440 *Biochemistry* **2011,** *50,* 440–442
DOI: 10.1021/bi101255e

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Rapid Report

Radical SAM Activation of the B_{12}-Independent Glycerol Dehydratase Results in Formation of 5′-Deoxy-5′-(methylthio)adenosine and Not 5′-Deoxyadenosine†

Jonathan M. Demick and William N. Lanzilotta*

Department of Biochemistry and Molecular Biology, A220B Davison Life Sciences Building, University of Georgia, Athens, Georgia 30602, United States

Received August 5, 2010; Revised Manuscript Received December 23, 2010

ABSTRACT: Activation of glycyl radical enzymes (GREs) by *S*-adenosylmethonine (AdoMet or SAM)-dependent enzymes has long been shown to proceed via the reductive cleavage of SAM. The AdoMet-dependent (or radical SAM) enzymes catalyze this reaction by using a [4Fe-4S] cluster to reductively cleave AdoMet to form a transient 5′-deoxyadenosyl radical and methionine. This radical is then transferred to the GRE, and methionine and 5′-deoxyadenosine are also formed. In contrast to this paradigm, we demonstrate that generation of a glycyl radical on the B_{12}-independent glycerol dehydratase by the glycerol dehydratase activating enzyme results in formation of 5′-deoxy-5′-(methylthio)adenosine and not 5′-deoxyadenosine. This demonstrates for the first time that radical SAM activases are also capable of an alternative cleavage pathway for SAM.

The identification of the "radical SAM" or "AdoMet radical" superfamily has fundamentally changed our view of the utility and functional diversity of the cofactor *S*-adenosylmethionine (AdoMet or SAM) (*1*−*4*). This report is focused on radical SAM "activases" involved in the generation of a catalytic glycyl radical on glycyl radical enzymes (GREs). Pyruvate formate lyase activating enzyme (PFL-AE) was one of the first radical SAM activases to be characterized and performs the activation of the GRE, pyruvate formate lyase (PFL) (*5*−*18*). The mechanistic paradigm that has been established for generation of the glycyl radical on essentially all GREs is shown in Figure 1A. Our group has previously reported the isolation of a GRE capable of dehydrating glycerol (*19*, *20*). Similar to PFL, the glycerol dehydratase (GD) was activated by another enzyme termed the glycerol dehydratase activating enzyme (GD-AE) and required AdoMet and strictly anoxic conditions (*19*). In this work, we have monitored the activation of the GD by the GD-AE and show that, as for several other GREs, a glycyl radical is formed. However, in contrast to the current paradigm, formation of the glycyl radical results in formation of 5′-deoxy-5′-(methylthio)adenosine (MTA). These data suggest that some radical SAM activases may use an alternative cleavage mechanism (Figure 1B) during activation of their GRE and suggest that a different radical intermediate may exist for certain radical SAM activases.

Activation of the GD by the GD-AE as monitored by EPR spectroscopy is shown in Figure 2. Formation of the glycyl radical requires a reduced iron−sulfur cluster (Figure 2A) and

