

Pyrrolysine Biosynthesis

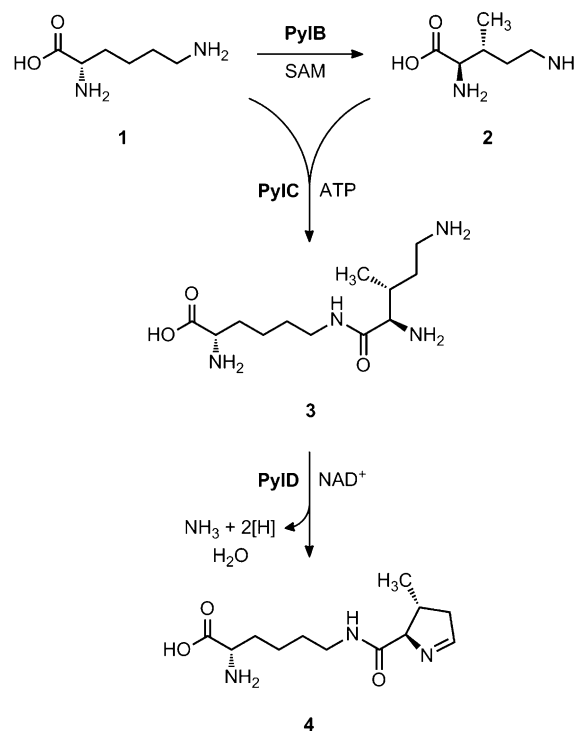
Crystal Structure of Methylornithine Synthase (PylB): Insights into the Pyrrolysine Biosynthesis**

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Dedicated to Professor Helmut Simon on the occasion of his 85th birthday

Pyrrolysine (**4**, abbreviated as Pyl or O, Scheme 1) is the 22nd amino acid that is encoded by the natural genetic code.^[1] In the archaeobacterial family *Methanosarcinaceae*, its incorporation into three proteins (MtmB, MtbB, and MttB) involved in the methylamine catabolic pathway is specified by the amber stop codon UAG.^[2] The unusual amino acid was discovered in 2002 by crystallography and mass spectrometry of MtmB, MtbB, and MttB.^[1a,b] A sophisticated bioinformatics strategy has shown a set of five genes (*pylBCDST*) to be both necessary and sufficient for the biosynthesis and utilization of **4**. More specifically, *pylT* and *pylS* code for a pyrrolysine tRNA and its cognate amino acyl tRNA synthetase, whereas three enzymes specified by *pylB*, *pylC*, and *pylD* catalyze pyrrolysine biosynthesis.^[1b] Incorporation experiments using [U-¹³C₆, U-¹⁵N₂]lysine (**1**) and recombinant *Escherichia coli* strains engineered for expression of *mtmB* and *pylBCDST* genes of *Methanosarcina barkeri*, showed that all the carbon and nitrogen atoms of **4** are derived from **1**. As shown in Scheme 1, **1** is believed to be converted into methylornithine (**2**) by PylB,^[3] which has sequence characteristics of the large superfamily of iron–sulfur enzymes, using *S*-adenosylmethionine (SAM) as a cofactor.^[4] The amide (pseudopeptide) **3** is formed by the PylC-catalyzed condensation of **1** and **2**. The oxidation and subsequent cyclization of **3** catalyzed by PylD affords **4**. However, parts of the pathway in Scheme 1 are based on indirect experimental data;^[3] thus, the intermediate **2** has not been directly detected, and the PylB protein has not been studied in vitro.^[5]

We have cloned and expressed the *pylB* gene of *M. barkeri* Fusaro and determined the structure of the recombinant PylB protein by X-ray crystallography at a resolution of 1.5 Å using single-wavelength anomalous dispersion for phasing. The monomeric protein folds into a single domain which is a member of the TIM barrel superfamily with close similarity to HydE and BioB (RCSB codes: 3CIW and 1R30, respectively;



