALDI-TOF/TOF MS). The peptide fragment (T194 to Y216) associated with the 34 Da mass-loss (m/z: 2430.1) was again identified in this experiment (Figure 4C) and their fragmentation patterns were identical, confirming the loss of H_2S followed by cyclic thioether formation (Supplementary figure 4 and 5). The unmodified species for the same peptide (predicted m/z 2464 Da) was not observed in this experiment. Identification of the same pattern of H_2S-loss from native THI4p, as observed in the *in vitro* reconstitution experiments, further validates THI4p as the sulfur donor for thiazole biosynthesis *in vivo*. In addition, the MS analysis identifies modified THI4p as the major species present *in vivo*, suggesting the absence of a repair-pathway in yeast for THI4p-H_2S. This suggests that the ratio of THI4p to thiamin produced should be 1:1. To test this hypothesis, the concentration of native THI4p in yeast cell free extract was determined by quantitative western blot analysis, using known quantities of recombinantly expressed THI4p to generate a calibration curve (Figure 4A) and thiamin concentration in yeast lysate and in the growth medium (secreted by yeast) was determined by the thiochrome assay (supplementary Figure 6). A 1:1.1±0.2 stoichiometry between THI4p and thiamin was demonstrated in yeast cultures growing at mid-log phase (four independent experiments), supporting its role as a single turn over enzyme (Figure 4D).

A mechanistic proposal for the sulfide transfer chemistry involved in ADT **5** formation that is consistent with these observations is outlined in Figure 4E. Addition of the thiol of Cys205 to intermediate **13,** formed as shown in Figure 1, followed by Fe^{+2} assisted elimination would give **14** and generate the active site dehydroalanine observed in the structure. It is also possible that the iron activates the sulfide transfer by direct interaction with the sulfur. The oxygen sensitivity of the reaction suggests that Fe^{+2} is the catalytically active iron oxidation state. Intermediate **14** is then converted to ADT **5** as shown in Figure 1.

The observations reported here strongly suggest that THI4p acts as a co-substrate rather than an enzyme. This is very unusual but is not without precedent. The best characterized example of a single turnover enzyme is the Ada protein which repairs O^6-methylguanine and methyl-phosphotriester lesions in DNA by transferring the methyl group to an active site cysteine. The resulting inactive enzyme serves as a signal to induce other DNA repair enzymes.[14–16] The possibility that inactive THI4p has other physiological function(s) remains to be explored. Interestingly, involvement of THI4p and its orthologs have been implicated in DNA protection and other stress related pathways.[17–19] The mechanism of this protection is not known. One possibility is that the abundant THI4p protects the cells by binding free cellular iron, which is known to cause oxidative damage via the generation of reactive oxygen species.

THI4p is a remarkable protein: It is a suicidal enzyme serving as a cosubstrate rather than an enzyme for the formation of the thiazole moiety of thiamin. This assembly involves a complex, unprecedented reaction sequence in which NAD serves as the source of the five carbon chain and THI4p serves as the sulfur source. The biological function of the modified protein, if any, remains to be elucidated.

Methods summary

THI4p and its mutants [pET28b vector, BL21(DE3) cell line] were overexpressed in LB and isolated using Ni-NTA chromatography as described previously[8]. To isolate unmodified THI4p for activity assays, M9 minimal medium was used for over expression (See supplementary materials for details). HPLC analyses of THI4p bound metabolites and the *in vitro* reconstitution assays were performed as previously described[8]. Assays of THI4p were performed in an anaerobic chamber (COY Laboratory Products Inc). A typical THI4p reconstitution reaction assay included ADP-ribose or NAD (final concentration 1 mM), glycine (final concentration 1 mM), freshly prepared $FeSO_4$ (final concentration 0.5 mM) and THI4p (300–500 μM). THI4p and its mutants were desalted into 50% methanol containing 0.1% formic acid for ESI-FTMS analyses. Chymotrypsin digestion/MALDI analyses of recombinant THI4p and its mutants were performed at the Proteomics Facility, Cornell University, using standard protocols. In-gel chymotrypsin digestion/MALDI-TOF/TOF MS analysis with THI4p isolated from yeast was carried out in the Russell laboratory at Texas A&M University and the ESI-FTMS analysis was carried out in the Dorrestein laboratory at UCSD. The crystal structures of THI4p (PDB ID 2GJC)[9] and THI1 (PDB ID 1RP0)[12] were refined against the deposited structure factor magnitudes using REFMAC from CCP4[20, 21]. The *Saccharomyces cerevisiae* strain BY4741 (purchased from Open Biosystems, Inc) was used for in vivo studies with THI4p. Vitamin-free medium A (medium YNB [Yeast Nitrogen Base] plus 0.01% uracil, 0.01% leucine, 0.005% histidine, 0.005% methionine, 2% glucose, and 0.03% G418) was used to grow the yeast strain to allow the expression of the endogenous THI4p. Endogenous THI4p concentration was determined using a quantitative western blot, while the concentration of thiamin was determined using the thiochrome assay.[10]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

