A “Radical Dance” in Thiamin Biosynthesis: Mechanistic Analysis of the Bacterial Hydroxymethylpyrimidine Phosphate Synthase

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Thiamin pyrophosphate is an important cofactor in all forms of life, where it plays a central role in the stabilization of the acyl carbanion biosynthon. Its biosynthesis involves separate syntheses of the thiazole and the pyrimidine heterocycles, which are then linked to form the cofactor. Thiamin thiazole biosynthesis is relatively well-understood. In prokaryotes, 1-deoxy-d-xylulose-5-phosphate, cysteine, and glycine or tyrosine are utilized by five proteins to construct the thiazole moiety, whereas in Saccharomyces cerevisiae, just one gene product converts NAD and glycine to thiazole, obtaining sulfur from a source yet unknown. In contrast, the mechanistic understanding of thiamin pyrimidine (HMP) biosynthesis, in both prokaryotes and eukaryotes, is still at an early stage. In yeast, a single gene product, THI3p, is implicated in HMP biosynthesis from PLP (pyridoxal 5'-phosphate) and histidine, however this reaction has not yet been successfully reconstituted in vitro. In bacteria and plants HMP-P synthase (ThiC) catalyzes the conversion of aminimidazole ribonucleotide (AIR, 1), an intermediate in the purine nucleotide biosynthesis pathway, to hydroxymethylpyrimidine phosphate (HMP-P, 2). In vivo and in vitro studies on the reaction catalyzed by ThiC, using labeled AIR, have revealed a rearrangement reaction of remarkable complexity (Scheme 1). The ThiC-catalyzed reaction has recently been reconstituted in a defined biochemical system. Spectroscopic, structural, and biochemical studies established this enzyme as a unique member of the [4Fe-4S] cluster dependent radical SAM (S-adenosylmethionine) superfamily.

Labeling studies in vivo and using cell free extract, have established the origin of all the thiamin pyrimidine carbon and nitrogen atoms in the AIR structure (Scheme 1). These studies relied on the ease of isolation of thiamin and therefore could only elucidate the fate of atoms incorporated into HMP-P. The complexity of living cells and cell free extract made it impossible to identify the fate of C1' and C3' of the AIR ribose (Scheme 1) as these atoms are not incorporated into thiamin. With the defined ThiC reconstitution system recently described, it is now possible to identify these reaction products. This identification is essential to understand the mechanism of the ThiC-catalyzed reaction. Enzymes belonging to the radical SAM superfamily of proteins initiate catalysis by hydrogen atom abstraction from the protein or substrate by the reactive S'-deoxyadenosyl radical (4), which triggers the rearrangement reaction by hydrogen atom abstraction from AIR (1). The colors in 1 and 2 show the origin of the atoms of HMP-P in the AIR structure.

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ThiC-catalyzed reaction was not detected by either method. To test for carbon monoxide production, an anaerobic carbon monoxide (CO) trapping assay,[19] which involves a specific change in the Soret-region absorbance of hemoglobin (Figure 1C) as a result of its association with CO to form carboxyhemoglobin, was adapted. With this assay, the formation of CO during the ThiC-catalyzed conversion of AIR to HMP-P was clearly detected. To confirm this result, the experiment was performed with different concentrations of AIR substrate, and the change in the signal was proportional to the concentration of AIR used in the reaction mixture (Figure 1D). We therefore conclude that the C3' of AIR is converted to carbon monoxide.

