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Reconstitution of ThiC in thiamine pyrimidine biosynthesis expands the radical SAM superfamily

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Abstract

4-Amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P) synthase catalyzes a complex rearrangement of 5-aminoimidazole ribonucleotide (AIR) to form HMP-P, the pyrimidine moiety of thiamin phosphate. The three-dimensional structures of HMP-P synthase and its complexes with the product HMP-P and a substrate analog imidazole ribotide were determined. The structure of HMP-P synthase reveals a homodimer in which each protomer comprises three domains: an N-terminal domain with a novel fold, a central ($\beta\alpha$)₈ barrel and a disordered C-terminal domain that contains a conserved CX₂CX₄C motif, suggestive of a [4Fe-4S] cluster. Biochemical studies have confirmed that HMP-P synthase is iron sulfur cluster dependent, that it is a novel member of the radical SAM superfamily and that HMP-P and 5'-deoxyadenosine are products of the reaction. Mössbauer and EPR spectroscopy confirm the presence of one [4Fe-4S] cluster. Structural comparisons reveal that HMP-P synthase is homologous to a group of adenosylcobalamin radical enzymes. This similarity supports an evolutionary relationship between these two superfamilies.

Thiamin pyrophosphate (ThDP, 1) is an essential cofactor in all forms of life and plays a key role in carbohydrate and amino acid metabolism¹. The biosynthesis of ThDP has been extensively studied in prokaryotes and involves the separate formation of thiazole phosphate (TMP, 2) and 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate (HMP-PP, 3) (Fig. 1a)^{1,2}. These are then coupled to form thiamin monophosphate (ThMP, 4). A final phosphorylation gives ThDP, the biologically active form of the cofactor. The biosynthesis of ThDP in *Saccharomyces cerevisiae* utilizes a completely different biochemical pathway for which the details are just beginning to emerge^{3–5}.

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AUTHOR CONTRIBUTIONS

A.C. performed all biochemical studies. Y.L. and Y.Z. performed all crystallographic studies.

T.L.G. assisted in protein purification and EPR spectroscopy. M.L. recorded and analyzed Mössbauer spectra.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Accession codes

Protein Data Bank: The coordinates for HMP-P synthase/HMP, HMP-P synthase/HMP-P and HMP-P synthase/IMR have been newly deposited under the accession codes 3EPM, 3EPO, and 3EPN, respectively.

The mechanistic enzymology of thiamin (5) thiazole (2) biosynthesis is now relatively well understood. The three identified pathways represented by *Escherichia coli*, *Bacillus subtilis* and *S. cerevisiae* have been reconstituted and most of the required enzymes have been mechanistically and structurally characterized^{2–6}. Biosynthesis of the thiamin thiazole in *E. coli* requires five gene products and utilizes 1-deoxy-D-xylulose 5-phosphate (DXP, 6), tyrosine (7) and cysteine (8). The *B. subtilis* pathway is similar to that of *E. coli* but utilizes glycine (9) rather than tyrosine. Biosynthesis of the thiamin thiazole in yeast requires a single enzyme and utilizes nicotinamide adenine dinucleotide (NAD, 10), glycine and an unknown sulfur source⁴.

In contrast, very little is yet known about the mechanistic enzymology of thiamin pyrimidine (4-amino-5-hydroxymethyl-2-methylpyrimidine; HMP, 11) formation; however, thiamin pyrimidine biosynthesis uses fundamentally different chemistry compared to that used for the biosynthesis of the nucleic acid pyrimidines. Labeling studies have established that HMP is derived from aminoimidazole ribonucleotide (AIR, 12) in bacteria (Fig. 1b)^{7,8} and from histidine (13) and PLP (14) in yeast (Fig. 1c)⁹. In each case, only a single gene is required for pyrimidine formation: *thiC* in bacteria, plants and algae and *THI5* in yeast. Detailed studies, using isotopically labeled AIR, have established the origin of most of the atoms of HMP in bacteria (Fig. 1b). These studies were first carried out *in vivo* and later reproduced and expanded upon using 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P, 15) synthase in cell free extract⁷. The labeling studies have established that the C2' carbon of the ribose is reduced to the methyl oxidation state and used to methylate the C2 position on the aminoimidazole and that the C4' carbon of the ribose is inserted into the C4-C5 double bond of the aminoimidazole to generate the aminopyrimidine. The methyl hydrogens are derived from the C2'H, C3'H and from the buffer. This remarkable rearrangement reaction is the most complex unresolved rearrangement in primary metabolism.

Radical *S*-adenosylmethionine (SAM, 16) enzymes utilize SAM to generate an 5'-deoxyadenosyl radical (17) that in turn serves as an oxidant for a wide variety of enzymatic reactions^{10,11}. For some enzymes SAM is regenerated at the completion of the reaction while for other enzymes SAM is a cosubstrate yielding 5'-deoxyadenosine (5-dAdo, 18) as a product. Bioinformatic studies have identified more than 600 possible members of the radical SAM superfamily¹². The radical SAM superfamily is characterized by a CX₃CX₂C motif that harbors a [4Fe4S] cluster. Upon reduction, the cluster serves as a source of an electron to produce the 5'-deoxyadenosyl radical.

