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Pyruvate is the source of the two carbons that are required for formation of the imidazoline ring of 4-demethylwyosine

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Abstract

TYW1 catalyzes the condensation of N-methylguanosine with two carbon atoms from an unknown second substrate to form 4-demethylwyosine, which is a common intermediate in the biosynthesis of all of the hypermodified RNA bases related to wybutosine found in eukaryal and archaeal $tRNA^{Phe}$. Of potential substrates examined, only incubation with pyruvate resulted in formation of 4-demethylwyosine. Moreover, incubation with C1, C2, C3, or C1,2,3-^{13}C-labeled pyruvate showed that C2 and C3 are incorporated while C1 is not. The mechanistic implications of these results are discussed in the context of the structure of TYW1.

Of the 151 modifications that have been documented in RNA, 92 occur in tRNA and many are conserved in all domains of life (1). The biosynthetic pathways leading to these modifications are often quite complex and ripe with novel chemistries.

Wybutosine (yW) and its derivatives are found in position 37 of tRNA encoding Phe in eukaryotes and archaea (2). yW and its derivatives are installed in a series of reactions (see Fig. 1) that all require *S*-adenosyl-L-methionine (SAM). The first step of the pathway is methylation of N-1 of G_{37} to generate N-methylguanosine (m^1G), which is converted to the tricyclic ring of 4-demethylwyosine (imG-14) in a reaction catalyzed by TYW1. imG-14 is converted to yW by the successive actions of TYW2, TYW3 and TYW4. TRM5 and TYW1 homologs are common to all organisms containing yW and yW derivatives. Thus imG-14 is a common intermediate to yW and yW derivatives in all organisms that produce the hypermodified base. Evidence for the biosynthetic pathway has accumulated through gene knock-out studies in yeast (3).

TYW1 has been classified as a member of the radical SAM superfamily (4) on the basis of a conserved CxxxCxxC motif, which provides 3 Cys thiolate ligands to form a catalytically essential $[4Fe\text{-}4S]^{+2/+1}$ cluster. The cluster presumably binds and reductively cleaves SAM to generate 5'-deoxyadenosyl radical (dAdo•) for a radical-mediated transformation, as is the case for other radical SAM proteins (5). In TYW1, the first step is presumed to be H-atom abstraction from the methyl group of m^1G to initiate radical mediated condensation with a two carbon donating second substrate, leading to formation of imG-14. The identity

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ASSOCIATED CONTENT

Supporting Information.

Detailed methods and materials can be found in the supporting materials. This material is available free of charge via the Internet at http://pubs.acs.org

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

of the second substrate has remained elusive, thus hampering mechanistic studies of TYW1, for which several high-resolution structures are known (6,7).

We have established a biochemical assay to study the source of the two carbon moiety that is required for the conversion of m^1G to imG-14. For reasons of ease of purification and stability, all of our experiments were carried out with the *M. jannaschii* homologs of TRM5 and TYW1. Biochemical function of TRM5, conversion of G to m^1G, has been documented (8); therefore, TRM5 could be utilized to generate m^1G-containing $tRNA^{Phe}$ *in situ*.

The recombinant *M. jannaschii* homolog of TYW1 was purified anaerobically and reconstituted with Fe and S (see Supplementary Information). The reconstituted protein was brown and had an absorbance spectrum that displayed a characteristic shoulder at 400 nm (Fig. S.3.). The m^1G-containing $tRNA^{Phe}$ (MJ_t16) was prepared by *in vitro* run off transcription (see Supplementary Information).

The assays were carried out anaerobically in the presence of $tRNA^{Phe}$, TYW1, TRM5, and SAM. The reaction mixtures contained all components that have been shown to be required for activity of TRM5 (SAM and Mg^{2+})(8). In addition, sodium dithionite was included to reduce the radical SAM cluster of TYW1. Incubations were carried out for 12 h at 60 °C, following which reactions were quenched and the RNA was extracted digested to nucleosides, and analyzed via LC-MS as described previously (9). The modified bases have distinct retention times and can be detected readily in the extracted ion chromatograms at *m/z*=298 (m^1G) or 322 (imG-14); these correspond to the $[M+H^+]$ ions of m^1G and imG-14, respectivly.