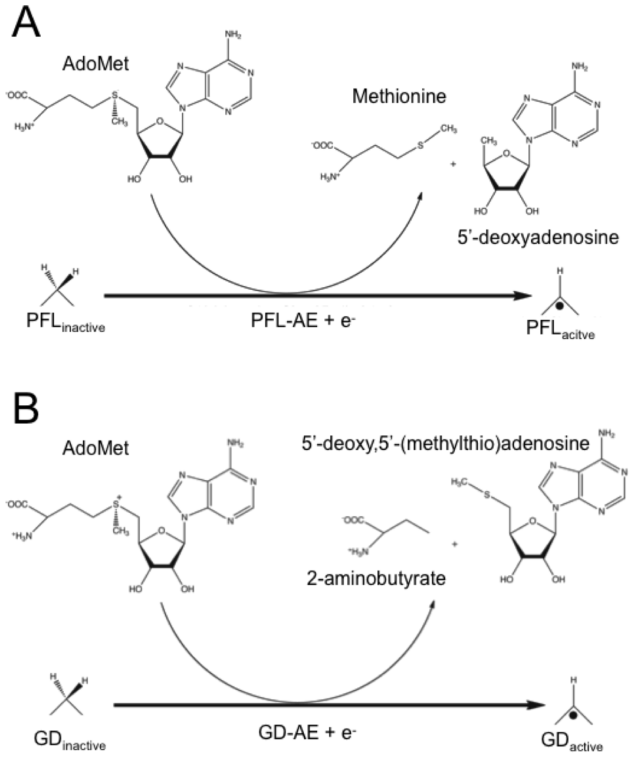

FIGURE 1: Proposed cleavage pathways during glycyl radical generation as catalyzed by radical SAM activases. The mechanism observed for the pyruvate formate lyase (A) and an alternative pathway proposed for the B_{12}-independent glycerol dehydratase (B) are depicted.

AdoMet. AdoMet also appears to perturb the *g* values of the Fe−S cluster signal (in Figure 2, compare panel A with panel B). Upon addition of the GD to the mixture of AdoMet and the GD-AE, containing a reduced Fe−S cluster, radical formation is observed (Figure 2C). The *g* values for the observed radical are identical to what has been observed for PFL and anaerobic ribonucleotide reductase (ARNR) and not that surprising given the high degree of sequence identity between these enzymes.

The complexity of the EPR signature for the GD-AE is consistent with the presence of the additional [4Fe-4S] cluster binding motifs in the primary structure of the GD-AE and multiple Fe−S clusters that may or may not be spin-coupled. The precise composition and electronic structure of the cluster or clusters that give rise to the EPR spectra are currently under investigation as well as the stoichiometry of the conversion of cluster spin to radical spin observed during activation.

While the formation of the glycyl radical was somewhat anticipated, the observation that the activation mechanism

†This work was also supported in part by a cooperative agreement with Metabolic Explorer and Petroleum Research (44563-AC4 to W.N.L.).
*To whom correspondence should be addressed. E-mail: wlanzilo@bmb.uga.edu. Phone: (706) 542-1324. Fax: (706) 542-1738.

 Published on Web 12/23/2010

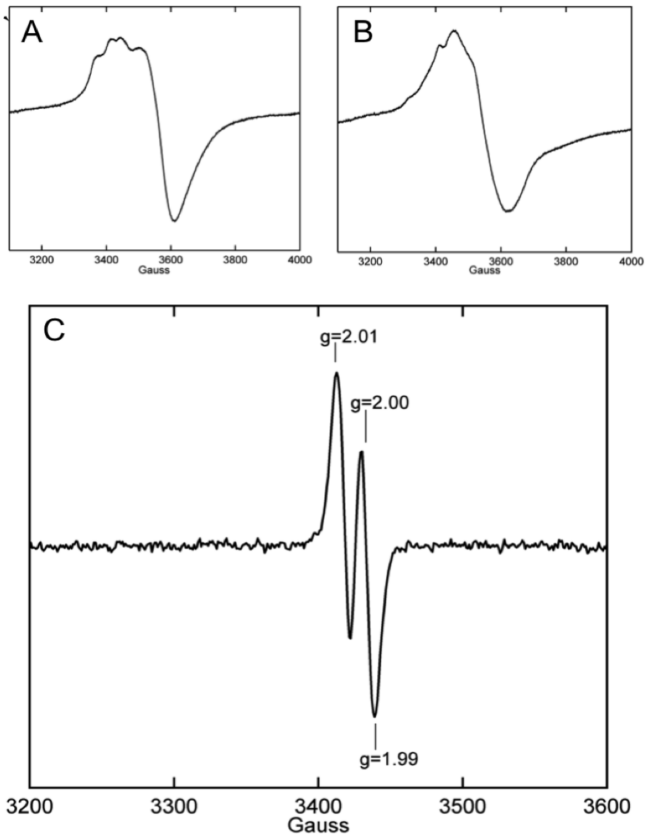

FIGURE 2: EPR spectra of the GD-AE alone (A), in the presence of 5 mM SAM (B), and in the presence of 5 mM SAM as well as the GD (C). EPR parameters and sample preparation are described in detail in the Supporting Information.