Prior to our studies, HMP-P synthase, the *thiC* gene product, was not reported to be a radical SAM enzyme, although some evidence existed. Previous studies showed a conserved CX₂CX₄C motif in HMP-P synthase and in *Salmonella enterica* the C581A, C584A and C589A mutant proteins were unable to biosynthesize the thiamin pyrimidine, suggesting that HMP-P synthase contained an iron-sulfur cluster¹³. This possibility was further supported by the observation that iron sulfur cluster biosynthetic mutants in *S. enterica* were thiamin requiring¹³ and by a recent study demonstrating that HMP-P synthase from *Arabidopsis thaliana* purifies with a chromophore that is consistent with a bound iron sulfur cluster¹⁴.

In this paper, we report the first successful reconstitution of purified bacterial HMP-P synthase, demonstrate that the enzyme is a previously unrecognized radical SAM enzyme utilizing SAM as a cosubstrate and producing HMP-P and 5'-deoxyadenosine as products, characterize the [4Fe-4S] cluster using Mössbauer and EPR spectroscopies and describe structures of the enzyme complexed with a substrate analog and with the product, HMP-P.

RESULTS

Chemical reconstitution of HMP-P synthase

Cell free extract from the *Caulobacter crescentus* HMP-P synthase overexpressing *E. coli* strain was subjected to chemical Fe-S cluster reconstitution by the addition of excess Fe^{2+} and S^{2-} under strictly anaerobic conditions. After removal of the excess Fe^{II} and S^{2-} by gel filtration, the resulting dark brown cell free extract was fractionated, again under strictly anaerobic conditions, by Ni-NTA affinity chromatography. The protein thus obtained was dark brown in color, with a UV-visible spectrum suggestive of the presence of an iron sulfur cluster (Fig. 2a and Supplementary Fig. 1a). Analyses of bound iron and sulfide revealed 7.2 ± 0.3 irons and 5.4 ± 0.4 sulfides associated with per monomer.

Activity of chemically reconstituted HMP-P synthase

Thiamin pyrimidine formation was initially assayed using the thiochrome assay (Supplementary Fig. 1b). In this assay, the product of HMP-P synthase is converted to thiamin phosphate enzymatically. This is then oxidized to the intensely fluorescent thiochrome phosphate (19), which can be readily detected by HPLC analysis with fluorescence detection.

The results of this assay are shown in Fig. 2b. The blue trace shows the HMP-P synthase activity in the Fe/S cluster reconstituted cell free extract. This activity is 14 fold higher than the activity observed with untreated HMP-P synthase in cell free extract (red trace) and supports the hypothesis that HMP-P synthase is an iron-sulfur cluster utilizing enzyme. The black trace shows the thiochrome phosphate present in the reconstituted sample in the absence of AIR. The thiochrome phosphate produced in this sample is due to the thiamin phosphate that copurifies with thiamin phosphate synthase¹⁵. The compound eluting at 9.3 min is thiochrome pyrophosphate (20) derived from enzyme-bound thiamin pyrophosphate in the cell free extract. The concentration of this compound is the same in all three samples.

Similar activity of HMP-P synthase was also achieved using the purified protein. This activity was dependent on SAM, AIR and reduction of the Fe/S cluster using dithionite (21) (Fig. 2c). The purified enzyme was rapidly inactivated by exposure to air.

Identification of the HMP-P synthase reaction products

The thiochrome assay for detecting the formation of HMP is highly sensitive and was very useful in searching for optimized reconstitution conditions; however, it is an indirect assay and does not give information on the phosphorylation state of HMP. This is because HMP and HMP-P are both substrates for the pyrimidine kinase and the phosphate is lost during thiamin formation¹⁶. In addition, we wished to determine the role SAM was playing in this reaction. With the optimized reconstitution of the HMP-P synthase in a defined system, the available levels of activity allowed us to assay directly for the products without thiochrome derivatization.

A reaction mixture containing AIR, SAM, dithionite and HMP-P synthase was directly analyzed by HPLC with UV detection. The resulting chromatogram for the complete reaction (blue trace) shows consumption of AIR and SAM and the production of two new species with retention times of 2.58 and 18.45 minutes (Fig. 2d,e). These reaction products were identified as HMP-P and 5'-deoxyadenosine, respectively, by comigration with authentic standards. For further verification, ¹H NMR and ESI-MS analyses were performed on HPLC purified products, which confirmed the structural assignments (Supplementary Figs. 1c,d and 2a). Control reactions lacking SAM and dithionite, run under identical reaction conditions, did not show the production of HMP-P or 5-dAdo. A reaction run in the absence of AIR shows reduced

levels of 5-dAdo formation suggesting that slow SAM reduction is occurring in the absence of AIR.

The reaction yield varied between protein preparations. Typically 75–150 μM HMP-P and 200–450 μM 5-dAdo are produced in a reaction containing 0.8–1.2 mM isolated HMP-P synthase (depending on the yield of the corresponding enzyme purification), 400 μM AIR and 800 μM SAM. Interestingly, the production of 5-dAdo was consistently higher (approximately 3-fold) than HMP-P and the product yields were enzyme concentration dependent (Supplementary Fig. 2b). Formation of HMP-P and 5-dAdo was very fast (Supplementary Fig. 3a) and was essentially complete within mixing time (30 s).