Four compounds, acetyl CoA, acetyl phosphate, phosphoenolpyruvate, and pyruvate, were tested as possible two carbon sources. Of these only incubation in the presence of pyruvate led to a reduction of the m^1G peak and appearance of imG-14 (*m/z*=322) in the extracted ion chromatogram (Fig. S.4). We found that methyl viologen, while not essential, stimulated activity; therefore it was included in all subsequent reactions. Control experiments established that imG-14 does not form in the absence of dithionite, TYW1, or pyruvate (Fig. S.4). Control reactions excluding SAM could not be carried out because it is a substrate for TRM5, which is utilized for synthesis of the N-methylguanosine containing $tRNA^{Phe}$ *in situ*.

To gain additional insights into the mechanism by which pyruvate, a three carbon compound, can be the source of the two carbons in the tricyclic system of yW, the experiments were repeated with several ^{13}C-labeled analogs. Extracted ion chromatographs at *m/z* of imG-14 (*m/z*=322) and at +1 and +2 are shown in Fig. 2. In the presence of unlabeled pyruvate, the extracted ion chromatograms show a peak at the expected *m/z*=322. Interestingly, when the reaction was carried out in the presence of [1-$^{13}C_1$]-pyruvate, an identical set of extracted ion chromatographs were obtained, indicating loss of C-1. When the assays were carried out with [2-$^{13}C_1$]- or [3-$^{13}C_1$]- pyruvate, however, the *m/z*=322 disappeared and a peak appeared in the extracted ions chromatogram at *m/z*=323. This suggests that both C2 and C3 of pyruvate are incorporated into the tricyclic ring of yW. Consistent with these labeling patterns, when the reaction was carried out in the presence of [1,2,3-$^{13}C_3$]-pyruvate, only a +2 shift to *m/z*=324 was observed. These results clearly demonstrate that the C-2 and C-3 of pyruvate are the source of the two carbon atoms that are required for synthesis of the tricyclic ring of yW. Note that the smaller peaks in the chromatograms at +1 are from the natural abundance of isotopes of imG-14, which is expected to be ~16% of the intensity of the $[M+H^+]$ peak in each instance. We also carried out LC-MS/MS runs where unlabeled or singly labeled imG-14 (*m/z*=322 or 323) were trapped and fragmented; in each case the $[MB_2^+]$ ion corresponding to the base carries the appropriate *m/z*, consistent with previous imG-14 fragmentation studies (3, 10).

Interpretation of the pyruvate labeling results with TYW1 can be carried out in the context of the two structures and mutagenesis results that are available in the literature (6,7). Six Cys residues are conserved in TYW1, three of which occur in the radical SAM signature sequence (C_{62}xxxC_{66}xxC_{69}, *M. jannaschii* numbering). The thiolate sidechains of these residues would be expected to bind three of the metal ions in the [4Fe-4S] cluster; the fourth iron is presumably coordinated to the α-amino and α-carboxylate of SAM, as has been shown previously for other radical SAM proteins (11). The radical SAM cluster is located on one side of a positively charged putative active site cleft, where tRNA is proposed to bind and flip the m^1G precursor for modification (7). The three additional conserved Cys residues (C26, C39, and C52) are located adjacent to the substrate binding site on the opposite side of the cleft from the SAM binding cluster. It has been proposed that these Cys residues could also form a [4Fe-4S] cluster. A conserved Lys residue (K41) *adjacent* to the second set of conserved Cys residues is also required for activity based on *in vivo* studies (6). When one considers *where* pyruvate would bind, it seems reasonable to propose that the pyruvate binding site is on the same side as the Lys, which it may form a Schiff base with to facilitate the chemistry.

