ThiC Is an [Fe-S] Cluster Protein That Requires AdoMet To Generate the 4-Amino-5-hydroxymethyl-2-methylpyrimidine Moiety in Thiamin Synthesis[†]

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ABSTRACT: Thiamin pyrophosphate is a required cofactor in all organisms. The biosynthesis of thiamin requires the independently synthesized 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate (HMP-PP) and 5-hydroxyethyl-4-methylthiazole phosphate (THZ-P) moieties. In bacteria, the pyrimidine moiety is derived from 5-aminoimidazole ribotide (AIR), and ThiC is the only gene product known to be required for this conversion in vivo. We report here the purification and characterization of the ThiC protein from *Salmonella enterica*. The data showed this protein generated HMP when AIR, *S*-adenosylmethionine (AdoMet), and an appropriate reducing agent were present. It is further shown that ThiC carries an oxygen labile [Fe-S] cluster essential for this activity.

Thiamin pyrophosphate (TPP) is an essential cofactor formed from 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate (HMP-PP) and thiazole monophosphate (THZ-P) moieties. In bacteria, HMP-PP and THZ-P are independently synthesized and combined by thiamin phosphate (TMP) synthase (ThiE, EC 2.5.1.3), yielding TMP prior to its conversion to TPP by the TMP kinase (ThiL, EC 2.7.4.16) (*1*) (Figure 1A). Synthesis of the THZ moiety has been reconstituted in vitro using components from Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli* and *Salmonella enterica*) bacteria (*2*−*7*). In contrast, our understanding of the biochemical steps leading to the synthesis of HMP is limited. Genetic and biochemical studies have shown that HMP is derived from 5-aminoimidazole ribotide (AIR) (*8*−*12*). In vivo labeling studies showed that all carbon and nitrogen atoms present in HMP are derived from AIR, as illustrated in Figure 1B (*8, 9, 13, 14*). These labeling data suggested that the conversion from AIR to HMP involves the breakage and re-forming of multiple bonds. Despite this complex intramolecular rearrangement, only one gene product (ThiC) has been shown to be essential for this conversion in vivo.

Formation of HMP was reported in cell-free extracts of an *E. coli* strain that overproduced ThiC (*14*). In the cited study, the ThiC-dependent conversion of AIR to the pyrimidine was measured by in situ conversion of the product to TMP, which was quantified by a thiochrome assay (*5*).

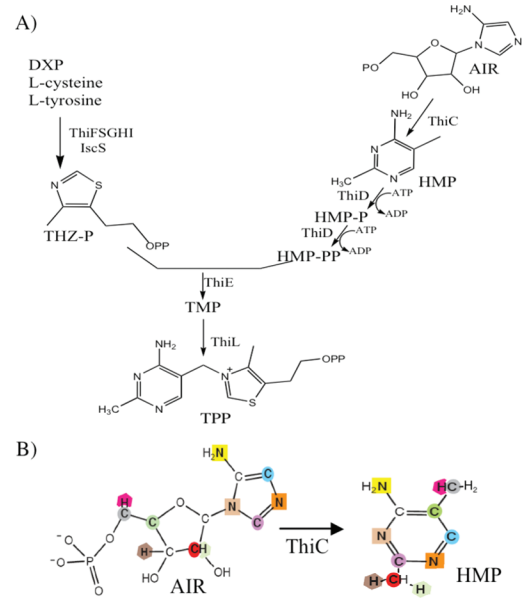

FIGURE 1: Thiamin biosynthesis in *S. enterica.* (A) Schematic of thiamin synthesis showing the independent formation of THZ-P and HMP-PP prior to thiamin synthase. Gene products involved are indicated by the relevant steps. (B) Labeling studies have shown the correlation of atoms present in AIR and HMP as indicated by shape and color (*8, 14*). Abbreviations: AIR, aminoimidazole ribotide; HMP, 4-amino-5-hydroxymethyl-2-methylpyrimidine; DXP, 1-deoxy-D-xylulose 5-phosphate; TMP, thiamin phosphate; TPP, thiamin pyrophosphate; THZ-P, thiazole monophosphate.

The ThiC-dependent activity detected in the cell-free extract was low and increased with the addition of dialyzed cell-free extract of a *thiC* mutant strain. These studies indicated that AIR, AdoMet, either NAD^+, NADH, or NADPH, and an additional cellular component were required for optimal ThiC activity. Purification of ThiC resulted in loss of all activity, and no evidence of an [Fe-S] cluster was found (*14*). Recently, the UV−visible spectrum of a plant-encoded ThiC was used to suggest the presence of an [Fe-S] cluster associated with the protein (*15*).