Having established the fates of the C1' and C3' atoms of AIR, we next investigated the role of the 5'-deoxyadenosyl radical in the reaction. As a member of the radical SAM superfamily, the 5'-deoxyadenosyl radical, generated by reduction of SAM, plays an intimate role in triggering the rearrangement of AIR to HMP-P. This radical may abstract a hydrogen atom directly from the substrate or, alternatively, abstract a hydrogen atom from the protein to generate a protein-bound radical,[16] which then reacts with the substrate. When it directly reacts with the substrate, the 5'-deoxyadenosyl radical may be used as a co-substrate or as a catalyst, in which case it is regenerated at the end of the reaction. To identify which hydrogen atom from AIR is abstracted by the 5'-deoxyadenosyl radical, four deuterium-labeled AIR isotopomers and an AIR di-deuterated at the 5' position of ribose were synthesized (Figure 2A) and the 5'-deoxyadenosine generated from each was purified by HPLC. Incorporation of a deuterium atom at the 5' position of 5'-deoxyadenosine results in a small upfield shift of the 5'1H NMR...
RIbose that was fully deuterated lytically H/D exchange to produce substrate. The synthesis used catalytic deoxyadenosyl radical abstracts hydrogen atoms for each HMP-P produced, rather than as a catalyst. This suggests that the same adenosyl radical abstracts hydrogen atoms from the 5' and the 4' positions of AIR.

To further test this unprecedented deoxyadenosyl radical reactivity, perdeuterated AIR was synthesized and tested as a substrate. The synthesis used catalytic H/D exchange to produce ribose that was fully deuterated (> 98%) at C2', C3', and C5' and partially deuterated at positions C4' (50%) and C1' (< 1%).

AIR was synthesized from this and subjected to the ThiC-catalyzed reaction. The resulting 5'-deoxyadenosine was analyzed by HPLC-coupled ESI-MS (Figure 2D; UD sample). The mass spectrum shows the production of [CH3-5']-deoxyadenosine, [CH3D-5']-deoxyadenosine and [CH2D-5']-deoxyadenosine in close to a 1:1:1 ratio. [CH3-5']-deoxyadenosine is likely to be produced by the quenching of the adenosyl radical by buffer components (uncoupled reaction), [CH3D-5']-deoxyadenosine is produced by the abstraction of a single deuterium and a hydrogen from the substrate, and the only way that [CH2D-5']-deoxyadenosine can be produced is by the sequential abstraction of two deuterium atoms from the substrate. The observed 1:1 distribution of mono- vs. bis-deuterated 5'-deoxyadenosine during the course of the ThiC-catalyzed reaction.

ThiC catalyzes one of the most complex rearrangement reactions in primary metabolism. Successful anaerobic purification of the ThiC enzyme and its reconstitution in a chemically defined system has set the stage for the elucidation

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**Scheme 2.** Mechanistic proposal for the rearrangement catalyzed by ThiC (R' = 5'-deoxyadenosyl radical, R-H = 5'-deoxyadenosine).
of the mechanism of this novel deep-seated rearrangement. Here we have demonstrated that C1’ and C3’ of AIR are converted to formate and carbon monoxide, respectively, and that HMP-P and 5’-deoxyadenosine are formed in a 1:1 ratio. Evidence for an organic radical associated with ThiC, upon treatment with SAM and dithionite, has been presented in the literature.[16] While the catalytic competence of this radical remains to be elucidated, our results indicate that under physiologically relevant reducing conditions, in the presence of AIR, the 5’-deoxyadenosyl radical generated at the ThiC active site reacts directly with the substrate to catalyze this rearrangement reaction and the 5’-deoxyadenosyl radical carries out two iterative hydrogen atom abstractions. This suggests that a hydrogen atom abstraction from C4’ or C5’ of the substrate initiates some reaction steps which ultimately regenerate the 5’-deoxyadenosyl radical followed by a second hydrogen atom abstraction leading to completion of the reaction. This strategy increases the catalytic versatility of the 5’-deoxyadenosyl radical allowing a single enzyme to catalyze the very complex rearrangement involved in conversion of AIR to HMP-P. A mechanistic proposal consistent with our observations thus far is outlined in Scheme 2. However, further experiments to determine the order of these hydrogen atom abstractions by the 5’-deoxyadenosyl radical and to trap intermediates on the reaction pathway are necessary to clarify our mechanistic analysis of this remarkable “radical dance”.

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