Our working model for the mechanism of TYW1, shown in Fig 3, is as follows. We propose that the conserved Lys residue forms a Schiff base with pyruvate. Reductive cleavage of SAM leads to formation of dAdo•, which either directly or perhaps through a protein side chain, propagates the radical to the m^1G generating a substrate radical. Radical addition to C-2 of pyruvate followed by homolytic scission of the C-1–C-2 bond would generate an intermediate, which through transimmination and subsequent deprotonation, forms imG-14. Decarboxylations, such as that proposed here, have been proposed in the reaction of pyruvate formate-lyase (12) and coproporphyrinogen synthyase (HemN) (13). The resulting formyl radical can either acquire an H-atom *or* undergo reduction and protonation to produce formate. Alternatively, oxidation of the formyl radical would generate CO_2. At this point, given the uncertainty about the involvement of enzyme-based radicals and the nature of the structure formed by the second set of conserved Cys residues, it is difficult to differentiate among these many possibilities. We note that while a Lys residue would be desirable, the transformations proposed in Fig. 3 could also occur with pyruvate alone; however, a Schiff base would provide an attractive electron sink to stabilize the intermediates. The *M. jannaschii* protein used in these studies lacks a flavin mononucleotide binding domain that appends the protein in higher organisms; its absence from archaeal proteins may indicate that it has an alternative mechanism for cluster reduction.

In summary, we have identified pyruvate as the second substrate for TYW1 in the production of imG-14 on the pathway to yW. We have successfully reconstitued activity *in vitro* using isotopically labeled pyruvate. The isotope labeling patterns unambiguously show that C-2 and C-3 of pyruvate are incorporated into the tricyclic base, whereas C-1 is lost. These observations, when taken with the X-ray crystal structures, conserved residues, and mutagenesis data in the literature (3, 6, 7, 14) provide support for the model proposed here, which will be useful in directing future mechanistic studies of the fascinating transformation catalyzed by TYW1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

yW	wybutosine
SAM	*S*-adenosyl-L-methionine
m^1G	N-methylguanosine
imG-14	4-demethylwyosine
dAdo•	5’-deoxyadenosyl radical