results in the formation of MTA and not 5′-deoxyadenosine is exciting because every other radical SAM activase investigated to date has been shown to produce methionine and 5′-deoxyadenosine during generation of the glycyl radical (*3*, *21*). Figure 3 shows the activation reaction as monitored by high-performance liquid chromatography (HPLC). No MTA peak was observed if either the GD or the GD-AE were excluded from the assay. Calibration of the MTA peak area against a standard curve revealed a stoichiometric relationship between the amount of GD added and the amount of MTA formed. To detect any amino acid products of activation, we applied Edman derivatization of the reaction mixture and HPLC. Consistent with our proposal, we observe simultaneous formation of 2-aminobutyrate and MTA (Supporting Information). Regardless of the radical intermediate that is formed as a result of reductive cleavage of AdoMet, our results are significant because it has been widely accepted that all radical SAM enzymes exclusively cleave the C5′−S bond. Figure 3 and Figure S2 of the Supporting Information show that, at least for the GD-AE, this is not the case. The prevailing wisdom states that reductive cleavage of SAM should result in a transient 5′-deoxyadenosyl radical that would rapidly abstract a hydrogen atom from the conserved glycine residue on the GD (*2*, *3*, *22*). In this case, the cleavage products are methionine and 5′-deoxyadenosine. Regardless of the radical intermediate, which is not the focus of this work, there is precedence for an alternative reductive cleavage event that leads to MTA formation (*23*) as we have observed. Although it is not a radical SAM enzyme, Dhp2 contains a [4Fe-4S] cluster that has recently been shown to catalyze the reductive cleavage of AdoMet (*23*) to form a 3-amino-3-carboxypropyl radical and

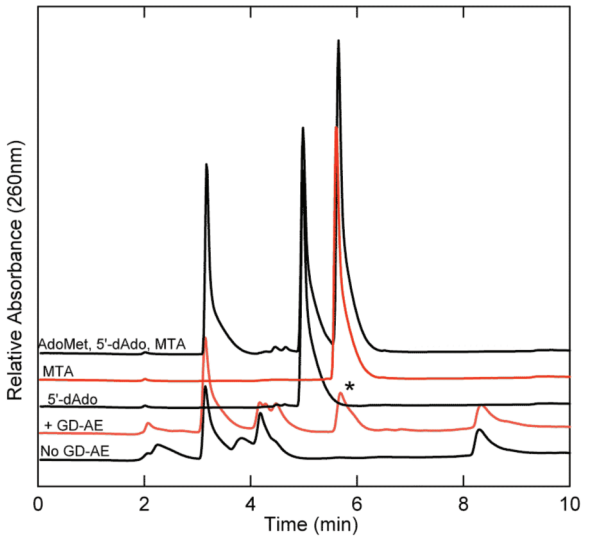

FIGURE 3: HPLC analysis of assay buffer containing a mixture of *S*-adenosylmethionine, 5′-deoxyadenosine, and 5′-deoxy-5′-(methylthio)adenosine (AdoMet, 5′-dAdo, MTA), only 5′-deoxy-5′-(methylthio)adenosine (MTA), only 5′-deoxyadenosine (5′-dAdo), or the complete assay mix required for activation of the GD with (+ GD-AE) or without (No GD-AE) the GD-AE. The asterisk highlights the appearance of a peak in the HPLC chromatogram when the reaction is quenched 15 s after the addition of the GD-AE.

MTA. By analogy, we propose that a radical intermediate, similar to what has been reported for Dhp2, abstracts a hydrogen atom to generate the glycyl radical on the GD. This is the only way to account for formation of MTA and 2-aminobutyric acid.

