Thiamine biosynthesis in *Escherichia coli*: isolation and initial characterisation of the ThiGH complex

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Abstract **In *Escherichia coli*, two of the proteins required for the biosynthesis of the thiazole moiety of thiamine (vitamin B_1) are ThiG and ThiH, encoded as part of the *thiCEFSGH* operon. In this study, a C-terminally hexahistidine-tagged ThiH (ThiH-His) was expressed in *E. coli* as a soluble protein from *thiGH-His-tag* and *thiFSGH-His-tag*-bearing plasmids. When isolated under anaerobic conditions, ThiG and ThiH-His co-purify as a large multimeric non-covalent complex. Electron paramagnetic resonance and UV–visible spectroscopy together with iron and sulfide analyses revealed the presence of an iron–sulfur cluster within this complex.**

Key words: Thiamine biosynthesis; Iron–sulfur protein; Electron paramagnetic resonance

1. Introduction

Thiamine (vitamin B_1) (Fig. 1.3) is an essential co-factor synthesised by both microorganisms and plants. The vitamin is assembled by coupling of 4-methyl-5-(β-hydroxyethyl)thiazole monophosphate (Fig. 1.1) and 4-amino-5-hydroxymethylpyrimidine pyrophosphate (Fig. 1.2) which are produced through separate pathways. In *Escherichia coli* the thiazole is derived from tyrosine, cysteine and 1-deoxy-D-xylulose-5-phosphate [1] (Fig. 1) but the mechanism by which these acyclic precursors are combined and cyclised to yield the thiazole remains uncertain. Four genes from the thiamine operon, *thiFSGH*, and two genes located elsewhere on the chromosome, *iscS* and *thiI*, are required for thiazole ring formation. Significant progress has been made in understanding the metabolic pathway by which sulfur is provided for thiazole [2–4]. Cysteine provides the sulfur atom, which is transferred to IscS, a pyridoxal phosphate (PLP) dependent cysteine desulfurase, to form an active site cysteine persulfide [5]. The sulfur atom can then be used to convert the C-terminal carboxylate of another enzyme, ThiS, into a thiocarboxylate. The formation of the ThiS thiocarboxylate is catalysed by ThiF, which activates the terminal glycine residue of ThiS as its acyl adenylate [4]. Once formed, the ThiS thiocarboxylate can react with residue Cys184 of ThiF to form a highly unusual acyl disulfide intermediate that is essential for the thiazole-forming reaction [2]. One other protein, ThiI, may also be involved in the sulfur transfer, although its role has yet to be fully elucidated [5–7].

Much less is known about the two remaining open reading frames (ORFs) required for thiazole biosynthesis, ThiG and ThiH. During mass spectrometric analysis of the protein products of the *thiCEFSGH* operon, ThiH was expressed from a pET derived plasmid which resulted in inclusion bodies. ThiH solubilised from this source gave the mass predicted by the encoding sequence [8]. The ThiH sequence contains a Cys-X-X-X-Cys-X-X-Cys motif that has been identified as characteristic of a family of iron–sulfur cluster-bearing enzymes [9]. Supporting evidence for the presence of an iron sulfur cluster in ThiH has been provided by studies of *Salmonella enterica* LT2, whose thiamine biosynthetic genes show a high degree of sequence similarity to those of *E. coli*. In these studies, two mutant strains of *Salmonella* with a thiamine dependent phenotype were isolated bearing mutations in *gshA* [10] and the *isc* gene cluster [11]. The mutated genes encode proteins that are important in maintaining the redox state of the cell and assembling iron–sulfur clusters into proteins, respectively. The effect of the *gshA* and *isc* mutations has been rationalised in terms of a readily oxidisable iron–sulfur cluster in ThiH, and suggests that the iron–sulfur cluster is essential for thiazole-forming activity, either as part of the catalytic activity or because it stabilises the protein [10].

