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Reaction of AdoMet with ThiC generates a backbone free radical

N. Cecilia Martinez-Gomez¹, Russell R. Poyner², Steven O. Mansoorabadi³, George H. Reed², and Diana M. Downs^{1,*}

¹Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

²Department of Biochemistry, University of Wisconsin, Wisconsin 53726

³College of Pharmacy and Department of Chemistry and Biochemistry, University of Texas, Austin, TX 78712

Abstract

ThiC is an [4Fe-4S] cluster protein that catalyzes the formation of 4-amino-5-hydroxymethyl-2-methylpyrimidine. EPR spectroscopic studies demonstrate that, upon interaction with AdoMet, active ThiC from *Salmonella enterica* generates a persistent free radical on the α -carbon of an amino acid residue. The EPR properties of the radical are consistent with any residue other than a Gly or Ala. Exposure to oxygen was accompanied by a fission of the radical-carrying polypeptide chain between the Gly436 and His437 residues in ThiC. Whether or not the backbone radical is part of the catalytic machinery, its presence provides evidence that ThiC employs free radical chemistry as expected for radical SAM enzymes.

ThiC is required for the biosynthesis of the 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate (HMP-PP) moiety of the essential cofactor thiamine pyrophosphate (TPP). The ThiC enzyme catalyzes rearrangement of 5-aminoimidazole-ribotide (AIR) to 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P) in a reaction that requires a reducing agent and AdoMet (1,2). The [4Fe-4S] cluster contained in anoxically purified ThiC is essential for activity. The conversion from AIR to HMP-P is a complicated intramolecular rearrangement that is not presently understood. Initial results suggested that the catalytic mechanism includes the formation of the 5'-deoxyadenosyl radical. While AdoMet is known to be required for ThiC activity, the protein does not have the CxxxCxxC motif characteristic of the radical SAM (AdoMet) superfamily of proteins (3). Instead the protein contains a Cys⁴⁹⁴ and C(S/T)MCxxx⁵⁸⁹ motif that is conserved in more than 100 ThiC homologues from diverse organisms (4).

The Fe-S cluster of ThiC undergoes reduction by dithionite (1). The EPR spectrum of the reduced protein exhibits an axial lineshape having turning points at $g=2.08$ and $g=1.94$ consistent with an [4Fe-4S]⁺¹ cluster (1). Estimates of unpaired spin concentrations using a CuEDTA standard (200 μ M) indicated that 60% of the protein had a reduced cluster. Anoxic addition of AdoMet (1 mM) to the reduced [4Fe-4S] cluster of ThiC reveals a new EPR signal centered at $g=2.002$. The position and width of this signal and its persistence at higher temperatures suggest that the new signal arises from an organic radical, specifically a protein radical. Spin quantitation indicates that the radical signal represents $\sim 7\%$ of the subunit concentration of the protein, or $\sim 11\%$ of the protein with reduced cluster. Reductive cleavage of AdoMet by [4Fe-4S]⁺¹ clusters is thermodynamically uphill, and the energetics could limit the amount of protein radical produced (5). The hyperfine splitting parameters of the signal of reductively activated ThiC after reaction with AdoMet were not consistent with a glycyl radical.

*To whom correspondence should be addressed. Diana Downs, email downs@bact.wisc.edu. Telephone: 608-265-4630. Fax: 608-262-9865

The experimentally observed spectrum is compared with a calculated spectrum in Figure 1. The calculation is based on a model of a radical centered on a backbone α -carbon having a β -methylene group and a β -amide NH. Hyperfine splitting parameters from the β - ^{14}N , β -methylene protons and the backbone NH are consistent with amino acid residues other than Gly and Ala. The spectrum is, however, sensitive to the dihedral angle of the β -methylene protons with respect to the spin bearing p orbital on the α -carbon. The dihedral angle is a property of the conformation of the side chain.

A ThiC sample prepared similarly but in D_2O gave an EPR spectrum that lacked the smaller doublet splitting on the main peaks. This same splitting was lost in the simulation by eliminating the β -amide proton (H3)(see supporting information). Splitting from 2 β protons is consistent with an α -carbon radical from an amino acid having a β -methylene containing side chain (i. e., not Gly or Ala).

Oxygen inactivates ThiC (1). Exposure of the AdoMet treated sample to oxygen rapidly destroys the EPR signal derived from the radical. Decay of the radical likely proceeds via formation of a transient peroxy radical adduct. For other members of the radical SAM superfamily of proteins that use a Gly radical in the catalytic cycle, inactivation by oxygen is accompanied by a fission of the radical-bearing polypeptide chain (6,7). The radical containing ThiC protein was exposed to oxygen and analyzed by SDS-PAGE. In addition to the full length protein (70 kD), two prominent, oxygen-dependent bands corresponding to peptides with molecular masses of ~ 52 kD and ~ 22 kD were detected. These bands, and two minor bands corresponding to masses of ~ 18 kD and ~ 15 kD were not present in the anoxic sample (Figure 2). Quantitation of the 55 kD band indicated $\sim 6\%$ of the ThiC peptide had been cleaved. N-terminal sequence analysis confirmed that the 52 kD peptide comprised the N-terminal portion of ThiC. The N-terminal sequence analysis for each of the remaining oxygen-specific bands identified the residues HVPMHMI as the N-terminus in all three bands, suggesting that the two smaller, fainter bands represented C-terminal truncations. This hypothesis is supported by the presence of a small amount of truncated ThiC (~ 7 kD) under anoxic conditions that is visible in moderately loaded gels. These data indicate that the oxygen fragmentation occurred between Gly436 and His 437 of the ThiC polypeptide. The cleavage site in the context of the ThiC sequence is shown in Figure 2. Oxygen mediated cleavage of a Gly centered radical in a protein typically generates an oxalyl terminus that does not undergo Edman degradation (6). Polypeptide cleavage by reactive species of oxygen can, however, lead to fragments that are susceptible to Edman based N-terminal sequencing (8,9).

