Thiamin Biosynthesis

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A "Radical Dance" in Thiamin Biosynthesis: Mechanistic Analysis of the Bacterial Hydroxymethylpyrimidine Phosphate Synthase**

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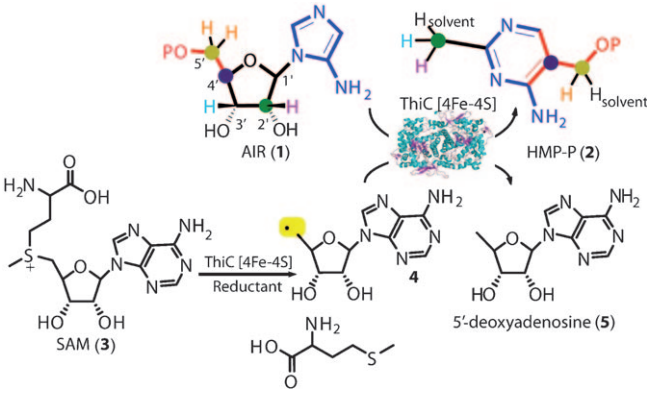
Thiamin pyrophosphate is an important cofactor in all forms of life, where it plays a central role in the stabilization of the acyl carbanion biosynthon.[1,2] Its biosynthesis involves separate syntheses of the thiazole and the pyrimidine heterocycles, which are then linked to form the cofactor. Thiamin thiazole biosynthesis is relatively well-understood.[3–7] 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, THI5p, 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 aminoimidazole ribonucleotide (AIR, **1**), an intermediate in the purine nucleotide biosynthesis pathway, to hydroxymethylpyrimidine phosphate (HMP-P, **2**).[8] 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).[9] 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.[10,11]

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).[9] 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



Scheme 1. The pyrimidine synthase-catalyzed conversion of AIR (**1**) to HMP-P (**2**; P = phosphate). Reduction of SAM (**3**) generates the 5-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.

recently described,[10] 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 5′-deoxyadenosyl radical (**4**, Scheme 1).[12–14] Here we also report the identification of the hydrogen atoms abstracted from AIR, initiating a remarkable "radical dance" leading to the conversion of AIR (**1**) to HMP-P (**2**).

To evaluate the fates of C1′ and C3′, [^{13}C-1′]-AIR and [^{13}C-3′]-AIR were synthesized. Using these as substrates, reactions were set up with ThiC, SAM, and dithionite. A SAM-free control reaction was also performed. After removal of the protein, HPLC analysis showed approximately 45% conversion of AIR to HMP-P while no conversion was detected in the control. ^{13}C NMR analysis of the reaction mixture generated using AIR labeled on C1 (Figure 1 A, B) showed a singlet at 170 ppm, which was absent in the control. Addition of sodium formate increased the intensity of this signal, confirming its assignment as the formate carbon. This demonstrates that C1′ of AIR is converted to formate. When identical experiments were performed with [^{13}C-3′]-AIR, no new signal was observed in the ^{13}C NMR spectrum, even though significant conversion of AIR to HMP-P (40–50%) was confirmed by HPLC analysis. This suggests that C3′ is not released as formate. Attempts were then made to detect formaldehyde in the reconstitution mixture, directly by ^{13}C NMR before[17] and after removal of the protein and using Purpald.[20] Generation of formaldehyde during the

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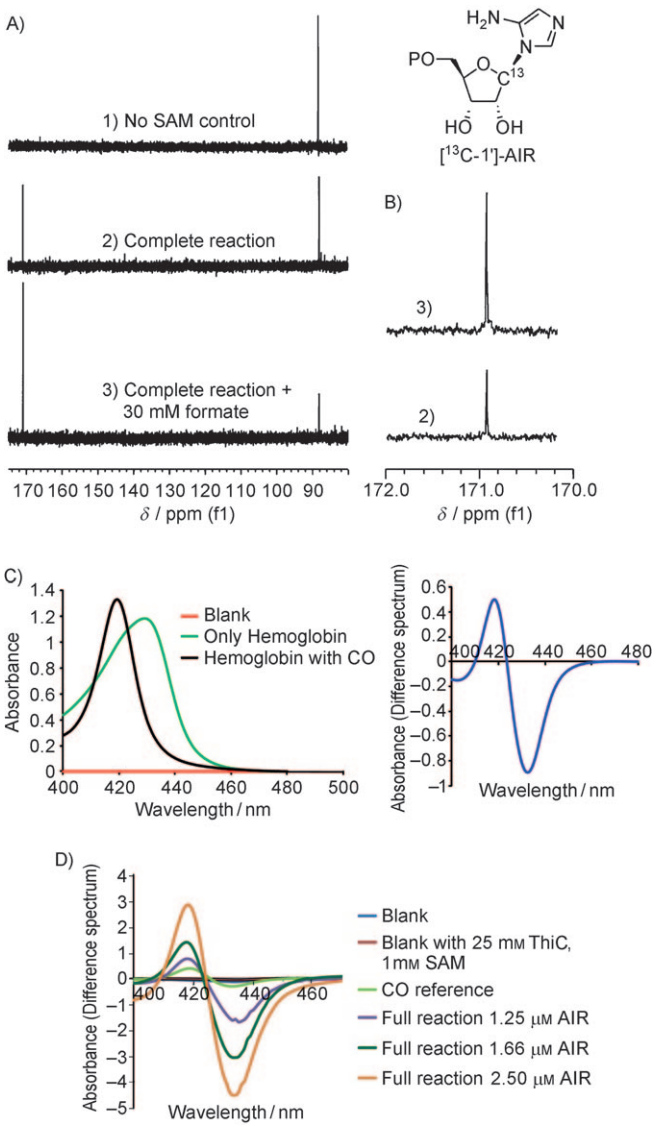