As a prerequisite to investigating the biochemical role of ThiG and ThiH in thiazole biosynthesis, we investigated their soluble expression, purification and characterisation. Here we report the soluble expression of ThiH with a $(His)_6$ tag attached to the C-terminus (ThiH-His) and show that it can be purified in a large (>400 kDa), multimeric, non-covalent complex with ThiG. Spectroscopic and analytical methods demonstrate that the ThiGH-His complex contains an iron–sulfur cluster.

2. Materials and methods

2.1. Materials

DNA manipulations were carried out by standard protocols. β-L-arabinose was purchased from Avocado; the other chemicals were of reagent grade and purchased from Sigma-Aldrich unless otherwise stated. Restriction enzymes were purchased from Promega; molecular biology reagents were obtained either from Promega or Qiagen. Pfu Turbo DNA polymerase was purchased from Stratagene. pBAD-TOPO and pBAD/HisA cloning systems were obtained from Invitro-

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Abbreviations: ThiH-His, ThiH with a $(His)_6$ tag attached to the C-terminus; EPR, electron paramagnetic resonance; BSA, bovine serum albumin; DTT, dithiothreitol; PLP, pyridoxal phosphate

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gen and pET-24d(+) from Novagen. Ampicillin (100 μg/ml) and kanamycin (30 μg/ml) were used to select for plasmids, as appropriate. Oligonucleotides were synthesised by Oswel (Southampton). The *E. coli* KG33 mutant was kindly provided by the *E. coli* Genetic Stock Center (Yale University). 4-amino-5-hydroxymethylpyrimidine was prepared by the method of Andersag [11]. Gel densitometry measurements were recorded using a Syngene Gene Genius imaging system.

2.2. Amplification and expression

Unless otherwise stated, the *thiH* present in the polymerase chain reaction (PCR) products described below encodes a C-terminally hexahistidine-tagged protein. DNA fragments carrying *thiGH* and *thiFSGH* were amplified from *E. coli* genomic DNA using Pfu Turbo and A-tailed using Taq polymerase. PCR products were ligated into pBAD-TOPO plasmid and subcloned into pET-24d(+) (Novagen) on an *Nco*1/*Bam*H1 restriction fragment and into pBAD/HisA by restricting the pET derived plasmids with *Nco*1/*Xho*1. The derived pET plasmid encoding ThiGH-His was named pRL800. The derived pET and pBAD plasmids encoding ThiFSGH-His were named pRL1000 and pRL1020, respectively.

For protein expression experiments, BL21(DE3) was transformed with the appropriate plasmid and plated onto 2YT agar at 37°C overnight. Single colonies were used to inoculate 2YT medium (100 ml) and grown at 37°C overnight. The overnight cultures were then used as 1% inocula into 2YT medium (4×1.25 l) and grown at 37°C until the OD at 600 nm reached 0.5–0.7. The cultures were then induced with either arabinose [0.2% (w/v)] or IPTG (0.5 mM) and the temperature decreased to 28°C. Five hours after induction, cells were harvested by centrifugation (Beckmann JA-14, 12000 rpm, 10 min at 4°C) and the cell pellet stored at −80°C until used.