REFERENCES

1. Rozenski J, Crain PF, McCloskey JA. The RNA Modification Database: 1999 update. Nucleic Acids Res. 1999; 27:196–197. [PubMed: 9847178]
2. RajBhandary UL, Chang SH. Studies on polynucleotides. LXXXII. Yeast phenylalanine transfer ribonucleic acid: partial digestion with ribonuclease T-1 and derivation of the total primary structure. J Biol Chem. 1968; 243:598–608. [PubMed: 5637712]
3. Noma A, Kirino Y, Ikeuchi Y, Suzuki T. Biosynthesis of wybutosine, a hyper-modified nucleoside in eukaryotic phenylalanine tRNA. EMBO J. 2006; 25:2142–2154. [PubMed: 16642040]
4. Sofia HJ, Chen G, Hetzler BG, Reyes-Spindola JF, Miller NE. Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: functional characterization using new analysis and information visualization methods. Nucleic Acids Res. 2001; 29:1097–1106. [PubMed: 11222759]
5. Frey PA, Hegeman AD, Ruzicka FJ. The Radical SAM Superfamily. Crit Rev Biochem Mol Biol. 2008; 43:63–88. [PubMed: 18307109]
6. Suzuki Y, Noma A, Suzuki T, Senda M, Senda T, Ishitani R, Nureki O. Crystal structure of the radical SAM enzyme catalyzing tricyclic modified base formation in tRNA. J Mol Biol. 2007; 372:1204–1214. [PubMed: 17727881]
7. Goto-Ito S, Ishii R, Ito T, Shibata R, Fusatomi E, Sekine SI, Bessho Y, Yokoyama S. Structure of an archaeal TYW1, the enzyme catalyzing the second step of wye-base biosynthesis. Acta Crystallogr D Biol Crystallogr. 2007; 63:1059–1068. [PubMed: 17881823]
8. Christian T, Hou YM. Distinct determinants of tRNA recognition by the TrmD and Trm5 methyl transferases. J Mol Biol. 2007; 373:623–632. [PubMed: 17868690]
9. Miles ZD, McCarty RM, Molnar G, Bandarian V. Discovery of epoxyqueuosine (oQ) reductase reveals parallels between halorespiration and tRNA modification. Proc Natl Acad Sci U S A. 2011; 108:7368–7372. [PubMed: 21502530]
10. Zhou S, Sitaramaiah D, Noon KR, Guymon R, Hashizume T, McCloskey JA. Structures of two new "minimalist" modified nucleosides from archaeal tRNA. Bioorg Chem. 2004; 32:82–91. [PubMed: 14990307]
11. Vey JL, Drennan CL. Structural insights into radical generation by the radical SAM superfamily. Chem Rev. 111:2487–2506. [PubMed: 21370834]
12. Knappe J, Neugebauer FA, Blaschkowski HP, Ganzler M. Post-translational activation introduces a free radical into pyruvate formate-lyase. Proc Natl Acad Sci U S A. 1984; 81:1332–1335. [PubMed: 6369325]
13. Layer G, Kervio E, Morlock G, Heinz DW, Jahn D, Retey J, Schubert WD. Structural and functional comparison of HemN to other radical SAM enzymes. Biol Chem. 2005; 386:971–980. [PubMed: 16218869]
14. de Crecy-Lagard V, Brochier-Armanet C, Urbonavicius J, Fernandez B, Phillips G, Lyons B, Noma A, Alvarez S, Droogmans L, Armengaud J, Grosjean H. Biosynthesis of wyosine derivatives in tRNA: an ancient and highly diverse pathway in Archaea. Mol Biol Evol. 27:2062–2077. [PubMed: 20382657]

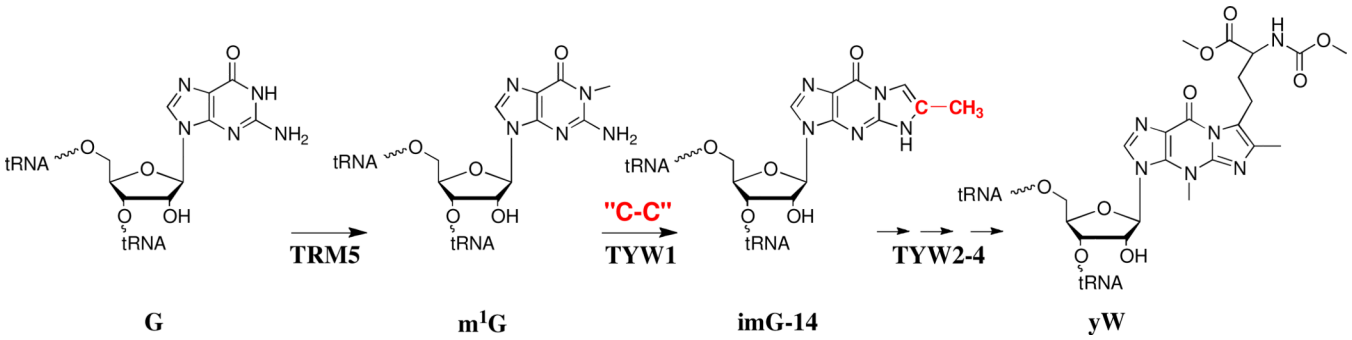

Figure 1.

The biosynthetic pathways of yW. G at position 37 is transformed to imG-14 through the actions of TRM5 and TYW1, which is subsequently converted to yW through the actions of TYW2, TYW3, and TYW4. The naming conventions used throughout this paper are for the free nucleoside.