***In vivo* reconstitution of HMP-P synthase**

Coexpression of Fe-S cluster utilizing proteins in *E. coli* containing pDB1282 plasmid is an established strategy for the *in vivo* reconstitution of the cluster. This plasmid encodes a set of proteins involved in iron sulfur cluster biogenesis (IscS, IscU, IscA, HscB, HscA and Fdx). Co-expression with pDB1282 significantly enhanced (4 fold) HMP-P synthase activity in cell free extract over a control experiment in which HMP-P synthase was expressed in the absence of pDB1282 (Supplementary Fig. 3b). Anaerobic purification led to pure protein, with 3.8 ± 0.3 irons and 4.4 ± 0.6 sulfides per monomer (Supplementary Fig. 3c). When 195 μM protein was incubated anaerobically with 200 μM AIR, 400 μM SAM and 5 mM dithionite, production of 110 ± 20 μM HMP-P and 230 ± 11 μM 5-dAdo was observed (Supplementary Fig. 4a).

Mössbauer spectroscopy

A sample of HMP-P synthase that was grown on media supplemented with the Mössbauer-active isotope ^{57}Fe , and isolated under strict anaerobic conditions contained only 1.7 Fe and 1.2 sulfides per monomer. The Mössbauer spectrum (Supplementary Fig. 4b) reveals a quadrupole doublet with parameters typical of a $[\text{4Fe-4S}]^{2+}$ cluster. The remaining Fe (two species) has parameters typical of high-spin Fe^{II} . We attribute the low stoichiometry (~ 0.2 $[\text{4Fe-4S}]^{2+}$ cluster per HMP-P synthase) to the rapid overexpression of HMP-P synthase despite the fact that the products of the *isc* genes from *A. vinelandii* were co-expressed. Therefore, we reconstituted anaerobically-isolated HMP-P synthase with additional $^{57}\text{Fe}^{\text{II}}$ and sulfide in the presence of DTT (22). This sample contained 7.2 Fe and 5.4 sulfide per HMP-P synthase. The 4.2-K/53-mT Mössbauer spectrum of this sample is shown in Fig. 3a (hashed marks). The spectrum is dominated by several well-resolved lines, in addition to a broad and featureless absorption, which ranges from -5 mm s^{-1} to $+5 \text{ mm s}^{-1}$ and accounts for $\sim 30\%$ of the total intensity. EPR spectroscopy of an identical sample reveals no signals attributable to Fe/S clusters with $S = 1/2$ ground state ($[\text{3Fe-4S}]^+$, $[\text{4Fe-4S}]^+$, and $[\text{2Fe-2S}]^+$), thereby ruling out the presence of these cluster types in this sample (*vide infra*). Consequently, the broad features are attributed to iron bound nonspecifically to HMP-P synthase¹⁷. The well-resolved lines can be simulated with three quadrupole doublets with the following parameters: $\delta_1 = 0.45 \text{ mm s}^{-1}$ and $\Delta E_{Q,1} = 1.12 \text{ mm s}^{-1}$ (43 % of total intensity, 3.1 Fe per HMP-P synthase, blue); $\delta_2 = 0.78 \text{ mm s}^{-1}$ and $\Delta E_{Q,2} = 3.37 \text{ mm s}^{-1}$ (18 % of total intensity, 1.3 Fe per HMP-P synthase, red); and $\delta_3 = 1.26 \text{ mm s}^{-1}$ and $\Delta E_{Q,3} = 3.02 \text{ mm s}^{-1}$ (14% of total intensity, 1.0 Fe per HMP-P synthase, green). The parameters of the first quadrupole doublet are typical of $[\text{4Fe-4S}]^{2+}$ clusters, suggesting that reconstituted HMP-P synthase has ~ 0.8 $[\text{4Fe-4S}]^{2+}$. We therefore conclude that HMP-P synthase harbors one $[\text{4Fe-4S}]$ cluster. The remaining two quadrupole doublets have parameters typical of Fe^{II} in a four-coordinate, sulfur-rich environment (doublet 2) and a five- or six-coordinate environment of hard N/O ligands (doublet 3)¹⁸. We speculate that one of the two Fe^{II} species may be bound in the mononuclear (His)₂-ligated site that was identified from the X-ray structure (*vide infra*), but we cannot assign it without further knowledge of the coordination environment of the mononuclear site.

EPR spectroscopy

The EPR spectrum of as-isolated HMP-P synthase is largely silent; however, a weak narrow signal is observed at $g = 2$ and is still present at 77 K, suggestive of the presence of an organic radical (Fig. 3b). At present, the nature of this radical and its catalytic competence are unknown. Upon reduction of as-isolated or reconstituted HMP-P synthase in the presence of 10 mM dithionite, an axial spectrum emerges (Fig. 3c), displaying approximate g -values of 2.02 and 1.93, suggestive of a $[4\text{Fe-4S}]^+$ cluster. Consistent with this assignment, the spectrum dramatically reduces in intensity at temperatures above 30 K, and is nearly unobservable above 50 K. Spin quantification indicates that the spectrum accounts for less than 0.1 equiv of spin per HMP-P synthase.