2.3. ThiGH-His purification

Protein concentrations were determined by the method of Bradford [12] or by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis and gel densitometry against bovine serum albumin (BSA) standards of known concentration. Where possible, protein samples were manipulated under anaerobic conditions using a Belle glove box (<2 ppm of O_2). Cell paste (~30 g) was transferred to the glove box, lysozyme (0.1 mg/ml), benzonase (5 U/ml) and PMSF (phenylmethylsulfonyl fluoride; 1 mM final concentration) were added followed by anaerobic buffer A [90 ml, 50 mM Tris–HCl, pH 8.0, 200 mM NaCl, 50 mM imidazole, 12.5% (w/v) glycerol]. The suspension was then withdrawn from the anaerobic box and rapidly lysed by sonication. The lysate was returned to the box, allowed to degas (10 min), PEI (polyethylene imine) added (0.2% final concentration) and cleared by centrifugation in gas-tight tubes (Beckmann JA-14 rotor, 12 000 rpm for 30 min at 4°C). The brown supernatant was applied to a Ni-charged Chelating Sepharose column (25 ml) which had previously been equilibrated with buffer A. The column was washed with buffer B [100 ml, 50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 50 mM imidazole, 12.5% (w/v) glycerol], then buffer A (50 ml) and proteins were eluted with an increasing gradient (0 to 50% over 4 column volumes) of buffer C (buffer A containing 500 mM imidazole). The most concentrated fractions were desalted on a Superdex-S75 column (AP Biotech, 3 cm I.D.×15 cm) equilibrated with anaerobic buffer D [50 mM Tris–HCl, pH 8.0, 5 mM dithiothreitol (DTT), 12.5% (w/v) glycerol] and the purest fractions, as judged by SDS–PAGE gel analysis, were pooled, glycerol added to a final concentration of 25% (w/v) and the protein concentrated to 3–6 mg/ml by ultrafiltration. Proteins were stored at −80°C in this buffer (buffer E).

2.4. Anaerobic analytical gel filtration

Purified ThiGH-His samples (250–300 μl) were applied to a Superdex 200 HR 10/30 pre-packed column (AP Biotech) equilibrated with anaerobic buffer D and eluted at 0.5 ml/min. Eluted proteins were analysed by SDS–PAGE.

2.5. Iron, sulfide and co-factor quantification

Iron and sulfide were analysed the methods of Fish [13] and Beinert [14], respectively, and were determined in triplicate. ThiGH-His samples were digested with trypsin (0.3 mg/ml, 37°C, overnight) to facilitate quantitative iron release before iron analysis. DTT was anaerobically removed from the protein samples prior to the sulfide assay using a NAP-10 column (AP Biotech) equilibrated with DTT-free buffer D. PLP was assayed by the method of Bates et al. [15].

2.6. UV–visible spectroscopy

UV–visible spectra were recorded in gas-tight cuvettes using a Perkin-Elmer Lambda 2 spectrophotometer at a ThiH-His concentration of 3 mg/ml in buffer E.

2.7. Electron paramagnetic resonance (EPR) spectroscopy

EPR spectra were recorded at X-band on a Bruker ELEXYS 500 spectrometer with an ER094X microwave bridge using an ER4122SHQ cavity. Low temperature experiments were performed using an ESR 900 liquid helium flow cryostat and ITC3 temperature controller (Oxford Instruments). Spectra were recorded at a ThiH-His concentration of 5–6 mg/ml in buffer E. Spectra of the reduced protein were obtained after incubation with dithionite (1 mM, 30 min). Double integration of spectra was done by comparison with a 1 mM sample of Cu(EDTA).

2.8. In vivo activity

pBAD/HisA and pRL1020 were transformed into *E. coli* KG33. Both transformants were grown in parallel in 2YT medium (10 ml) containing ampicillin (100 μg/ml). When the OD at 600 nm reached ~1.3, cells were pelleted and washed by resuspending in Davis and Mingioli medium [16] (2×5 ml) containing arabinose (0.2%) and ampicillin (100 μg/ml). Pellets were resuspended in medium to give an OD_{600} of 0.790 and five serial 10-fold dilutions were prepared for both samples. Equal volumes (5 μl) were plated on Davis and Mingioli solid medium containing agar (1.5%), ampicillin (100 μg/ml), D-glucose (0.2%), β-L-arabinose (0.2%), L-tyrosine and L-cysteine (0.2 mM) and 4-amino-5-hydroxymethylpyrimidine (50 μM). Colonies were allowed to grow at 37°C for 48 h.