In this report, we describe the purification and functional assay of the *S. enterica* ThiC protein. We show that, when purified anoxically, ThiC contained an oxygen labile [Fe-S] cluster that was essential for ThiC to convert AIR to HMP.

Strain DM7474 (*E. coli*; SG-13009 pREP4 pMD34) was used to produce ThiC-His_6. When purified under anoxic conditions, 12 mg of ThiC protein was obtained from 30 g of cells. The ThiC protein sample (6 mg/mL) had a brown color with an UV−visible spectrum characteristic of [Fe-S] cluster proteins, including a shoulder at 410 nm (Figure 2A,

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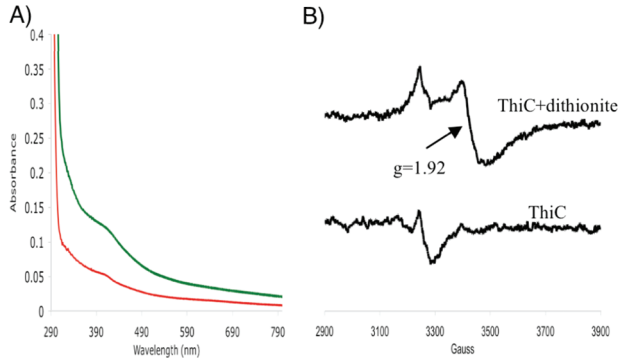

FIGURE 2: Spectroscopic analysis detects an [Fe-S] cluster in ThiC. (A) UV−visible spectra. The UV−visible spectra of ThiC (10 μM) as purified (green) and after being exposed to oxygen for 5 h (red) are shown. (B) EPR spectra. ThiC (100 μM) was incubated with 2.7 mM dithionite (10 min), and the EPR spectra were recorded at 10 K. A $g = 1.92$ signal is present only upon reduction with dithionite. Conditions: field center, 3300 G; scan width, 400 G; microwave frequency, 9.26 GHz; microwave power, 1 mW; modulation frequency, 100 kHz; modulation amplitude, 16 G; time constant, 0.3 s; scan time, 240 s; and gain, 8000.

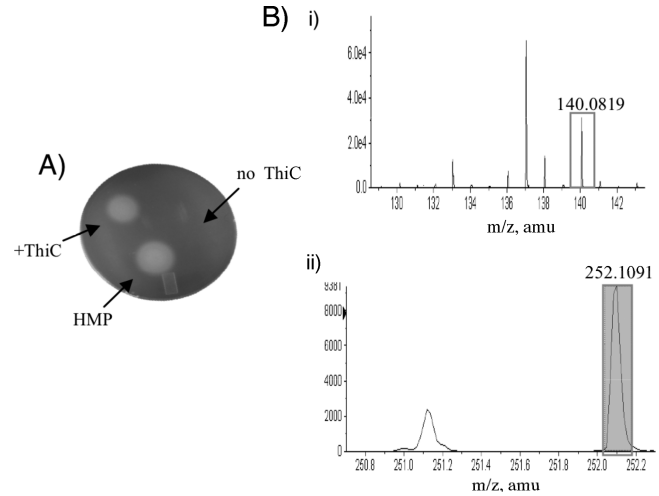

FIGURE 3: ThiC reaction generates HMP. (A) Bioassay of reaction products. A layer of soft agar seeded with a culture of strain DM7185 (*thiC*) was spread on minimal medium. Reaction mixtures from an assay with or without ThiC protein as indicated were spotted on the agar and incubated for 18 h at 37 °C. HMP was spotted as a control. Turbid zones indicated growth. (B) HPLC−MS analysis of reaction products. Units on the *y* axis correspond to intensity counts. (i) In the HPLC elution profile, at 6.49 min there was a peak with the accurate mass of HMP (*m*/*z* 140.0819). (ii) Likewise, at 2.79 min a peak with the accurate mass of 5′-deoxyadenosine (*m*/*z* 252.10) was detected. Both signals were compared to authentic standards. In addition to ThiC, the reaction mixture contained 0.8 mM NADPH, Fpr (12 mM), FldA (10 mM), 2 mM AIR, 1.2 mM AdoMet, 1.6 mM ATP, and 2.6 mM $MgCl_2$.

green). The magnitude of the signal at 410 nm decreased when the sample was exposed to oxygen (Figure 2A, red) or when dithionite was added to an anoxic sample (data not shown). The protein contained 3.8 ± 0.6 and 3.0 ± 0.8 mol of iron and sulfide, respectively, per mole of protein. The theoretical A_{400}/A_{280} ratio of [4Fe-4S] cluster/subunit proteins is 0.16−0.19 (*f*). The A_{400}/A_{280} ratio of four independently reconstituted ThiC preparations was 0.17 ± 0.3 (*f*), consistent with the presence of a [4Fe-4S] cluster/subunit ThiC (ε_{280} = 75.33 mM^{-1} cm^{-1}). A typical ThiC preparation was >90% pure as judged by visualization on a SDS−PAGE gel (Supporting Information).