Structure of HMP-P synthase

The crystal structure of HMP-P synthase from *C. crescentus* was determined initially at 2.8 Å resolution. The overall structure is a homodimer with one dimer per asymmetric unit. Each protomer contains a bound metal ion, assigned as Zn^{II} based on the peak height in difference electron density maps, and an HMP molecule, which in the beginning was thought to be a possible product and therefore added during crystallization. Each protomer consists of three domains (Fig. 4a). Domain 1 comprises the first 213 residues. This domain contains a novel fold consisting of six α -helices and five β -strands (Supplementary Fig. 5a,b). The secondary structural elements form a thin blanket-like structure that folds over domains 2 and 3. Domain 2 comprises residues 214–510 and has a $(\beta\alpha)_8$ barrel fold in which β -strands 6–13 make up the barrel core. Each β -strand is followed by one α -helix except $\beta 8$ ($\alpha 9$ and $\alpha 10$) and $\beta 9$ ($\alpha 11$, $\alpha 12$, $\alpha 13$ and $\alpha 14$). The bottom of the barrel (N-terminal end of the β -strands) is covered by β -strands 1 and 2 of domain 1. Domain 3 comprises residues 511–548. These residues form an antiparallel three helix bundle followed by a loop that extends into the top of the $(\beta\alpha)_8$ barrel of the adjacent protomer. The protein contains 76 additional C-terminal residues that are disordered in our structure. Two additional structures with different ligands and with resolutions of 2.0 Å were also determined. The complexes crystallized with a slightly smaller unit cell and did not contain the transition metal. All three structures showed the same general structural features with a disordered C-terminus. Refinement statistics are given in Supplementary Table 1.

The HMP-P synthase homodimer has approximate dimensions of $100 \text{ Å} \times 60 \text{ Å} \times 48 \text{ Å}$ (Fig. 4a). Dimer formation joins the two $(\beta\alpha)_8$ barrels in an approximately antiparallel arrangement and buries a total of 2800 Å^2 (12.6%) of solvent accessible surface per monomer. The interface is mostly hydrophobic with 72.7% of the atoms nonpolar. The main interactions involve helices $\alpha 16$, $\alpha 17$, $\alpha 18$ (the last three helices in the $(\beta\alpha)_8$ barrel) and the three helix bundle ($\alpha 19$, $\alpha 20$, $\alpha 21$) at the beginning of domain 3.

HMP-P synthase active site

The HMP-P synthase active site was identified using complexes with HMP-P and imidazole ribotide (IMR, 23) (Supplementary Fig. 5c–e). The active site is within a cavity at the C-terminal end of the $(\beta\alpha)_8$ barrel, with 1300 Å^2 of solvent accessible area and 1900 Å^3 of solvent accessible volume (Fig. 4a). Most of the residues in the active site cavity are highly conserved among the HMP-P synthase orthologs (Supplementary Fig. 6). A large peak assigned as a Zn^{II} with tetrahedral geometry is located within the active site and is coordinated by the N ϵ 2 of His417, the N ϵ 2 of His481, and two water molecules with unusually long distances (Supplementary Fig. 7a,b). While the identity of the transition metal remains uncertain, the B-factor during refinement is consistent with an assignment of Zn^{II} .

The HMP-P site is located near the N-terminus of helix $\alpha 12$ and the C-terminus from the adjacent protomer. Hydrophobic residues Met248, Leu250, Val274 and Tyr440 line one side of the active site cavity. Atoms N1 and N2 of pyrimidine moiety form hydrogen bonds with

the side chains of Glu414 and Ser474, respectively, assuming that the former is protonated. The phosphate-binding site is located at the N-terminus of α 12 and is stabilized by the helix dipole (Supplementary Fig. 5e). Hydrogen bonds form with the amide nitrogen atom of Gly335, O η of Tyr277, N ϵ 2 of His313, O γ of Ser333 and N η 1 of Arg377.

The phosphate binding site of IMR is similar to that of HMP-P, and the imidazole and ribose moieties generally overlap the pyrimidine binding site of the HMP-P structure. Atom N3 of the imidazole ring forms a hydrogen bond with the side chain of Asp347, assuming that it is protonated. The two hydroxyl groups of the ribose moiety of IMR are hydrogen bonded to the O η of Tyr440 and O δ of Asn219.

Modeling of a [4Fe-4S] cluster

Thus far four crystal structures of radical SAM enzymes with bound SAM are available from the Protein Data Bank: biotin (24) synthase (BioB; PDB ID 1r3o)¹⁹, HemN (PDB ID 1olt)²⁰, MoaA (PDB ID 2fb3)²¹, and LAM (PDB ID 2a5h)²². Each displays a ($\beta\alpha$)₈ barrel or modified β -barrel fold in which SAM is ligated through its amino and carboxylate groups to a [4Fe-4S] cluster. When these four radical SAM enzymes are superimposed, the [4Fe-4S] clusters and SAM binding sites overlap well. Even though the CX₃CX₂C motifs of these enzymes are located near the N-terminus and the CX₂CX₄C motif of HMP-P synthase is located near the C-terminus, we superimposed the structure of biotin synthase onto HMP-P synthase in order to predict the location of its [4Fe-4S] site. After superposition, the [4Fe-4S] cluster is located in an open part of the HMP-P synthase active site with very few bad contacts between either the cluster or the amino acid residues of the cluster binding loop. Interestingly, the [4Fe-4S] cluster and its binding loop are juxtaposed with the final C-terminal residue visible in the HMP-P synthase X-ray structure. This final residue immediately precedes the conserved CX₂CX₄C motif of HMP-P synthase. Therefore, we used the biotin synthase backbone as a guide for threading the residues of the putative HMP-P synthase [4Fe-4S] binding domain. Following manual adjustment and energy minimization, the modeled [4Fe-4S] binding domain showed no bad contacts and had good geometry with the cysteine residues of the Cys561, Cys564, and Cys569 providing ligands for the iron atoms (Fig. 4b,c). Furthermore, the modeled cluster positions the SAM molecule near the ribose ring of the substrate analog IMR in an orientation, from which the adenosyl radical could abstract a hydrogen atom from the substrate ribose or from the protein to generate the organic radical shown in Fig. 3c.