3. Results

3.1. Expression, purification and characterisation of the ThiGH-His complex

As initial attempts to express ThiH alone resulted in the accumulation of insoluble inclusion bodies (data not shown), the potential of co-expressing ThiH with other thiamine biosynthetic proteins was examined. Two fragments were amplified by PCR, encoding ThiGH and ThiFSGH. In each case, a 6×His tag was appended to the C-terminus of ThiH, to facilitate the subsequent purification of the expressed protein. This strategy has been successfully employed for the isolation of other proteins containing an oxygen-sensitive iron–sulfur cluster [17–20]. Co-expression of ThiGH-His from pET-24d(+) resulted in ThiG and ThiH-His accumulating in the soluble fraction as indicated by SDS–PAGE analysis (Fig. 2A, lane 1). However, this expression system consistently gave a low yield of cell paste (~5–15 g of cells from 5 l) and other expression systems were therefore examined. A similar expression level was obtained when ThiFSGH-His were expressed from the same host plasmid (Fig. 2A, lane 2), but the yield of cell paste was improved (~25 g from 5 l). A further slight improvement was achieved by subcloning ThiFSGH-His into pBAD/HisA, which routinely gave a yield of 25–30 g of cell paste from 5 l.

ThiH-His and any associated protein were purified by nickel affinity chromatography under anaerobic conditions (<0.2 ppm O_2) from either pRL800, pRL1000 or pRL1020 in BL21(DE3). The protein eluted from this column had a brown colour and a marked tendency to precipitate. Removal of the imidazole-containing buffer by prompt desalting helped to prevent the precipitation, as did the use of glycerol-containing buffers.

SDS–PAGE analysis of the proteins isolated from pRL800/BL21(DE3) showed that in addition to ThiH-His, another protein with an apparent molecular weight of 26 kDa was present. Gel densitometry indicated that this band was present

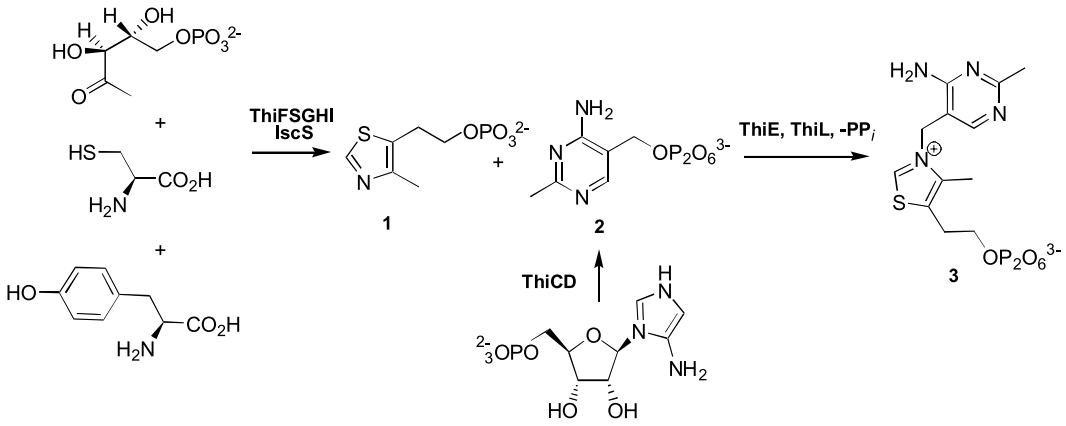

Fig. 1. Thiamine biosynthesis in *E. coli*. 1: 4-Methyl-5-(β-hydroxyethyl)thiazole monophosphate; 2: 4-amino-5-hydroxymethylpyrimidine pyrophosphate; 3: thiamine pyrophosphate; PP_i: pyrophosphate.

at approximately 0.2 mol equivalents of the ThiH-His. When proteins were isolated from pRL1000 or pRL1020 transformants expressing ThiFSGH-His, there was a substantial increase in the amount of the 26 kDa band, to approximately 0.8–1.0 mol equivalents of ThiH-His (Fig. 2A, lanes 4–6). N-terminal protein sequencing of this 26 kDa band showed that the first seven amino acids corresponded to the sequence of ThiG. Electrospray mass spectrometry confirmed the presence of two species with masses consistent with the expected masses of ThiH-His (found 44 142.7 Da, calculated 44 143.0 Da) and ThiG (found 26 893.3 Da, calculated 26 896.1 Da).