Figure 1. Determining the fate of C1′ and C3′ of AIR: A) NMR analysis of the pyrimidine synthase reaction mixture using AIR labeled with ^{13}C at the 1 position of the substrate: 1) ^{13}C signal for C1′ of AIR (88 ppm) in the SAM-free control reaction; 2) full reaction mixture showing a new signal at 171 ppm along with the C1′ signal of unreacted AIR; 3) addition of 30 mM sodium formate to the reaction mixture enhances the intensity of the new peak at 171 ppm. B) An expanded view of the NMR spectra around the formate peak at 171 ppm for samples (2) and (3). C) UV/Vis spectrum of hemoglobin (green) and carboxyhemoglobin (black) and the resulting difference spectrum. D) The ThiC-catalyzed reaction run in the presence of hemoglobin shows that carboxyhemoglobin production is dependent on the AIR concentration.

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

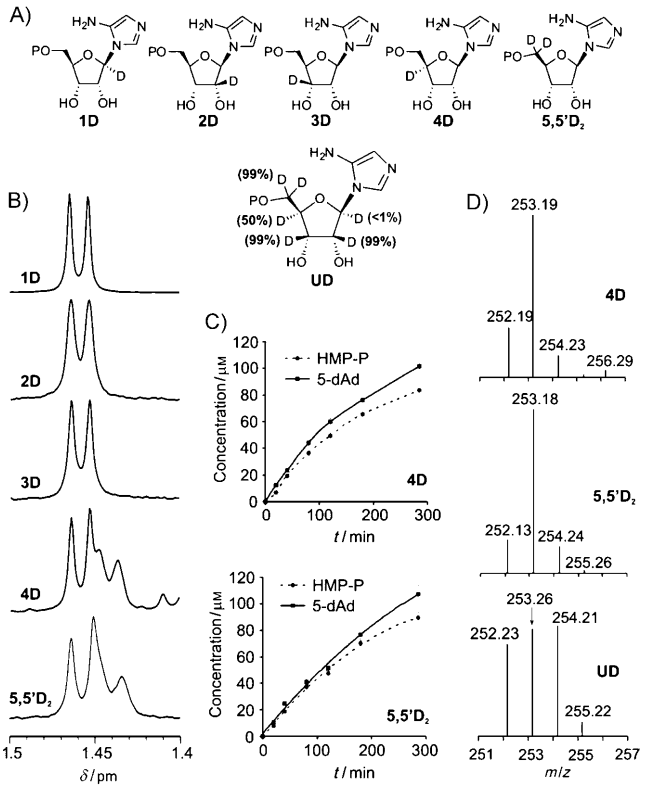


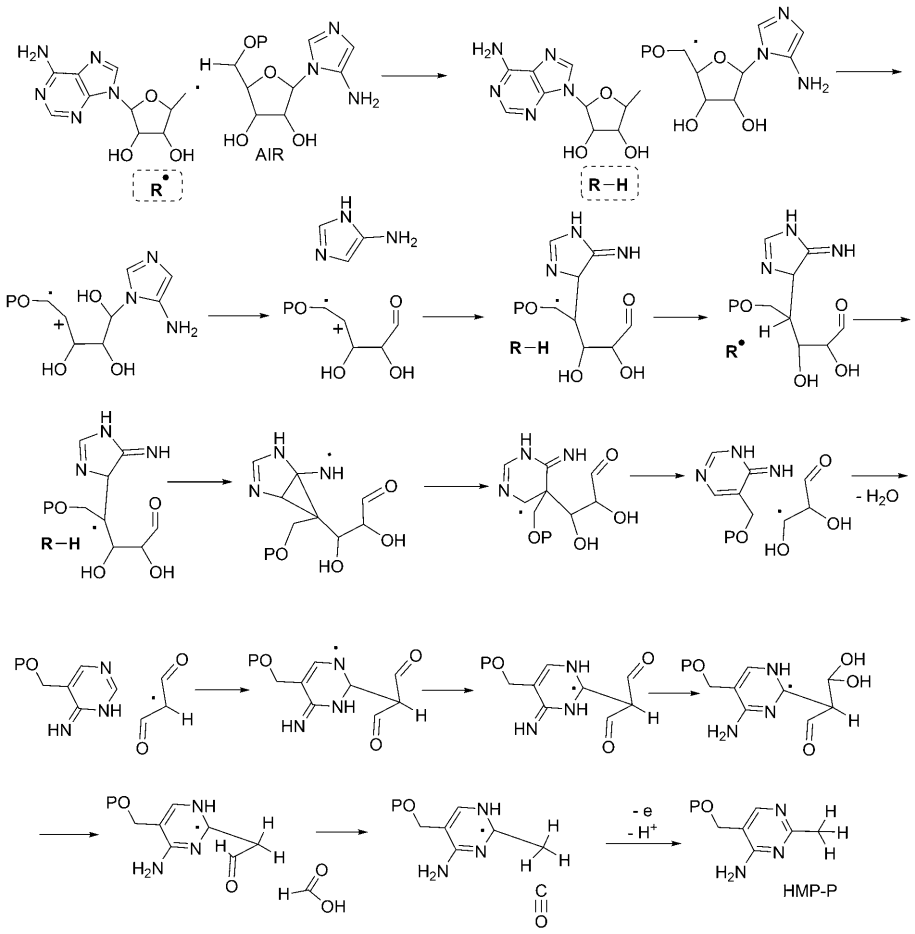
Figure 2. Hydrogen atom abstraction from AIR occurs at C4′ and at C5′. A) Deuterium-labeled substrates synthesized for this study. B) ^1H NMR signal for the 5′-methyl group of 5-deoxyadenosine isolated from the ThiC reactions with the AIR isotopomers. Incorporation of deuterium in the methyl group for [4-D]-AIR (**4D**) and [5,5′-D_2]-AIR (**5,5′D_2**) is noted by the presence of the additional, upfield shifted broader doublet. C) Rate of formation of 5′-deoxyadenosine and HMP-P measured over 5 h reveal a 1:1 product ratio. D) MS analysis of 5′-deoxyadenosine produced in the early phase of the reaction reveal predominant monodeuteration (*m*/*z* 253) with [4-D]-AIR and [5-D]-AIR and 50% dideuteration (254 Da) with [UD]-AIR.

signal.[15] Therefore, ^{1}H NMR analysis of the isolated 5′-deoxyadenosine was used to detect deuterium incorporation. Surprisingly, we observed deuterium incorporation into 5′-deoxyadenosine when [5,5′-D_2]-AIR (**5,5′D_2**) or [4-D]-AIR (**4D**) was used as the substrate. No deuterium incorporation was associated with the 1′, 2′, or 3′-labeled substrates (Figure 2B).