Structural similarities to other proteins

A DALI search (www.ebi.ac.uk/dali) using the entire HMP-P synthase structure as the query failed to identify the radical SAM enzymes or any other structural homologs. However, a DALI search using only the ($\beta\alpha$)₈ barrel domain (residues 214 – 510) identified three of the radical SAM enzymes as structural homologs (Supplementary Table 2). Of these BioB gave the highest Z-score (13.0), while LAM and HemN gave DALI Z-scores of 5.9 and 5.1, respectively. Biotin synthase has a conventional ($\beta\alpha$)₈ barrel whereas LAM and HemN have modified barrels referred to as β -crescents, which probably accounts for the differences in Z-scores.

In addition to the radical SAM enzymes described above, the DALI search identified a group of adenosylcobalamin (AdoCbl, 25) radical enzymes that are also structural homologs with Z-scores ranging from 16.0 to 21.0 (Supplementary Table 2). The top two DALI Z-scores correspond to glutamate (26) mutase from *Clostridium cochlearium*²³ (GM, PDB identifier 1cb7) and lysine (27) 5,6-aminomutase from *Clostridium sticklandii*²⁴ (5,6-LAM, PDB identifier 1xrs). Furthermore, GM (Fig. 5a) and LAM (Fig. 5b) have tandem ($\beta\alpha$)₈ barrels that superimpose well on the HMP-P synthase homodimer. The catalytic domain (A) and the AdoCbl binding domain (B) are separate chains generating an (AB)₂ heterotetramer.

The top 10 matches also include three other tandem ($\beta\alpha$)₈ barrel structures that superimpose well with the HMP-P dimer: a methionine (28) synthase domain from *Thermotoga maritima* (MetH, PDB identifier 1q7m, Z = 18.2)²⁵, methylmalonyl-coenzyme A (29) mutase from *Propionibacterium shermanii* (MCM, PDB identifier 1req, Z = 17.3)²⁶, and carbon monoxide dehydrogenase corrinoid/iron-sulfur protein (Z = 16.0) (CoFeSP, PDB identifier 2h9a, Z = 16.0)²⁷. MCM (Fig. 5c) displays nonequivalent ($\beta\alpha$)₈ barrels only one of which contains an active site. Fused to this domain is an AdoCbl binding domain that is positioned near the active site. CoFeSP (Fig. 5d), which utilizes methylcobalamin (MeCbl; 30) as a cosubstrate, also displays nonequivalent ($\beta\alpha$)₈ barrels with one active site. The MeCbl binding domain is fused to the noncatalytic domain and positioned near the active site of the catalytic domain. While there are structures of all domains of MetH, there is no intact structure that shows the relationship between the MeCbl binding domains and the ($\beta\alpha$)₈ barrel substrate-binding domains.

DISCUSSION

The bacterial HMP-P synthase catalyzes a remarkable rearrangement reaction of AIR to form the pyrimidine moiety of thiamin (Fig. 1b). Our initial attempts at reconstituting this reaction in a cell free extract from an *E. coli* HMP-P synthase overexpression strain yielded very low activity. However, this activity was sufficient to complete an *in vitro* labeling study that identified the origin of most of the atoms of HMP as well as the cofactor requirements of the reaction⁷. The reaction showed an absolute requirement for AIR and was enhanced by SAM, reduced nicotinamide (31) and by the addition of cell free extract from wild type *E. coli*.

Active enzyme was obtained by treating *E. coli* cell free extract containing *C. crescentus* HMP-P synthase with Fe^{II} and sulfide under anaerobic conditions followed by anaerobic purification by Ni-NTA chromatography. Similar preparations were obtained by overexpressing HMP-P synthase in an *E. coli* strain that also overexpressed the enzymes required for iron/sulfur cluster biosynthesis. Our efforts to reconstitute active HMP-P synthase were greatly facilitated by the availability of a highly sensitive assay using the other thiamin biosynthetic enzymes needed to convert minute amounts of biosynthesized HMP-P to the intensely fluorescent thiochrome phosphate (Supplementary Fig. 1b). The use of this assay to optimize the reconstitution resulted in a biochemically-defined HMP-P synthesizing system, requiring only AIR and SAM as the substrates, dithionite as the reducing agent and purified iron sulfur cluster containing HMP-P synthase. While the thiochrome assay does not identify the phosphorylation state of the pyrimidine as the phosphate is lost during thiamin phosphate formation, the optimized reconstitution yielded sufficient product for direct HPLC analysis. In this way, the products of the reaction were identified as HMP-P and 5-dAdo. Currently, this system is not catalytic and the observed yield of HMP-P production is ~10% (chemical reconstitution) or ~50% (*in vivo* reconstitution) with respect to the enzyme added. Incorporation of excess AIR or SAM did not result in any further enhancement of the product yield. However this reconstitution of active, purified HMP-P synthase is a major advance. It has enabled us to experimentally demonstrate that HMP-P synthase is a radical SAM enzyme, to characterize the [4Fe-4S] cluster using Mössbauer and EPR spectroscopies and opens up the system for detailed mechanistic characterization.