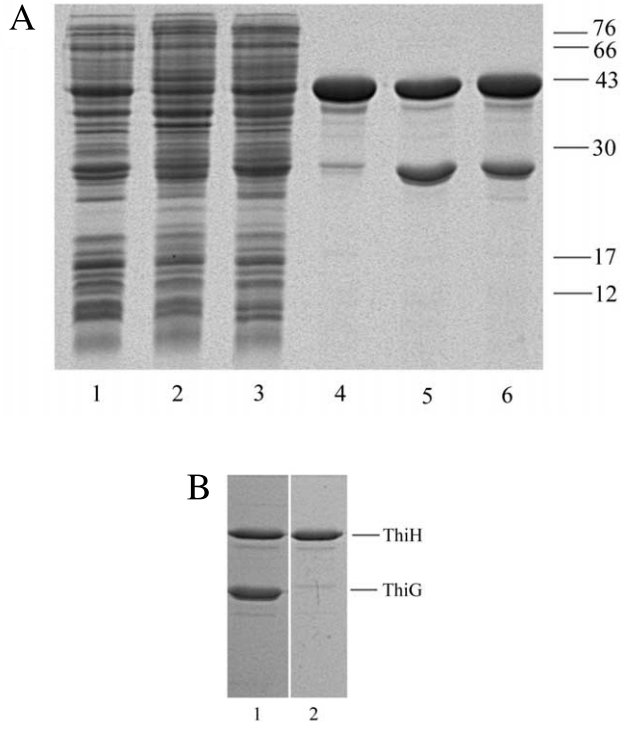

Fig. 2. SDS–PAGE analysis of protein samples. A: Purification of ThiGH-His from *E. coli* BL21(DE3). Lanes 1–3: cleared lysates; lanes 4–6: purified proteins. Lanes 1 and 4: from pRL800; lanes 2 and 5: from pRL1000; lanes 3 and 6: from pRL1020. B: Fractions from analytical gel permeation chromatography. Lane 1: fraction eluted at 23 min (~440 kDa); lane 2: fraction eluted at 30 min (~44 kDa).

The possibility of a complex between ThiG and ThiH-His was further investigated by analytical gel permeation chromatography. Two peaks were observed at 23 and 30 min, corresponding to apparent molecular weights of 440 and 44 kDa, respectively. SDS–PAGE analysis confirmed that the 440 kDa complex contained both ThiG and ThiH-His, whilst the second peak contained only monomeric ThiH-His (Fig. 2B, lanes 1 and 2). As the plasmids pRL1000 and pRL1020 encoded four ORFs (ThiFSGH-His), the possibility of ThiF or ThiS co-purifying with ThiH-His was also examined. Differentiating ThiG and ThiF by SDS–PAGE is complicated by the closeness of their predicted masses (26 896 Da and 26 970 Da, respectively), but neither SDS–PAGE analysis nor electrospray mass spectrometry of the purified protein samples indicated that protein species other than ThiG and ThiH-His were present.

Protein samples purified from pRL800 and pRL1000 were assayed for iron and sulfide content. Because of the co-purification of ThiG, the concentration of ThiH-His was estimated by gel densitometry against a series of BSA standards that had been analysed by SDS–PAGE. ThiGH-His from pRL1020 was found to contain 1.0 Fe and 2.0 S atoms per mol ThiH-His polypeptide chain, while that from pRL800 was found to contain 0.5 Fe and 0.7 S atoms per mol ThiH-His polypeptide chain. Assays for PLP in purified ThiGH-His

