Published in final edited form as:

Angew Chem Int Ed Engl. 2016 March 7; 55(11): 3724–3728. doi:10.1002/anie.201510635.

C3'-Deoxygenation of paromamine catalyzed by a radical SAM enzyme: Characterization of AprD4 and its reductase partner AprD3**

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Abstract

C3'-deoxygenation of aminoglycosides results in their decreased susceptibility to phosphorylation thereby increasing their efficacy as antibiotics. However, the biosynthetic mechanism of C3'-deoxygenation is unknown. To address this question, aprD4 and aprD3 genes from the apramycin gene cluster in *Streptomyces tenebrarius* were expressed in *E. coli* and the resulting gene products were characterized in vitro. AprD4 is shown to be a radical SAM enzyme catalyzing homolysis of SAM to 5'-deoxyadenosine (5'-dAdo) in the presence of paromamine. [4'-²H]-Paromamine was prepared and used to demonstrate that its C4'-H is transferred to 5'-dAdo by AprD4, during which the substrate is dehydrated to a product consistent with 4'-oxolividamine. In contrast, paromamine is reduced to a deoxy product when incubated with AprD4/AprD3/ NADPH. These results indicate that AprD4 is the first radical SAM diol-dehydratase and along with AprD3 is responsible for 3'-deoxygenation in the biosynthesis of aminoglycosides.

Graphical Abstract

Dehydration of 1,2-diol by radical reaction: C3'-deoxygenation of paromamine is catalyzed by two enzymes, a radical *S*-adenosylmethionine (SAM) enzyme AprD4 and a NAD(P)H dependent dehydrogenase AprD3. AprD4 catalyzes dehydration of the 3,4-dihydroxyl moiety of paromamine and AprD3 reduces the nascent keto intermediate to complete the C3'-deoxygenation.

**This work was supported by grants from the National Institutes of Health (GM035906) and the Welch Foundation (F-1511)

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Supporting information for this article is available on the WWW under

Keywords

biosynthesis; aminoglycosides; radical SAM enzymes; enzyme catalysis; radical mechanism

The aminoglycosides represent an important class of antibiotics in the treatment of Gram negative bacterial infections.^{1–3} Their biological activity is a result of their ability to bind the bacterial 30S ribosome and subsequently inhibit protein synthesis. Many members of this family are characterized by a 2-deoxystreptamine (2-DOS, **1**) core that is decorated with a variety of carbohydrates necessary for activity (see Figure 1). In a number of aminoglycosides, such as apramycin (**2**),⁴ tobramycin (**3**),⁵ nebramycin 5' (**4**)⁶ and lividomycin B (**5**)⁷, a 3'-deoxyhexose is found linked via a glycosidic bond to the C4 position of the 2-DOS core. The absence of a C3' hydroxyl group in these species leaves them more effective as antimicrobial agents, because they are less susceptible to modification by aminoglycoside phosphotransferase, APH(3'), which is a known mechanism of resistance in some bacteria.¹ While the biosynthesis of 2-DOS has been investigated in detail,^{8–10} much less is known regarding assembly of the sugar appendages,^{11,12} and in particular the mechanism of C3'-deoxygenation despite its importance to the development and improvement of clinically useful aminoglycoside antibiotics.¹³

The gene clusters responsible for the biosynthesis of apramycin (**2**), tobramycin (**3**), and lividomycin B (**5**) in *Streptomyces* and *Streptoalloteichius* species have been described,^{14–17} and significant progress has been made in elucidating the reactions involved in the tobramycin pathway.^{18,19} While no gene in the tobramycin cluster has been implicated in catalyzing C3'-deoxygenation,^{14,15} recent investigations including gene knockout studies²⁰ into the apramycin and lividomycin B pathways have identified the AprD4/AprD3 and LivW/LivY pairs as likely candidates for the activity. For example, incorporation of the *aprD4* and *aprD3* genes into the kanamycin (see **6**) producing strain *S. kanamyceticus* led to the isolation of 3'-deoxykanamycins (e.g., **7**),¹⁹ which are not normally produced. Likewise, previous studies have also shown the conversion of neamine (**9**) to nebramine (**10**) in cell-free extracts of *S. venezuelae* expressing AprD4 and AprD3.¹⁹ However, no conversion of kanamycin B (**8**) to tobramycin (**3**) was observed under the same conditions, leading to the suggestion that the substrate for AprD4/AprD3 may be a pseudodisaccharide (e.g., **9**) rather than a pseudotrisaccharide (e.g., **8**).¹⁹ Since apramycin (**2**) and tobramycin (**3**) are produced together in the same bacterial strains, it is possible that AprD4/AprD3 serves as the C3'-deoxyoxygenation machinery for both pathways.