References

1. Jordan, F. Thiamin enzymology. In: Mander, L.; Liu, H-W., editors. Comprehensive Natural Products Chemistry. Elsevier; 2010. p. 561-598.

2. Begley TP. Cofactor biosynthesis: an organic chemist's treasure trove. Nat. Prod. Rep. 2006; 23:15–25. [PubMed: 16453030]

3. Begley, TP.; Ealick, SE. Thiamin biosynthesis. In: Mander, L.; Liu, H-W., editors. Comprehensive Natural Products Chemistry. Elsevier; 2010. p. 547-560.

4. Jurgenson CT, Begley TP, Ealick SE. The structural and biochemical foundations of thiamin biosynthesis. Annu Rev Biochem. 2009; 78:569–603. [PubMed: 19348578]

5. Hazra AB, Han Y, Chatterjee A, Zhang Y, Lai RY, Ealick SE, Begley TP. Missing Enzyme in Thiamin Thiazole Biosynthesis: Identification of TenI as a Thiazole Tautomerase. J Am Chem Soc. 2011; 133:9311–9319. [PubMed: 21534620]

6. Kriek M, Martins F, Leonardi R, Fairhurst SA, Lowe DJ, Roach PL. Thiazole synthase from Escherichia coli: an investigation of the substrates and purified proteins required for activity in vitro. J. Biol. Chem. 2007; 282:17413–17423. [PubMed: 17403671]

7. Praekelt UM, Byrne KL, Meacock PA. Regulation of THI4 (MOL1), a thiamine-biosynthetic gene of Saccharomyces cerevisiae. Yeast. 1994; 10:481–490. [PubMed: 7941734]

8. Chatterjee A, Jurgenson CT, Schroeder FC, Ealick SE, Begley TP. Biosynthesis of thiamin thiazole in eukaryotes: conversion of NAD to an advanced intermediate. J. Am. Chem. Soc. 2007; 129:2914–2922. [PubMed: 17309261]

9. Jurgenson CT, Chatterjee A, Begley TP, Ealick SE. Structural insights into the function of the thiamin biosynthetic enzyme Thi4 from Saccharomyces cerevisiae. Biochemistry. 2006; 45:11061–11070. [PubMed: 16964967]

10. Chatterjee A, Jurgenson CT, Schroeder FC, Ealick SE, Begley TP. Thiamin biosynthesis in eukaryotes: characterization of the enzyme-bound product of thiazole synthase from Saccharomyces cerevisiae and its implications in thiazole biosynthesis. J. Am. Chem. Soc. 2006; 128:7158–7159. [PubMed: 16734458]

11. Chatterjee A, Schroeder FC, Jurgenson CT, Ealick SE, Begley TP. Biosynthesis of the thiamin-thiazole in eukaryotes: identification of a thiazole tautomer intermediate. J. Am. Chem. Soc. 2008; 130:11394–11398. [PubMed: 18652458]

12. Godoi PH, Galhardo RS, Luche DD, Van Sluys MA, Menck CF, Oliva G. Structure of the thiazole biosynthetic enzyme THI1 from Arabidopsis thaliana. J. Biol. Chem. 2006; 281:30957–30966. [PubMed: 16912043]