While the precise identity of the radical intermediate in the GD-AE activation mechanism is currently being investigated, this is the first time that MTA and 2-aminobutyrate have been observed as reaction products during the activation of a GRE by a radical SAM activase. The data clearly demonstrate that different reductive cleavage pathways exist for radical SAM enzymes. Moreover, these results are extremely surprising given the level of identity in the primary structures for PFL, ARNR, and the GD. Whether radical SAM activating enzymes use the alternative mode of AdoMet cleavage during radical generation is now an important question that needs to be investigated further. Given the structural similarities between the GD and PFL at the level of both primary and tertiary structure, it is difficult at this time to determine exactly why the GD-AE follows a different reductive cleavage pathway during glycyl radical formation. Ultimately, to address this question, some structural information will be required.

PFL-AE, anerobic ribonucleotide reductase activating enzyme (ARNR-AE), and the GD-AE all contain the hallmark adenosine and catalytic cluster (CX_3CX_2C) binding motifs. However, in contrast to both PFL-AE and ARNR-AE, the GD-AE also contains two additional ferredoxin-like [4Fe-4S] cluster binding domains (*20*). The latter observation is also true for at least two other radical SAM enzymes involved in GRE activation, specifically, BssD and hydroxyphenylacetate decarboxylase activase (*24*, *25*). At this time, the chemical products of glycyl radical formation in the latter two systems have not been investigated. The physiological role and exact composition of the additional clusters in these enzymes have not been fully investigated, but a reasonable hypothesis is that the additional domain is required for the GD-AE to accept electrons from a physiological donor, as proposed for hydroxyphenylacetate

decarboxylase activase based on mutagenesis results (*25*). The electron donor for the GD-AE is most likely a low-potential ferredoxin. This is in contrast to PFL-AE for which it has been shown that electrons for PFL-AE are provided by reduced flavodoxin (*8*). While the source of the low-potential electron required for reductive cleavage of AdoMet may seem like a minor point, this may lead to a different structural arrangement in the active site. Moreover, it is not known whether other AdoMet radical activating enzymes containing the additional ferredoxin-like [4Fe-4S] cluster binding domains also work by reductive cleavage of AdoMet to yield MTA. In any case, our findings provide evidence for an exciting new hypothesis, specifically that, in contrast to the current paradigm, radical SAM activases containing additional Fe−S clusters may operate by a different reductive cleavage mechanism. At present, PFL-AE is the only structure available for any radical SAM activase. A structure of a radical SAM activase with the additional ferredoxin-like [4Fe-4S] cluster binding domains will certainly provide some insight into the atomic details of AdoMet binding, but additional parameters such as the kinetics and thermodynamics of electron transfer and the reductive cleavage event must also be investigated. Whether one reductive cleavage mechanism is chosen over the other and the occurrence of the additional Fe−S cluster-containing domains in radical SAM activases throughout Nature are likely to have important evolutionary implications. For example, one might inquire about whether PFL-AE is truly a model enzyme for all radical SAM activases or whether PFL-AE is actually an exception to the rule.

ACKNOWLEDGMENT

We thank Dr. Lance Wells and Robert L. Bridger for performing the MS analysis; they are funded in part by an equipment grant from the Georgia Research Alliance to Dr. Wells. We also thank Dr. Jeff Urbauer for critical review.