Scheme 1. Biosynthesis of pyrrolysine.^[3]

for details see the Supporting Information). In addition to the typical (β/α)₈ fold (residues 57–335), PylB comprises three helices at the N-terminus (HA–HC) and a C-terminal helix (HE) which stretches across the N-terminal pole of the barrel. The C-terminal barrel pole is occluded by loops connecting S1/H1, S6/H6, and S8/H8 (Figure 1 A, and Supporting Information, Figure S2). A central cavity containing an iron–sulfur cluster, SAM, the reaction product **2**, and several localized water molecules appears to be sealed from bulk solvent (Figure 2 A). The loop connecting S1/H1 contains a CxxxCxxC motif (amino acid residues 71–78) that is a conserved feature of many radical SAM enzymes.^[6] The three cysteine residues coordinate the [4Fe–4S] cluster that is located at the C-terminal pole of the barrel, inside the central cavity. The fourth iron ion of the cluster points into the cavity, where it is coordinated by the methionine residue of SAM (Figure 1). In close parallel to results obtained with the HemN protein,^[7] the blurry appearance of the electron-density lobe representing the sulfonium center (Figure 1 B) is interpreted as a superposition of biosynthetic SAM (major component) and its *R* epimer (minor component) that can be

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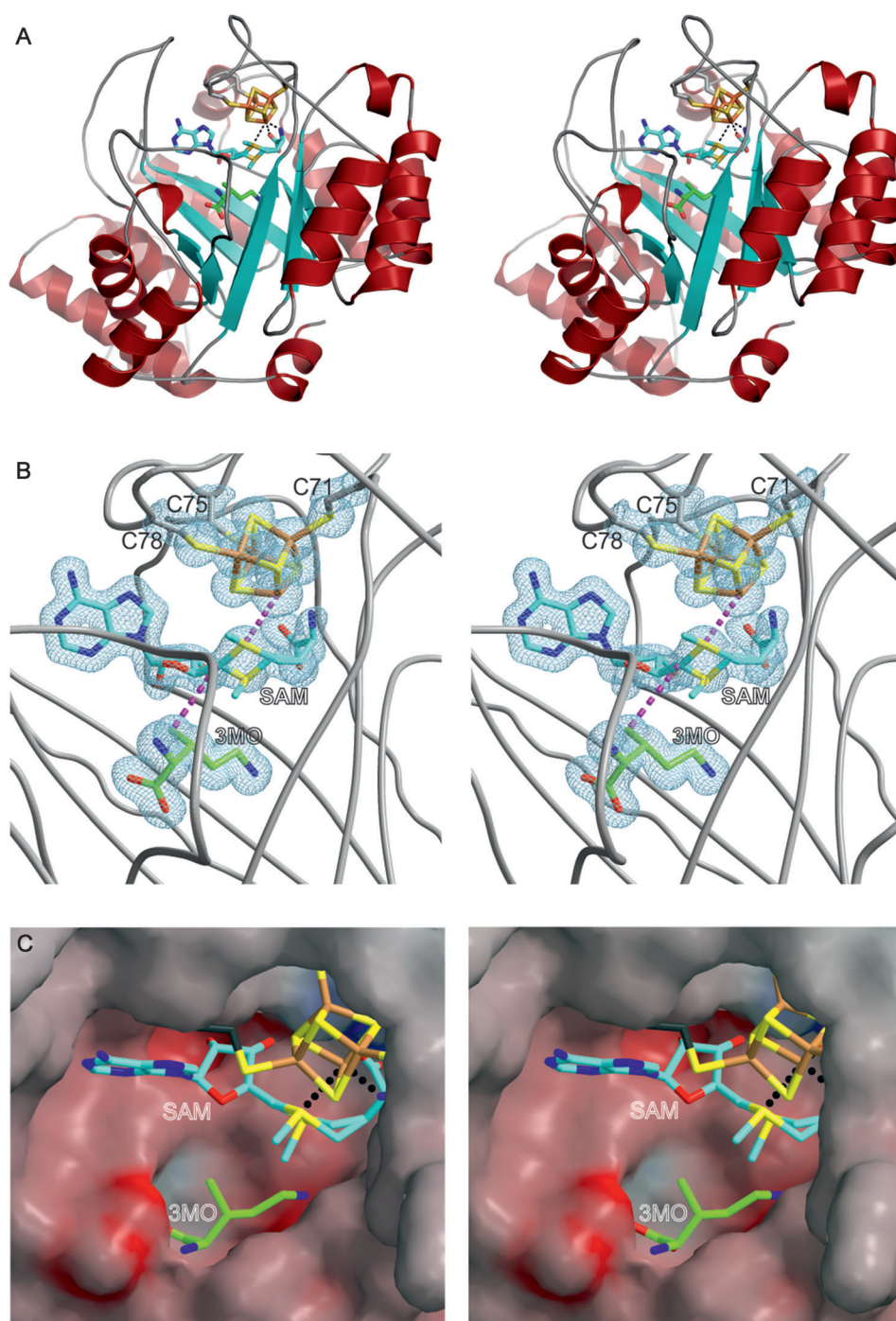