13. Faou P, Tropschug M. Neurospora crassa CyPBP37: a cytosolic stress protein that is able to replace yeast THI4p function in the synthesis of vitamin B1. J Mol Biol. 2004; 344:1147–1157. [PubMed: 15544818]

14. Teo I, Sedgwick B, Kilpatrick MW, McCarthy TV, Lindahl T. The intracellular signal for induction of resistance to alkylating agents in E. coli. Cell. 1986; 45:315–324. [PubMed: 3009022]

15. Sedgwick B, Robins P, Totty N, Lindahl T. Functional domains and methyl acceptor sites of the Escherichia coli ada protein. J. Biol. Chem. 1988; 263:4430–4433. [PubMed: 3162236]

16. Demple B, Sedgwick B, Robin P, Totty N, Waterfield MD, Lindahl T. Active site and complete sequence of the suicidal methyltransferase that counters alkylation mutagenesis. Proc. Natl. Acad. Sci. USA. 1985; 82:2688–2692. [PubMed: 3887409]

17. Machado CR, Praekelt UM, de Oliveira RC, Barbosa AC, Byrne KL, Meacock PA, Menck CF. Dual role for the yeast THI4 gene in thiamine biosynthesis and DNA damage tolerance. J. Mol. Biol. 1997; 273:114–121. [PubMed: 9367751]

18. Ruiz-Roldán C, Puerto-Galán L, Roa J, Castro A, Di Pietro A, Roncero MI, Hera C. The Fusarium oxysporum sti35 gene functions in thiamine biosynthesis and oxidative stress response. Fungal. Genet. Biol. 2008; 45:6–16. [PubMed: 17964830]

19. Medina-Silva R, Barros MP, Galhardo RS, Netto LE, Colepicolo P, Menck CF. Heat stress promotes mitochondrial instability and oxidative responses in yeast deficient in thiazole biosynthesis. Res Microbiol. 2006; 157:257–281.

20. Collaborative Computational Project-Number 4. The CCP-4 suite: programs for protein crystallography. Acta. Crystallogr. D. 1994; 50:760–763. [PubMed: 15299374]