Based on the oxygen fragmentation experiment, His437 is a candidate for the radical bearing residue following reaction of reduced ThiC with AdoMet. His437 is part of a motif EGPGHVP that is conserved in over 100 ThiC homologues. The EPR properties of the radical are not unique to a His α -carbon radical but are entirely compatible with such a species. The captodative stabilization effect credited with stabilization of the Gly radical would also occur for a radical on any α -carbon in the backbone (10). As noted above, Gly radicals lack the β -methylene protons whose splitting are evident in the spectrum, and Ala radicals would have another β -proton splitting that is not observed. The reported crystal structure of *C. crescentus* ThiC was obtained with an apoprotein lacking the Fe-S cluster. However, the structure derived from modeling of the Fe-S domain using the structure of biotin synthase as a guide suggests proximity of His417 (corresponds to *S. enterica* His437) to the site of binding of the substrate analog, imidazole ribotide and to the proposed binding site of AdoMet (2). In the structural model, the imidazole side chain is proposed to be a ligand for a metal ion (2).

Members of the radical SAM superfamily of proteins are involved in a variety of biochemical reactions including, metabolism and synthesis of vitamins, cofactors, and antibiotics (3). The unique iron in the [4Fe-4S] cluster (i. e., the one that is not liganded to a Cys thiolate) is available

to bind to AdoMet. Reductive cleavage of AdoMet generates the 5'-deoxyadenosyl radical which can then initiate free radical chemistry in substrates directly as in lysine 2,3-aminomutase (2,3-LAM) (11) or via intermediate protein radicals as in pyruvate formate lyase (PFL) (11, 12). It is likely that an early step in the activation of ThiC or in the catalytic cycle is the reductive cleavage of AdoMet generating the 5'-deoxyadenosyl radical (5). Once formed the 5'-deoxyadenosyl radical could react directly with the substrate as in 2,3-LAM or with the protein as in PFL-PFL activase complex. In the latter, the Fe-S cluster and AdoMet cleavage are carried out by the separate PFL activase enzyme (12). In PFL, the radical on the α -carbon of Gly-734 is a storage form of the radical and downstream thiyl radicals on Cys419 and Cys418 are believed to interact directly with the substrate (12,13).

Based on the unique placement of Cys residues in the Fe-S binding motif and the atypical AdoMet binding motif (1), ThiC appears to be an outlier in the radical SAM family. There aren't, at present, other examples of radical SAM proteins that have both a resident Fe-S cluster and use a protein radical as part of their catalytic cycle. At this point any connection between the backbone radical generated by reaction with AdoMet and the catalytic mechanism is lacking. Although the backbone radical might represent an alternative to a Gly radical as a "storage" form of free radical equivalents, it is also possible that the backbone radical is a product of a highly specific, but adventitious side reaction. Furthermore we have also seen that the as isolated protein contains a very weak radical signal (data not shown) consistent with the observations made by Chatterjee *et. al* (2). As noted above backbone radicals except for those on Gly or Ala residues would give similar signals, so it is not possible to know if these are the same radicals. Spectroscopically, this AdoMet-dependent ThiC radical provides an exceptionally clean example of an α -carbon radical in a polypeptide setting. Whether or not the backbone radical is part of the catalytic machinery, the AdoMet dependent generation of this radical is evidence that the complicated rearrangement catalyzed by ThiC does indeed employ free radical chemistry as expected for radical SAM enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

EPR spectrum of ThiC backbone radical. A) EPR spectrum at 40 K of reconstituted ThiC in the presence of AdoMet. The spectrum is an average of 4 scans with microwave power 4 mW, modulation amplitude 4 Gauss. B) Resolution enhanced version of spectrum A. C) Simulation of B assuming coupling to two β -protons ($A_{\text{iso}} \text{ H1} = 15 \text{ G}$, $A_{\text{iso}} \text{ H2} = 0.2 \text{ G}$); one β -amide ^{14}N ($A_{^{14}\text{N}} [0 \text{ G}, 15 \text{ G}, 0 \text{ G}]$); and one β -amide proton ($A_{\text{H}} [8.4 \text{ G}, 7.4 \text{ G}, 0.2 \text{ G}]$). Additional details regarding the fitting parameters are given in supporting information.

Figure 2.

Oxygen Fragmentation of the polypeptide chain. Reduced ThiC+AdoMet gassed with O₂ for 20 minutes or kept under anoxic conditions as indicated. The samples were resolved by SDS-PAGE. A) for the high molecular weight bands (4-12%), and B) for the low molecular weight bands (12%). 2 µg protein was applied per lane, visualization was by Commassie blue staining. The sequence of ThiC surrounding the cleavage site is depicted. Starred residues are conserved in over 100 ThiC homologs. The line between Gly436 and His437 indicates the site of oxygen dependent cleavage.