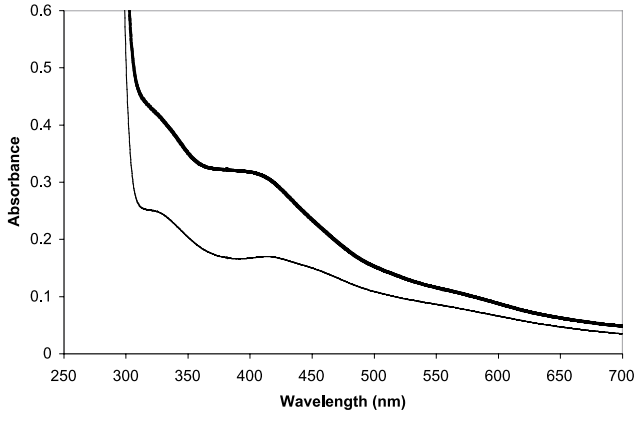

Fig. 3. UV–visible spectra of protein samples. Each sample was 3 mg/ml in ThiH-His (68 μM). Heavy line: protein isolated from pRL1020/BL21(DE3); light line: protein isolated from pRL800/BL21(DE3). Both spectra were recorded in buffer E.

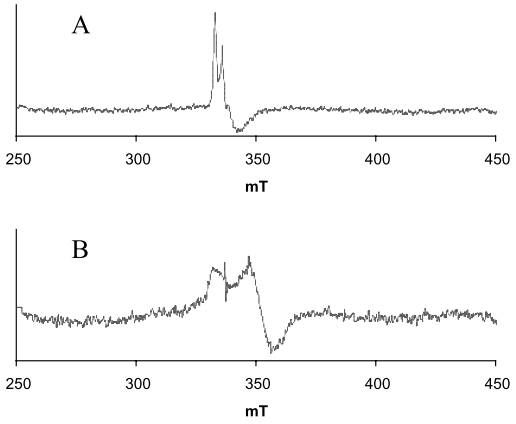

Fig. 4. X-band EPR spectra of ThiGH-His isolated from pRL1020/BL21(DE3). A: As isolated (5.0 mg/ml in ThiH-His). B: Sample reduced with dithionite, after subtraction of a background buffer spectrum. The sharp signal at 338 mT is an artefact of the background subtraction. The spectra were recorded under the following conditions: temperature, 10 K; microwave frequency, 9.4335 GHz; microwave power, 1.997 mW; modulation amplitude, 0.4 mT; receiver gain, 1×10^4.

from pRL1020 were negative (less than 0.001 mol PLP per mol ThiH-His).

3.2. Spectroscopic properties of the iron–sulfur centre of ThiGH-His

The UV–visible spectrum of proteins isolated from pRL800 or pRL1020 are shown in Fig. 3. They exhibit a characteristic absorption maximum at 410 nm with a shoulder present at 340 nm. The spectra resemble those reported for anaerobically isolated PFL-AE [21] or LipA [17] and are consistent with a mixture of [3Fe–4S] and $[4Fe–4S]^{2+}$ clusters in the samples [17,21,22]. EPR spectra of ThiGH-His isolated from pRL1020 are shown in Fig. 4. The spectrum of the as isolated protein (Fig. 4A) showed a signal typical of a [3Fe–4S] centre that integrates to 0.7 μM. Upon reduction with dithionite, a $S=1/2$ species characterised by an axial EPR signal with $g_{\|}=2.02$ and $g_{\perp}=1.92$, integrating to 1.1 μM, was observed (Fig. 4B). This is consistent with the formation of a $[4Fe–4S]^{+}$ cluster upon reduction and similar behaviour has been observed for the iron–sulfur cluster of LipA [17], spore photoproduct lyase [20] and anaerobic ribonucleotide reductase activating enzyme [23].

3.3. In vivo activity

E. coli KG33 is auxotrophic for 4-methyl-5-(β-hydroxyethyl)thiazole and is known to contain a lesion in ThiH [24]. Transformation with pRL1020 (encoding ThiFSGH-His) resulted in vigorous growth on thiamine deficient medium, whereas the control sample, *E. coli* KG33 transformed with pBAD/HisA, grew poorly (data not shown). This complementation experiment indicates that ThiH-His encoded by pRL1020 is active and enables the cells to produce thiazole.