Sequence analysis has led to the assignment of AprD3 as an NAD-dependent dehydrogenase and AprD4 as a member of the radical SAM family of enzymes.^{14,15,18} The radical SAM enzymes are characterized by the presence of a [4Fe-4S] cluster that serves to reductively homolyze *S*-adenosyl-L-methionine (SAM) and thereby generate methionine (**17**) and a 5'-deoxyadenosyl radical (5'-dAdo•, **18**, see Scheme 2) that initiates a radical-mediated transformation of substrate to product.^{21–23} The catalytic [4Fe-4S] cluster is typically coordinated by a highly conserved CxxxCxxC motif,²⁴ however, in the case of AprD4, a non-canonical CxxxCxxx motif is observed instead. While a small but growing number of radical SAM enzymes such as PhnJ,²⁵ HmdB,²⁶ QueE,²⁷ Phd2²⁸ and ThiC²⁹ do indeed

exhibit atypical [4Fe-4S] binding motifs, the noncanonical motif of AprD4 still leads to questions as to whether AprD4 operates as a radical SAM enzyme as well as the mechanism by which C3'-deoxygenation is effected during the biosynthesis of amino-glycosides. Herein, we report the purification and biochemical characterization of AprD4 and AprD3 from *S. tenebrarius* and demonstrate their ability to catalyze 3'-deoxygenation of paromamine (**11**) to lividamine (**13**) *in vitro*.

Both the *aprD3* and *aprD4* genes were amplified by polymerase chain reaction from *S. tenebrarius* genomic DNA, cloned into a pET28 vector, and heterologously overexpressed in *Escherichia coli* (Figures S1, S2). The His₆-tagged AprD4 was isolated from cell lysates using a Ni-NTA affinity column and then subjected to anaerobic incubation with Fe(NH₄)₂(SO₄)₂ and Na₂S in a Coy anaerobic chamber with an atmosphere of >97% N₂ and 3% H₂ to reconstitute the [4Fe-4S] cluster. Iron³⁰ and sulfur analysis³¹ of the reconstituted AprD4 revealed the presence of 9.2 ± 0.6 equivalents of iron and 8.2 ± 0.7 equivalents of sulfide per AprD4 monomer. These results indicated the presence of two [4Fe-4S] clusters bound to each AprD4 monomer. Likewise, the UV-vis spectrum of the reconstituted AprD4 exhibited a shoulder around 420 nm, which is typical of [4Fe-4S]²⁺-containing proteins.^{32,33} Furthermore, addition of sodium dithionite (2 mM) to the reconstituted enzyme led to a decrease in the absorbance at 420 nm implying reduction of the clusters (Figure S2).

Coincubation of reconstituted AprD4 (0.01 mM) with 1 mM paromamine (**11**) and 2 mM SAM in the presence of 10 mM dithiothreitol (DTT) and 2 mM dithionite (50 mM NH₄HCO₃ buffer, pH 7.8) at room temperature led to the formation of 5'-dAdo (**19**) observed by HPLC with UV detection at 260 nm (see Figure 2A, trace b). While exclusion of dithionite from the reaction did not lead to detectable levels of 5'-dAdo formation, the replacement of dithionite with either methyl viologen and NADPH or the *E. coli* flavodoxin/flavodoxin reductase/NADPH system were effective (see Figure S4). In contrast, benzyl viologen and NADPH was not suitable for enzyme activity. Likewise, when the incubation was performed without prior reconstitution of the enzyme, no 5'-dAdo was observed (see Figure 2A, trace e). Finally, although uncoupled formation of 5'-dAdo was discernible in the absence of paromamine, the extent of SAM turnover to 5'-dAdo was significantly lower than that of the full reaction (see Figure 2A, traces d and b). These results strongly imply that AprD4 is able to catalyze radical homolysis of SAM consistent with its assignment as a radical SAM enzyme.^{21–23}

The AprD4 reaction was also monitored by HPLC using a Corona charged aerosol detector (CAD),³⁴ because the pseudo-disaccharide substrate paromamine (**11**) and its products lack a UV chromophore. However, despite the observed conversion of SAM to 5'-dAdo, neither significant consumption of paromamine nor new product formation was noted, unless 10 μM AprD3 and 1 mM NADPH were also included in the reaction mixture (see Figure 2B, traces b & g). In the latter case, a new peak with a retention time of 7.2 min together with the consumption of paromamine was detected by Corona CAD. Electrospray ionization mass spectrometry (ESI MS) of the new product revealed a signal at 330.1638 *m/z* consistent with the sodium adduct of lividamine (**13**, calc. 330.1636 [M+Na]⁺, Figures 3E and S3). These findings led to the hypothesis that AprD4 operates as a radical-mediated dehydratase with

AprD3 serving as its reductase partner to effect the overall reduction of paromamine to lividamine (**11**→**12**→**13**, see Scheme 1).