When purified under oxic conditions, the ThiC protein sample (4 mg/mL) was colorless and had no spectroscopic feature that would indicate the presence of an [Fe-S] cluster (data not shown). Together, these results suggested that ThiC contained an oxygen labile [Fe-S] cluster. All subsequent manipulations of the protein were performed under anoxic conditions (<2 ppm O_2) unless otherwise stated.

The ThiC protein was reduced with 2.7 mM dithionite, transferred to an EPR tube, and frozen in liquid nitrogen. The spectra recorded at 10 K had a $g = 1.92$ signal, characteristic of [4Fe-4S] cluster proteins reduced to the +1 state (Figure 2B) (*16*).

Purified ThiC was assayed in vitro for activity. ThiC (11 nmol) was reduced with dithionite (2.7 mM) for 30 min, and substrates were added in the following order to a final volume of 100 μL: AdoMet (1.2 mM), AIR (2 mM), $MgCl_2$ (2.6 mM), and ATP (1.6 mM). The reaction mixture was incubated at 37 °C for 8 h, boiled for 2 min, and cleared by centrifugation (16000*g* for 2 min at 4 °C). The supernatant was removed from the anaerobic chamber and tested for its ability to support the growth of a strain lacking ThiC (DM7185) (*17*). Figure 3A shows results from a representative bioassay, with authentic HMP as a control.

The qualitative results from the bioassay were extended by HPLC−MS (MALDI-TOF) analysis of the reaction product(s). An ion signal with an accurate mass of *m*/*z* 140.0819 was detected in the reaction mixture containing ThiC (Figure 3B). This ion was identical to a control standard of HMP both in mass and in retention time and was dependent on ThiC. The amount of HMP formed was small compared to the amount of substrate provided. Consistently, spin quantitation analysis showed a 1:100 correspondence between the amount of $[4Fe\text{-}4S]^+$ cluster observed and the protein present, suggesting that only 1% of the protein had a reduced cluster. This result could indicate that the majority of the protein population is not loaded with a [4Fe-4S] cluster and/or the clusters are not being efficiently reduced.

In this study, we demonstrated that ThiC was able to convert AIR to HMP and required AdoMet to do so. The data indicated the ThiC protein contained an oxygen labile [Fe-S] cluster that was essential for activity and explained the need for strictly anoxic conditions in all manipulations. Under standard conditions (10 K), and in the presence of dithionite, the cluster in ThiC had a signal typical of a $[4Fe\text{-}4S]^+$ cluster.

In a chemically defined system, ThiC required AIR and AdoMet for product formation. In addition, a suitable reducing system such as the Fpr/FldA/NADPH system or dithionite was necessary to generate active ThiC. HMP was detected as the final product of the ThiC reaction. The formation of HMP-P could not be eliminated since the phosphate group may be unstable under the acidic conditions used for purification. Previous studies are most consistent with HMP-P being the true product of the ThiC reaction (*18, 19*), and future studies are needed to clarify this.

The requirement of AdoMet for ThiC activity narrows the possible mechanisms involved in the reaction. ThiC does not contain the characteristic CXXXCXXC motif that defines members of the radical AdoMet superfamily of proteins. However, it contains a C(S/T)MCXXXXC motif near the C-terminus of the protein that is conserved in every ThiC

homologue representing more than 100 diverse organisms (*17*). A single additional cysteine is similarly conserved. Further, the primary sequence of ThiC has a conserved motif rich in glycine residues (GIVSRGGS). Glycine-rich sequences have been suggested to encode the amino acids necessary to bind AdoMet in members of the AdoMet superfamily of proteins (*20*). AdoMet is not acting as a methylating agent in thiamin biosynthesis (*14*) and may be either a substrate or cofactor needed to generate the 5′-deoxyadenosyl radical. Analysis of the reaction mixture detected the ThiC-dependent formation of 5′deoxyadenosine [by mass spectroscopy (Figure 3B)] and methionine (by bioassay), suggesting AdoMet is being cleaved in the reaction. Further studies aimed at the quantification of both products of this cleavage will help define the specific role of AdoMet in the ThiC-catalyzed reaction.

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SUPPORTING INFORMATION AVAILABLE

Detailed materials and methods. This material is available free of charge via Internet at http://pubs.acs.org.

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