To determine if the 5′-deoxyadenosyl radical is used as a co-substrate or as a catalyst, we determined the product ratio (HMP-P:5′-deoxyadenosine) of the ThiC-catalyzed reaction over a period of 5 h using [5,5′-D_2]-AIR (**5,5′D_2**) and [4-D]-AIR (**4D**) as substrates. A product ratio of nearly 1:1 was observed throughout the course of the reactions (Figure 2C). Flavodoxin–flavodoxin reductase and NADPH were used to reduce the Fe–S cluster of ThiC, since the use of dithionite as the reducing agent was associated with high levels of uncoupled 5′-deoxyadenosine production. Although NMR analysis was used initially to detect deuterium incorporation in 5′-deoxyadenosine, it was not suitable for quantitative analyses. In addition to the signals from monodeuterated and non-deuterated 5′-deoxyadenosine being convoluted, prolonged incubation of the reaction mixture (16 h) was required to generate enough 5′-deoxyadenosine for its isolation and characterization by NMR spectroscopy. Enhanced levels of uncoupled 5′-deoxyadenosine production towards the later part of the reaction introduces a higher population of unlabeled 5′-deoxyadenosine in a sample prepared in this manner. Therefore, HPLC-coupled ESI-MS analysis of the 5′-deoxyadenosine was performed to allow sensitive product detection at the earlier points of the reaction, where uncoupled 5′-deoxyadenosine production was minimal, and revealed monodeuterated 5′-deoxyadenosine as the predominant product in both cases (Figure 2D). These results demonstrate that the 5′-deoxyadenosyl radical is used as a co-substrate, which abstracts two 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%)[17] 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 [CH_3-5′]-deoxyadenosine, [CH_2D-5′]-deoxyadenosine and [CHD_2-5′]-deoxyadenosine in close to a 1:1:1 ratio. [CH_3-5′]-deoxyadenosine is likely to be produced by the quenching of the adenosyl radical by buffer components (uncoupled reaction), [CH_2D-5′]-deoxyadenosine is produced by the abstraction of a single deuterium and a hydrogen from the substrate, and the only way that [CHD_2-5′]-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 (*m*/*z* 253 and 254, respectively) is consistent with the deuteration of the substrate (98% at C5′, 50% at C4′). Since HMP-P and 5′-deoxyadenosine are formed in a 1:1 ratio, these results confirm that two hydrogen atoms, one from C4′ the other from C5′ of AIR are incorporated into a single 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



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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