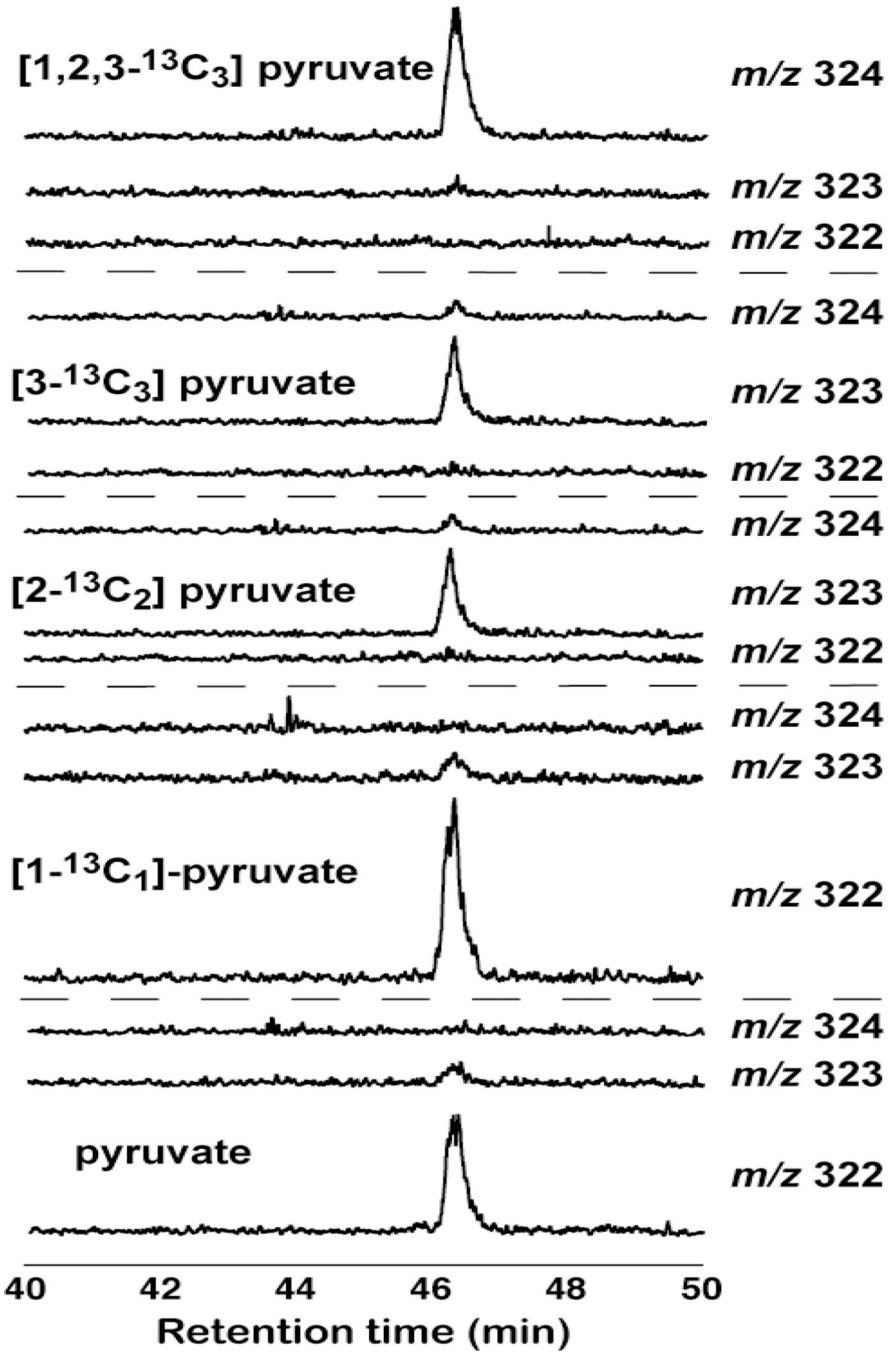

Figure 2.
Extracted ion chromatograms showing incorporation of C-2 and C-3 carbon atoms of pyruvate into 4-demethylwyosine.