The large radical SAM superfamily was established on the basis of a common CX₃CX₂C sequence using bioinformatics¹². The conserved cysteine residues are usually located near the N-terminus of the protein, but are occasionally found near the middle; however, the [4Fe-4S] cluster and SAM are in the same place in the known structures. HMP-P synthase was not originally included in this superfamily. The observation of a different conserved cysteine pattern near the C-terminus and the prediction that these residues form a separate swapped SAM binding domain, together with the reconstitution of the [4Fe-4S] cluster and the

observation of 5'-deoxyadenosine as a product, demonstrate that HMP-P synthase is a novel member of the superfamily.

Sequence alignments of HMP-P synthase orthologs reveal that HMP-P synthases from anaerobes are shorter than from aerobes, lacking up to 130 residues in the N-terminus and 30 residues in the C-terminus. The $(\beta\alpha)_8$ core domain appears to be highly conserved, and the three cysteine residues near the C-terminus are absolutely conserved (Supplementary Fig. 6a). In anaerobic organisms the [4Fe-4S] binding loop is followed by only about 20 residues, while in aerobic organisms there are about 50 residues. Likewise, the N-terminal domains, which fold over the bottom (N-terminal end) and side of the $(\beta\alpha)_8$ barrel, contain only about 70 residues for anaerobes and about 210 residues for aerobes suggesting that the extra residues provide protection for the oxygen sensitive iron-sulfur cluster¹³.

The AdoCbl radical enzyme superfamily and radical SAM superfamily are functionally related, both using a 5'-deoxyadenosyl radical for carrying out molecular rearrangements, suggesting the possibility of an evolutionary relationship²⁸. Structural evidence for an evolutionary relationship was provided by the observation that both superfamilies utilize $(\beta\alpha)_8$ barrels²⁹. The prediction of a separate SAM-binding domain for HMP-P synthase and a common tandem $(\beta\alpha)_8$ barrel interface provides further structural evidence for such a link between these two superfamilies (Fig. 5). This might have occurred through a common ancestor with separate cofactor and catalytic subunits in which a SAM-binding domain and a AdoCbl-binding domain could exchange followed by gene fusions that eventually committed an enzyme to one or the other of the cofactors.

The structural and biochemical studies described here demonstrate that the bacterial HMP-P synthase is a radical SAM enzyme. In these enzymes, a reduced [4Fe-4S] cluster reduces SAM to give the adenosyl radical. This radical then participates in the isomerization of AIR to HMP-P. The production of 5-dAdo as a product of the reaction suggests that the hydrogen atom abstracted by 5-dAdo is not returned to the product. Aside from the results of the labeling experiments shown in Fig. 1b, we do not yet know the mechanism of this novel rearrangement reaction. Because of the large number of possibilities, we will refrain from proposing a mechanism until the catalytic competence of the protein-bound radical has been established, the site of initial hydrogen atom abstraction has been identified and the fates of the ribose C1' and C3' carbon atoms, both of which are absent in HMP-P, have been determined.

METHODS

Expression and purification of *C. crescentus* HMP-P synthase

The gene coding for HMP-P synthase was amplified by PCR from *C. crescentus* genomic DNA and cloned into the expression vector pDESTF1 (Invitrogen). The plasmid was transformed into the *E. coli* Rosetta (DE3) expression strain (Novagen), which contained a second plasmid to supplement the *argU*, *argW*, *glyT*, *IleX*, *leuW*, *metT*, *proL*, *thrT*, *thrU*, and *tyrU* tRNAs. Expression of HMP-P synthase was induced at $OD_{600} = 0.6-0.8$ with 0.5 mM isopropyl-beta-D-thiogalactopyranoside (32) and incubation was continued overnight at 298 K. Cells were harvested by centrifugation at 5000 rpm for 10 min and kept at -80°C . Purification of the selenomethionine (SeMet, 33)-substituted protein is described in the Supplementary Methods.

Purification of Fe-S cluster reconstituted HMP-P synthase

3.5 mL of the filtered clarified lysate on ice was transferred into the glove box for reconstitution of the Fe-S cluster under strictly anaerobic conditions. Freshly prepared 500 mM dithiothreitol (DTT) stock solution was added to obtain a final concentration of 10 mM. Ferrous ammonium sulfate (34) was added to a final concentration of 10 mM in small aliquots, ensuring thorough

mixing. Sodium sulfide was then added to the same final concentration in a similar fashion. The mixture was allowed to incubate on ice for 30 minutes and then 3 mL of this solution, dark in color, was loaded onto a Bio-Rad 10 DG desalting column, pre-equilibrated with 200 mM Tris-HCl buffer, pH 7.6, containing 4 mM MgCl₂ and 1 mM DTT. Later, it was further subjected to Ni-NTA affinity purification as described in the Supplementary Methods.

HMP-P biosynthesis reconstitution assay

Assays were set up in a glovebox under strictly anaerobic conditions. A typical assay mixture contained 800 μ M SAM, 400 μ M AIR, 10 mM dithionite and 200 μ L of protein (purified or in cell free extract). The enzyme concentration varied between 0.8–1.2 mM among preparations for purified HMP-P synthase. Total protein concentration in cell free extract varied between 35–40 mg mL⁻¹. Products were detected directly or with a thiochrome assay as described in the Supplementary Methods.

Mössbauer and EPR spectroscopies

Mössbauer spectra were recorded on a spectrometer from WEB research (Edina, MN) operating in the constant acceleration mode in a transmission geometry. Spectra were recorded with the temperature of the sample maintained at 4.2 K in an externally applied magnetic field of 53 mT oriented parallel to the γ -beam. The quoted isomer shifts are relative to the centroid of the spectrum of a foil of α -Fe metal at room temperature. Data analysis was performed using the program WMOSS from WEB research.