SUPPORTING INFORMATION AVAILABLE

Materials and detailed experimental procedures as well as additional HPLC and mass spectroscopy data. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Sofia, H. J., Chen, G., Hetzler, B. G., Reyes-Spindola, J. F., and Miller, N. E. (2001) *Nucleic Acids Res. 29*, 1097–1106.
2. Cheek, J., and Broderick, J. B. (2001) *J. Biol. Inorg. Chem. 6*, 209–226.
3. Frey, P. A., Hegeman, A. D., and Ruzicka, F. J. (2008) *Crit. Rev. Biochem. Mol. Biol. 43*, 63–88.
4. Gambarelli, S., Luttringer, F., Padovani, D., Mulliez, E., and Fontecave, M. (2005) *ChemBioChem 6*, 1960–1962.
5. Frey, M., Rothe, M., Wagner, A. F., and Knappe, J. (1994) *J. Biol. Chem. 269*, 12432–12437.
6. Parast, C. V., Wong, K. K., Lewisch, S. A., Kozarich, J. W., Peisach, J., and Magliozzo, R. S. (1995) *Biochemistry 34*, 2393–2399.
7. Reddy, S. G., Wong, K. K., Parast, C. V., Peisach, J., Magliozzo, R. S., and Kozarich, J. W. (1998) *Biochemistry 37*, 558–563.
8. Sawers, G., and Watson, G. (1998) *Mol. Microbiol. 29*, 945–954.
9. Becker, A., Fritz-Wolf, K., Kabsch, W., Knappe, J., Schultz, S., and Volker Wagner, A. F. (1999) *Nat. Struct. Biol. 6*, 969–975.
10. Knappe, J., and Wagner, A. F. (2001) *Adv. Protein Chem. 58*, 277–315.
11. Krebs, C., Broderick, W. E., Henshaw, T. F., Broderick, J. B., and Huynh, B. H. (2002) *J. Am. Chem. Soc. 124*, 912–913.
12. Walsby, C. J., Hong, W., Broderick, W. E., Cheek, J., Ortillo, D., Broderick, J. B., and Hoffman, B. M. (2002) *J. Am. Chem. Soc. 124*, 3143–3151.
13. Walsby, C. J., Ortillo, D., Broderick, W. E., Broderick, J. B., and Hoffman, B. M. (2002) *J. Am. Chem. Soc. 124*, 11270–11271.
14. Gelius-Dietrich, G., and Henze, K. (2004) *J. Eukaryotic Microbiol. 51*, 456–463.
15. Lehtio, L., and Goldman, A. (2004) *Protein Eng., Des. Sel. 17*, 545–552.
16. Buis, J. M., and Broderick, J. B. (2005) *Arch. Biochem. Biophys. 433*, 288–296.
17. Walsby, C. J., Ortillo, D., Yang, J., Nnyepi, M. R., Broderick, W. E., Hoffman, B. M., and Broderick, J. B. (2005) *Inorg. Chem. 44*, 727–741.
18. Peng, Y., Veneziano, S. E., Gillispie, G. D., and Broderick, J. B. (2010) *J. Biol. Chem. 285*, 27224–27231.
19. O'Brien, J. R., Raynaud, C., Croux, C., Girbal, L., Soucaille, P., and Lanzilotta, W. N. (2004) *Biochemistry 43*, 4635–4645.
20. Raynaud, C., Sarcabal, P., Meynial-Salles, I., Croux, C., and Soucaille, P. (2003) *Proc. Natl. Acad. Sci. U.S.A. 100*, 5010–5015.
21. Nicolet, Y., Amara, P., Mouesca, J. M., and Fontecilla-Camps, J. C. (2009) *Proc. Natl. Acad. Sci. U.S.A. 106*, 14867–14871.
22. Broderick, J. B. (2010) *Nature 465*, 877–878.
23. Zhang, Y., Zhu, X., Torelli, A. T., Lee, M., Dzikovski, B., Koralewski, R. M., Wang, E., Freed, J., Krebs, C., Ealick, S. E., and Lin, H. (2010) *Nature 465*, 891–896.
24. Leuthner, B., Leutwein, C., Schulz, H., Horth, P., Haehnel, W., Schiltz, E., Schagger, H., and Heider, J. (1998) *Mol. Microbiol. 28*, 615–628.
25. Yu, L., Blaser, M., Andrei, P. I., Pierik, A. J., and Selmer, T. (2006) *Biochemistry 45*, 9584–9592.