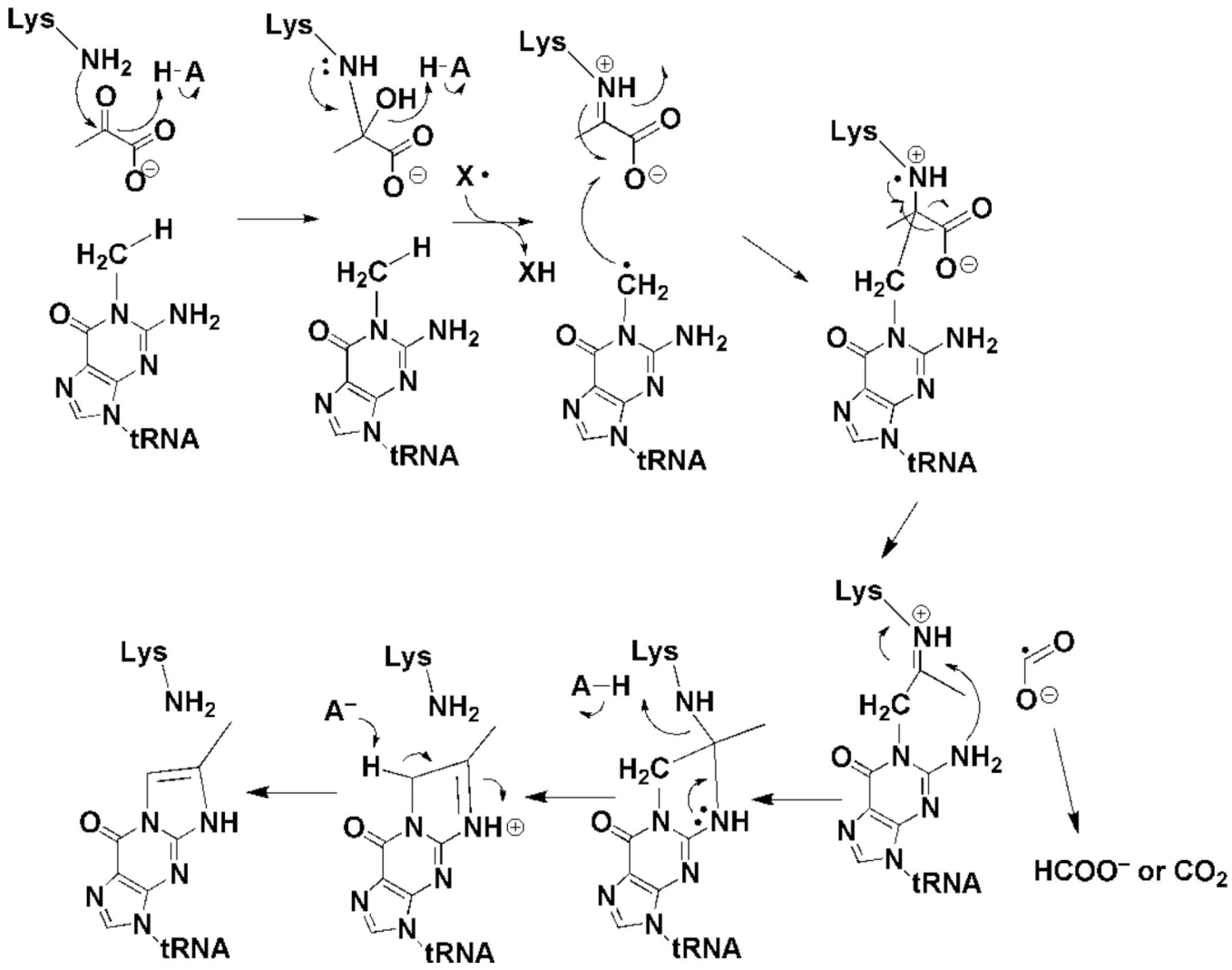

Figure 3.
Putative mechanism for the transformation of N-methylguanosine to 4-demethylwyosine.