Low-temperature X-band EPR spectra were recorded in perpendicular mode on a Bruker (Billerica, MA) Elexsys E-560 instrument. Sample temperature was maintained with an ITC503S temperature controller and an ESR900 liquid helium cryostat, both from Oxford Instruments (Concord, MA). Samples, which contained 500 μ M HMP-P synthase in a total volume of 500 μ L were loaded into EPR tubes and frozen in liquid N₂ inside of a Coy (Grass Lake, MI) anaerobic chamber.

Crystallization of HMP-P synthase

The SeMet HMP-P synthase/HMP crystals were grown using the sitting drop vapor diffusion method at 298 K by mixing 5.0 μ L of the protein solution ($\sim$ 10 mg mL⁻¹) with 4.0 μ L of a well solution (30% PEG4000, 100 mM Tris-HCl, pH 8.2, 200 mM Li₂SO₄, 2.0 mM DTT) and 1.0 μ L of 100 mM HMP. Plate crystals appeared within three days and took one to two weeks to reach their maximum size of 0.05 mm $\times$ 0.1 mm $\times$ 0.01 mm. The crystals were monoclinic, space group P2₁, with unit cell dimension $a = 63.3$ Å, $b = 103.4$ Å, $c = 95.4$ Å and $\beta = 91.6^\circ$. Assuming two HMP-P synthase molecules of 68 kDa in the asymmetric unit, the Matthews coefficient V_M is 2.29 Å³ Da⁻¹, corresponding to a solvent content of 46.4%³⁰. For cryoprotection, the crystals were transferred to a cryoprotectant containing 30% PEG4000, 100 mM Tris-HCl, pH 8.2, 200 mM Li₂SO₄, 2.0 mM DTT, and 15% glycerol, immediately frozen in the cryostream, and stored under liquid nitrogen prior to data collection. Crystallization of the additional complexes is described in the Supplementary Methods.

X-ray data collection and processing

All of the HMP-P synthase X-ray diffraction data were collected at the Northeastern Collaborative Access Team (NE-CAT) beamline 24-ID-C of the Advanced Photon Source (APS) at a wavelength of 0.97918 Å. The data were integrated and scaled using HKL2000³¹. Data collection details are described in the Supplementary Methods. The data collection statistics are summarized in Supplementary Table 1.

Structure determination and refinement

The crystal structure of HMP-P synthase from *C. crescentus* was solved by single wavelength anomalous diffraction (SAD) methods at 2.8 Å resolution using SeMet-substituted HMP-P/HMP crystal. Se sites were using ShelxD^{32,33}, and were refined with MLPHARE³⁴. RESOLVE³⁵ was used for initial model building and the model was completed using O³⁶, and Coot³⁷. Structure refinement was performed using CNS³⁸. The model quality was assessed at intervals with PROCHECK³⁹. The structures of the complexes were refined at 2.0 Å resolution. Additional details are provided in the Supplementary Methods. For the HMP-P synthase structures, 84.8% to 88.9% of the non-glycine residues are in the most favored region of the Ramachandran plot, with no residue in the disallowed region. The final refinement statistics are shown in Supplementary Table 1.

Structural Analysis

Structure-based sequence alignments were done either using the protein structure comparison server SSM⁴⁰ at the European Bioinformatics Institute or using the program LSQKAB⁴¹ of the CCP4 suite. The sequence alignment figure was prepared with ESPRIPT⁴². The Protein-Protein Interaction Server^{43,44} was used to analyze protein-protein interfaces for accessible surface area, hydrogen bonds, and salt bridges. Figures were prepared with PyMOL⁴⁵ and CCP4mg⁴⁶.

Synthesis of IMR (23)

The chemical synthesis of IMR is outlined in (Supplementary Fig. 7c) and described in the Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The biosynthesis of thiamin pyrophosphate **(a)** Overall bacterial pathway. Aminoimidazole ribonucleotide **12** is converted to 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate **15** by HMP-P synthase (ThiC), which is phosphorylated by ThiD to give HMP pyrophosphate **3**. The thiazole moiety **2** is biosynthesized from 1-deoxy-D-xyulose 5-phosphate **6**, cysteine **8** and dehydroglycine **35**. The dehydroglycine is generated from glycine (ThiO) in *B. subtilis* and from tyrosine (ThiH) in *E. coli*. The pyrimidine and thiazole are coupled by ThiE to give thiamin phosphate **4** and ThiL catalyzes the final phosphorylation. **(b)** Conversion of AIR **12** to the thiamin pyrimidine in bacteria and plants. The color coding indicates the source of non-hydrogen atoms in HMP-P as demonstrated by labeling studies. **(c)** Biosynthesis of thiamin

pyrimidine in fungi. In fungi the pyrimidine moiety is derived from histidine **13** and pyridoxal 5'-phosphate **14** using a single enzyme, THI5p. The color coding indicates the source of non-hydrogen atoms. **(d)** The HMP-P synthase reactions. When Fe-S cluster loaded HMP-P synthase is reduced with dithionite, it reduces SAM **16** to generate methionine **28** and the 5'-deoxyadenosine (5-dAdo) radical **17**, which is required by HMP-P synthase to convert AIR **12** to HMP-P **15**.

Figure 2.