21. Murshudov GN, Vagin AA, Lebedev A, Wilson KS, Dodson EJ. Efficient anisotropic refinement of macromolecular structures using FFT. Acta Crystallogr. D. 1999; 55(Pt 1):247–255. [PubMed: 10089417]
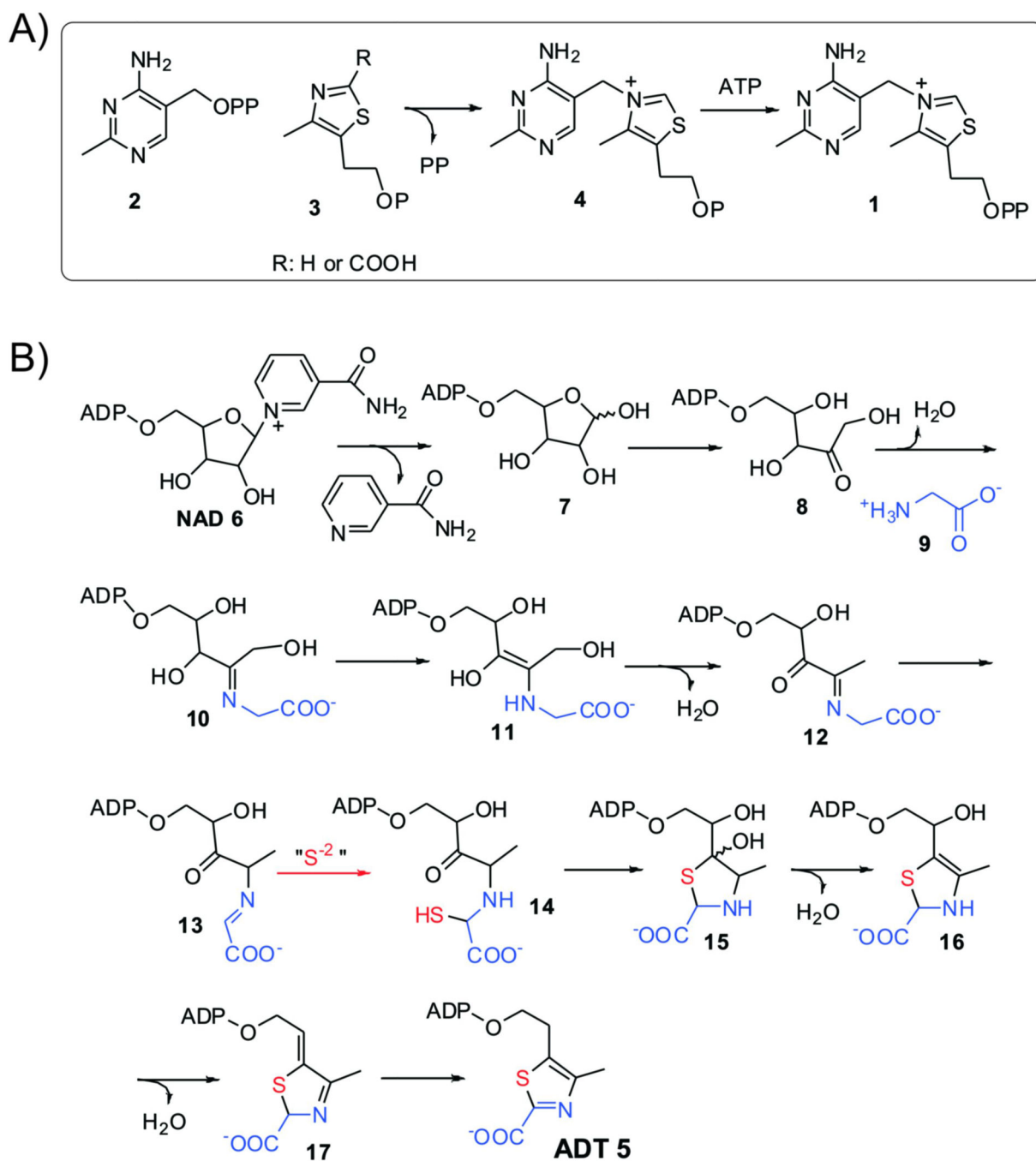
Figure 1.
A) The late steps in thiamin pyrophosphate biosynthesis. B) Mechanistic proposal for the biosynthesis of thiamin-thiazole in eukaryotes catalyzed by THI4p.
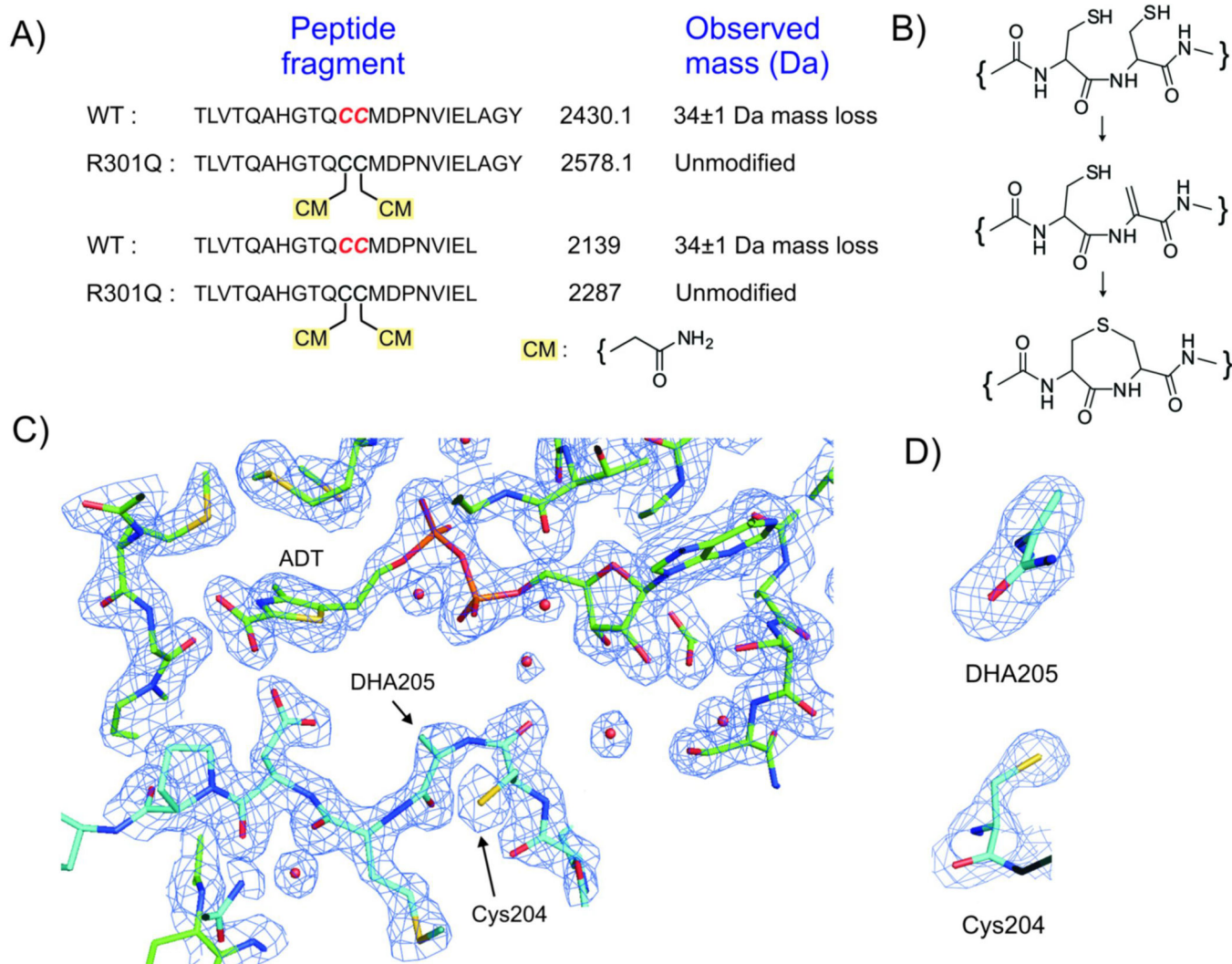
Figure 2.
Identification of the site of the M-34Da modification in wtTHI4p. A) The peptide fragments originating from wtTHI4p and the R301Q mutant containing the site of modification. B) A mechanistic hypothesis to explain the mass loss and lack of reactivity with iodoacetamide of the modified