Figure 1. A) Stereoview ribbon drawing of PylB with SAM sandwiched between the reaction product **2** (3MO) and a [4Fe-4S] cluster. B) Stereoview of $2F_o - F_c$ electron density map (blue mesh, 1σ) for the active site of PylB. Putative trajectories for electron transfer (short-dashed violet line, upper right) and hydrogen-atom transfer (long-dashed violet line, lower left) are indicated. C) Potential surface of the PylB active site viewed from the C-terminal pole of the β barrel; the [4Fe-4S] cluster, SAM, and **2** (3MO) are shown as stick models. The coordinates and structure factors of PylB have been deposited in Protein Data Bank (PDB) with code number 3T7V.

formed under ambient conditions by spontaneous epimerization.^[8]

The high occupancy of **2** inside the active-site cavity of PylB is surprising and had to be generated by PylB catalysis,

since the compound is not a natural metabolite of *E. coli*, nor has it been added as a component of the growth medium or buffers used during purification and crystallization. Clearly, **1** was converted into **2** in vivo and product **2** had to be tightly bound to form a complex with PylB even after the three-step purification procedure and subsequent crystallization. This demonstrates that PylB is expressed in an active form in *E. coli*. However, in our in vitro studies we could not find any detectable turnover (see the Supporting Information), indicating that PylB might be a single-turnover or suicide enzyme at least in the absence of PylC and/or PylD. Although the structures of the substrate and product of PylB had been previously deduced by elegant proteomics conjectures, biosynthetic **2** has not been physically detected and characterized up to now.^[3,9] The structure of the novel metabolite is now directly visualized in the electron density and its stereochemistry is unequivocally confirmed as *2R,3R*. The carbon atoms 2–5 and the δ amino group of the amino acid are all related by approximate *anti* conformations, but the carboxylic acid group is in *gauche* conformation. The reaction product **2** and the SAM cofactor are both imbedded by a complex network of hydrogen-bond and hydrophobic interactions with the inner wall of the active-site cavity (Supporting Information, Figure S3).

The apparent rigidity of the active site invited an attempt to model the substrate **1** into the cavity, after virtual removal of the enzyme product. Free-energy minimization starting from various different conformations of **1** afforded a robust model (Figure 2B). The ϵ amino group of modeled **1** is located close to the position of the cognate δ amino group of **2**. The carboxy and α amino

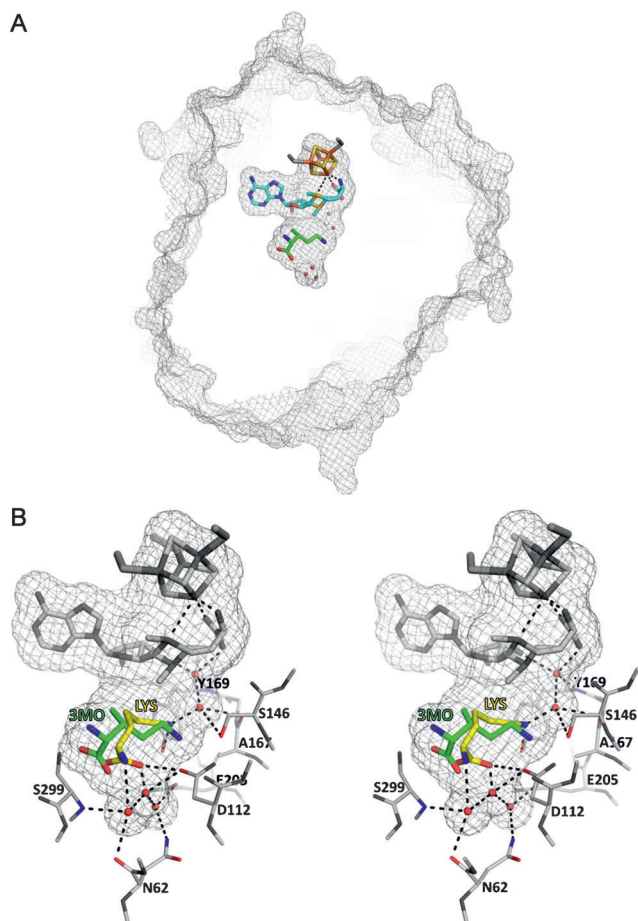


Figure 2. Connolly surfaces of PylB with the [4Fe-4S] cluster, SAM, and **2** (3MO), shown as stick models and enclosed water molecules shown as red spheres. A) Overview. B) Stereoview of the active site with amino acid residues interacting with modeled L-lysine (**1**, LYS) through indirect hydrogen bonds.

group of the modeled molecule **1** are rotated by comparison with the cognate structure elements in **2**.