HMP-P synthase activity. **(a)** UV-Visible absorption spectrum of isolated, Fe-S cluster reconstituted, HMP-P synthase (25 μ M; black trace) and its change upon reduction with 200 μ M dithionite (gray trace). **(b)** *In vitro* reconstitution of HMP-P biosynthesis, analyzed by HPLC after converting HMP-P **15** to the fluorescent thiochrome phosphate **19**. Enhanced product yield is observed when HMP-P synthase is subjected to chemical Fe-S cluster reconstitution. Blue and black traces represent reactions utilizing Fe-S cluster reconstituted and untreated HMP-P synthase lysate respectively; red trace represents a reaction lacking AIR **12**. The thiochrome pyrophosphate **20** peak arises from thiamin pyrophosphate in lysate and stays constant. **(c)** Activity of Fe-S cluster reconstituted, purified HMP-P synthase. Sample 1 is reaction mixture containing HMP-P synthase, AIR, SAM, dithionite. Sample 2 is reaction 1 incubated aerobically. Samples 3, 4 and 5 are reaction 1 without SAM, AIR and dithionite respectively. **(d)** HPLC analysis of the HMP-P synthase reactions. Blue trace represents full reaction [HMP-P synthase, AIR, SAM and dithionite]; green trace, red trace and black trace represent the full reaction without AIR, SAM and dithionite respectively. Reaction products HMP-P **15** (r_t = 2.5 min) and 5-dAdo **18** (r_t = 18.45 min) were identified by comigration with authentic standards and subsequent spectroscopic characterization. Three sections of the chromatogram (1.5–2.7 min, 5–6 min, 18.3–18.6 min) are presented to clearly demonstrate the changes.

Figure 3.

EPR and Mossbauer spectroscopy of HMP-P synthase. **(a)** 4.2-K/53-mT Mössbauer spectrum of a sample of HMP-P synthase overexpressed in bacteria grown on ^{57}Fe -supplemented media and further reconstituted with ^{57}Fe , sulfide, and DTT (hashed marks). The solid lines are simulations with three quadrupole doublets using the parameters quoted in the text, representing $[4\text{Fe-4S}]^{2+}$ clusters (blue), and two distinct high-spin Fe^{II} species (red and green). **(b)** EPR spectrum of an as-isolated sample of HMP-P synthase that was coexpressed with Isc cluster. Presence of an organic radical is noted with parameters mentioned in the text. **(c)** EPR spectrum of reconstituted HMP-P synthase. The sample (500 μM) was reduced with 10 mM dithionite before anaerobically loading into an EPR tube and freezing. The spectrum was obtained under the following conditions: microwave power, 101 μW ; receiver gain, 3; modulation amplitude, 10 G; temperature, 13 K; microwave frequency, 9.5 GHz.

Figure 4.

Structure of HMP-P synthase. **(a)** The HMP-P synthase homodimer. The protomer consists of three domains. The N-terminal domains are colored in shades of blue, the $(\beta\alpha)_8$ core domains are colored in shades of green and the C-terminal domains are colored in shades of red. HMP-P is shown as ball-and-stick. The final 66 amino acids are disordered; however, the final ordered residues, which immediately precede a conserved CX_2CX_4C motif, extend into the active site of the adjacent protomer. The C-terminal tail is anchored to the adjacent protomer by a three helix bundle motif located at the beginning of the C-terminal domain. **(b)** Stereoview of the HMP-P synthase active site with modeled SAM and the [4Fe-4S] cluster. The atoms are color coded by atom type (green = C, blue = N, red = O, yellow = S and orange = Fe). The substrate analog IMR 22 from the crystal structure is shown. Residues Cys561, Cys564 and Cys 569, SAM and the [4Fe-4S] cluster were modeled using biotin synthase as a guide. Hydrogen bonds are indicated by dotted lines. **(c)** Superposition of the $(\beta\alpha)_8$ domains from HMP-P synthase and biotin synthase (PDB ID 1r3o). HMP-P synthase is shown in blue and biotin synthase is shown in silver. The [4Fe-4S] cluster and SAM from biotin synthase are shown in ball-and-stick.

Figure 5.

Cartoons depicting the domain assemblies of cobalamin-dependent enzymes with HMP-P synthase-like protomers and dimer interfaces. Each chain within a molecule is color coded differently. **(a)** Glutamate mutase from *C. sticklandii*. GM contains two identical catalytic subunits and two identical AdoCbl binding subunits, which cap the catalytic domains, forming an $(AB)_2$ heterotetramer. **(b)** Lysine 5,6-amino mutase. 5,6-LAM forms an $(AB)_2$ heterotetramer with an assembly similar to that of GM. **(c)** Methylmalonyl coenzyme A mutase from *P. shermanii*. The catalytic and AdoCbl binding domains are fused (A). The blue subunit (B) does not contain a catalytic site. **(d)** Carbon monoxide dehydrogenase corrinoid/iron-sulfur protein. The blue subunit contains a $(\beta\alpha)_8$ domain (B), with no catalytic site, fused to an MeCbl binding domain, which caps the active site of the catalytic domain (A). **(e)** HMP-P synthase from *C. crescentus*. HMP synthase is a homodimer. The $(\beta\alpha)_8$ core domain is fused to the predicted [4Fe-4S] binding domain. This domain is disordered in the crystal structure but the final ordered residues extend into the active site of the adjacent protomer and are preceded by a three helix bundle that anchors the C-terminus to the adjacent protomer.