peptide. C) Active site of THI4p with bound ADT. The separation of the sulfur atom of ADT and the C_β atom of the dehydroalanine residue is 5.3 Å. The loop Gln203 – Pro208 is from a fourfold-related monomer and has carbon atoms colored cyan. Water molecules are shown as red spheres. The electron density map ($2F_o$-F_c contoured at 3σ) clearly shows the loss of sulfur from Cys205 to form the dehydroalanine residue. D) Magnified electron density of residue DHA205 and residue Cys204.
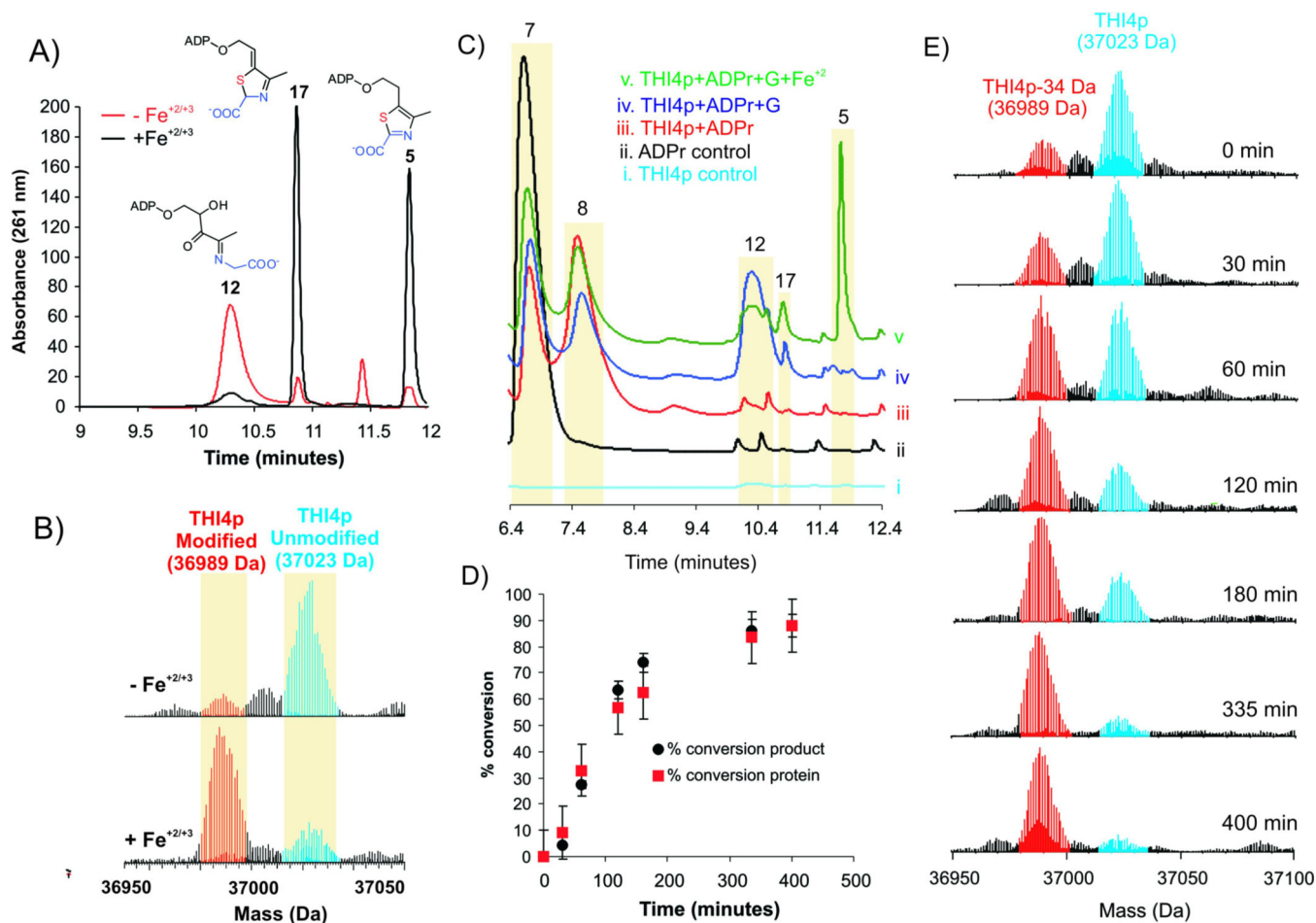
Figure 3.
Reconstitution of the biosynthesis of ADT **5**. A) HPLC analysis of the metabolites associated with wtTHI4p overexpressed in M9 minimal medium ±100 μM iron. B) ESI-FTMS analysis of wtTHI4p overexpressed in M9 minimal medium ±100 μM iron shows iron-dependent modification (M = −34 Da) of the protein. C) HPLC analysis of wtTHI4p catalyzed partial and full reactions and the relevant control reactions. Incubating THI4p with ADPr **7**, glycine and iron(II) results in the production of ADT **5**. D) Time course for the reaction showing a 1:1 ratio of protein modification and thiazole production (error bars indicate s.d.) E) MS analysis of wtTHI4p over the time course of the reaction showing the progressive conversion of the enzyme to the M-34 Da species.
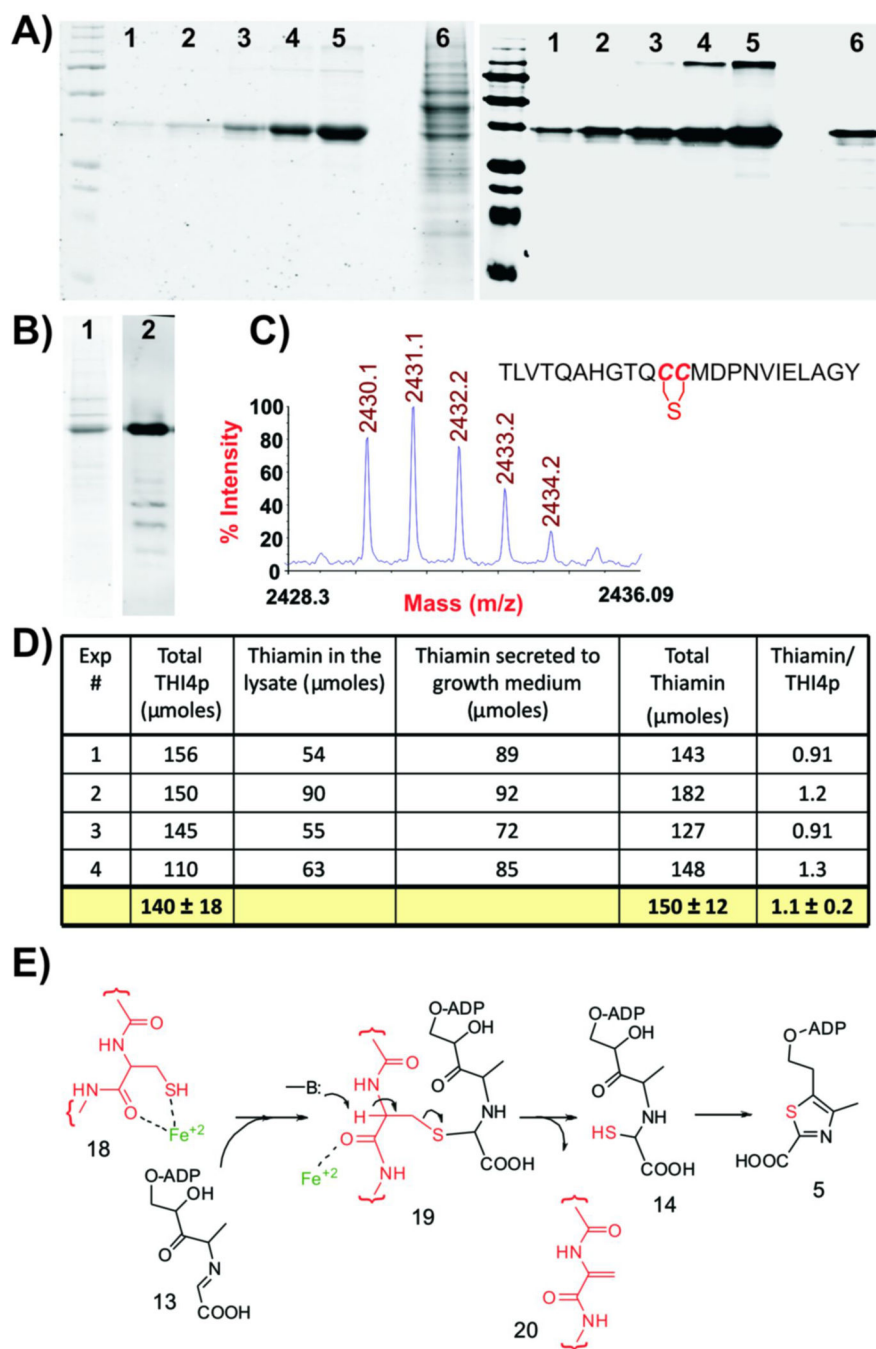
Figure 4.
Characterization of native THI4p from *S. cerevisiae*. A) Analysis of THI4p in yeast cell free extract using a western blot (right) and coomassie blue (left). Lanes 1–5 contain increasing concentrations (0.5, 1, 2, 4, and 8 μM) of (His)$_6$-THI4p. Lane 6 contains yeast crude lysate. B) Native THI4p, isolated from yeast cell free extract, analyzed by SDS-PAGE/coomassie blue (1) and western-blot (2). C) In gel chymotrypsin digestion/MALDI-TOF analysis of isolated native THI4p demonstrates that the peptide containing the C204-C205 region has the same modification as observed with THI4p expressed in *E. coli*. D) Quantitation of

THI4p and thiamin produced in a culture of yeast, growing in vitamin free defined medium, demonstrates that THI4p is a substrate rather than a catalyst. E) Proposed mechanism for the iron-mediated sulfur transfer reaction involved in the formation of intermediate 14 (Figure 1).