4. Discussion

ThiH-His from *E. coli* has been purified in a complex with ThiG from cells expressing either ThiGH-His or ThiFSGH-His. Thus, for the first time a purified soluble form of this enzyme could be obtained and characterised. By purifying the protein under anaerobic conditions, the protein could be isolated with a proportion of its oxygen-sensitive iron–sulfur cluster present. EPR and UV–visible spectroscopic evidence indicates that the anaerobically purified protein contains a [3Fe–4S] cluster. EPR signals suggested that a $[4Fe–4S]^{+}$ cluster was formed upon reduction with sodium dithionite. The concentration of ThiH-His in these samples was 5 mg/ml, which is 114 μM, compared with EPR signal integrations of 0.7 μM and 1.1 μM for the [3Fe–4S] and [4Fe–4S] cluster signals, respectively; however the Fe content was only 1.0 per molecule, so that the maximum possible concentration of clusters was ca 30 μM. It can often be difficult to reduce [4Fe–4S] clusters fully and we observed at least 6% of the potential clusters by EPR. From these properties and the shared Cys-X-X-X-Cys-X-X-Cys motif, it is clear that the iron–sulfur centre in this complex shows many similarities to those of other members of the 'radical SAM' family of enzymes [9,20,25].

The nature of the expression system used to produce the protein sample had a considerable influence on the iron and sulfide stoichiometry and on the amount of ThiG that co-purified with ThiH-His. A subsection of the thiamine biosynthetic operon, encoding ThiFSGH-His, was inserted into expression vectors (pRL1000 and pRL1020). This resulted in isolated protein fractions with the highest iron and sulfide stoichiometries, of 1.0 and 2.0 per ThiH-His polypeptide chain, respectively. Protein isolated from this system also contained the highest proportion of ThiG, approximately 0.8–1 equivalents of the ThiH-His. Thus, the presence of *thiFS* in the plasmid affected the expressed form of ThiGH-His, although it is unclear whether the presence of *thiFS* is affecting DNA transcription, the translation or stability of the mRNA or whether there is a direct interaction between the expressed proteins. Mass spectrometric and SDS–PAGE analysis of the ThiGH-His-containing fractions did not provide any evidence for other proteins, such as ThiF or ThiS, forming a tight complex with ThiG or ThiH-His.

The recent studies of Ollagnier-de-Choudens et al. [26,27] have identified a PLP dependent desulphurase activity in suitably reconstituted biotin synthase, a member of the 'radical SAM' family. As the sulfur for thiamine biosynthesis is thought to be provided by ThiS thiocarboxylate, it is uncertain whether PLP is required for thiazole formation. Assays for PLP in purified ThiGH-His from pRL1020 did not show the presence of any PLP. Alternatively, this co-factor may be required but loosely bound to ThiGH-His, then lost during purification (as happens with biotin synthase).

The complementation of the thiazole auxotroph *E. coli* KG33 demonstrates that ThiGH-His encoded by these plasmids is active in the production of thiazole and hence thiamine, but we have not yet been able to assay the protein reported herein for in vitro thiazole-forming activity. As both ThiG and ThiH are required for thiazole biosynthesis [24], it is tempting to speculate that the ThiGH complex may in fact be the functional thiazole synthase. The spectroscopic properties, oxygen sensitivity, iron and sulfide content and the presence of the iron–sulfur cluster binding motif in the ThiH sequence raises the intriguing question of the relationship of ThiGH to the 'radical SAM' family of enzymes [9]. The precise mechanism of the thiazole cyclisation is at present unclear and a more detailed understanding of the role of the ThiGH complex and the iron–sulfur cluster in the thiazole

biosynthetic pathway will require the development of an in vitro activity assay.

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