Based on the experimental structure of **2** and the modeled structure of **1** inside the cavity, it is possible to discuss the reaction trajectory in the reverse direction, that is, the PylB-catalyzed conversion of **2** into **1** (Scheme 2). It is established that catalysis with an iron-sulfur SAM enzyme is initiated by the transfer of an electron from or via an iron-sulfur cluster to the sulfonium motif of SAM. The resulting radical undergoes fragmentation under formation of methionine and a 5'-deoxyadenosyl radical (**5** \cdot , Supporting Information, Scheme SS2) which serves as the actual catalytic transponder.^[4] For the conversion of **2** into **1** (i.e., the reverse reaction), the rules of organic chemistry suggest an initial abstraction of a hydrogen atom from the position 3 methyl group of **2** by **5** \cdot

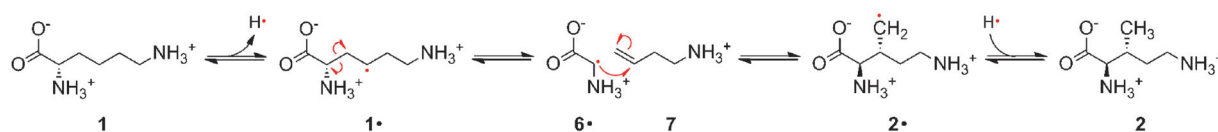
which would result in the formation of 5'-deoxyadenosine (**5**) and the radical species **2** \cdot . Radical **2** \cdot could then isomerize under formation of **1** \cdot , which could abstract a hydrogen atom from **5** under regeneration of **5** \cdot and formation of **1**. Conveniently, the methyl group in **2** is located in close proximity (about 4.6 Å) to the 5'-position of the ribose moiety of SAM. This is in line with distances found between **5** \cdot and the reactants in other members of the iron-sulfur SAM protein superfamily.^[10] The skeletal rearrangement converting **2** into **1** (by way of the reverse reaction) could proceed through a fragmentation-recombination sequence involving the formation of a glycol radical (**6** \cdot) and aminobutene (**7**), similar to the reaction mechanism of glutamate mutase (Supporting Information, Scheme SS1).^[11] In line with these mechanistic considerations, the C4 methylene group of modeled **1** adopts a position that is similar to that of the methyl group of **2** in the experimental X-ray structure. The large active-site cavity essentially occupies the half of the protein molecule that is associated with the C-terminal pole, well in line with the general observation of active sites located at the C-terminal poles of TIM barrel enzymes.^[12] However, the crystal structure fails to suggest a specific path for the release of the reaction product from the active site of PylB into the bulk solvent. Product release might involve conformational dynamics of the loops connecting the S1/H1, the S6/H6, and the S8/H8 secondary structure elements; however, the S1/H1 loop appears rigid due to its coordination to the iron-sulfur cluster. An exit pathway in the direction of the N-terminal pole of the β barrel would also require substantial conformational dynamics including, but not limited to, a rearrangement of helix HE.

Based on the unique way of incorporation of **4** into its target proteins (through the recognition of amber codons mediated by the *pylT* tRNA, without any requirement for additional, specific sequence features in the neighborhood),^[13] the pyrrolysine system has the potential to revolutionize the methodology for the production of proteins with non-natural amino acid substitutions by in vivo or in vitro translation. Whereas this approach depends on the tRNA and aminoacyl tRNA synthetase specified by *pylT* and *pylS*, PylB may have some potential for the production of certain non-natural amino acids, especially in isotope-labeled form. Already, it has been shown by in vivo experiments with recombinant *E. coli* strains that D-ornithine as an artificial substrate for the PylBCD machinery is conducive to the formation of norpyrrolysine.^[3,9]

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Scheme 2. Hypothetical reaction mechanism for PylB.

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