As a first test of this hypothesis, reaction mixtures excluding AprD3 were analyzed by ESI MS to check for low concentrations of the putative dehydration product of paromamine that went undetected by HPLC/Corona CAD. As shown in Figure 3A, a peak was observed at 328.15 *m/z* consistent with the sodium adduct of such an intermediary species (**12**). To further support assignment of this MS signal to the putative ketone intermediate, the AprD4 reaction in the absence of AprD3 was run in the presence of 10 mM phenylhydrazine for 4 hr prior to ESI MS analysis. In this case, a new peak at 418.20 *m/z* was observed whereas the signal at 328.15 *m/z* was significantly reduced suggesting formation of a phenylhydrazone derivative (**14**, see Figure 3B). Moreover, treatment of the AprD4-only reaction with NaBH₄ or NaBD₄ resulted in a shift of the signal at 328.15 *m/z* to 330.17 and 331.17 *m/z*, respectively (see Figures 3C and D). These results support the hypothesis that AprD4 catalyzes the first of two half-reactions resulting in the dehydration of paromamine to form a keto intermediate such as **12**.

Based on these results, at least two mechanisms can be envisioned for the AprD4-catalyzed dehydration of paromamine (**11**) as shown in Scheme 2. Consistent with radical SAM chemistry, both mechanisms begin with initial hydrogen atom abstraction from C4' of paromamine (**11**→**20**) by the 5'-dAdo• radical (**18**) generated by the reductive homolysis of SAM. The resulting substrate radical (**20**) may then be deprotonated to form a ketyl radical **21/22** that subsequently undergoes β-elimination of the 3'-OH group to yield the enol radical **23** (route A). This mechanism is similar to proposals for (*R*)-2-hydroxy-acyl-CoA dehydratase³⁵ and ribonucleotide reductase.³⁶ One-electron reduction of the resulting enol radical intermediate would then complete the dehydration reaction (e.g., **23/24**→**12**). Alternatively, the initially formed substrate radical **20** may instead undergo a radical-induced 1,2-hydroxyl shift to form the *gem*-diol radical **25** (route B) in direct analogy to the mechanisms of the B₁₂-dependent dioldehydratases and ethanolamine ammonia lyase.^{37–39} Following reduction, the *gem*-diol intermediate **26** would then be converted to **12** via the elimination of water.

In order to further investigate the mechanism of AprD4-catalyzed dehydration and establish the site of hydrogen atom abstraction from **11**, the [4'-²H] isotopolog of paromamine was prepared (see Supporting Information for details). When 10 μM AprD4 was incubated with 1 mM [4'-²H]-paromamine, 2 mM SAM, and 2 mM dithionite in 50 mM NH₄HCO₃ (pH 7.8) for 8 hr at room temperature, formation of 5'-dAdo was again observed and this time with single-deuterium incorporation beyond natural abundance as determined by ESI MS (see Figure S5). A complimentary assay with unlabeled paromamine (**11**) and AprD4 using the dideuterated [5'-²H₂]-SAM isotopolog⁴⁰ in the incubation revealed hydrogen transfer from **11** to [5'-²H₂]-5'-dAdo• to yield [5'-²H₂]-5'-dAdo as the product (Figure S6). These observations further substantiate the proposed early steps of AprD4 catalysis shown Scheme 2, whereby AprD4 acts as a radical SAM enzyme that initiates a radical-mediated dehydration of paromamine via hydrogen atom abstraction from the C4' position.

In summary, the results reported herein provided preliminary evidence for the assignment of functions of AprD4 and AprD3 in the biosynthesis of C3'-deoxyaminoglycosides. AprD4 appears to be the first example of a radical SAM diol-dehydratase and catalyzes the radical-mediated dehydration of paromamine (**11**). This transformation is reminiscent of the radical-mediated dehydration reactions catalyzed by the B₁₂-dependent diol-dehydratases^{38,39} as well as the B₁₂-independent glycerol dehydratase,⁴¹ which employs a radical SAM activating enzyme to facilitate turnover.^{41a,42} However, the reactive 5'-dAdo• radical (**18**) is consumed *in vitro* to give 5'-dAdo (**19**) during the AprD4 catalytic cycle, whereas the radical initiator is typically regenerated in the case of other radical-mediated dehydratases.^{38,39}

AprD3 is shown to be a dehydrogenase acting as the reductase counterpart to AprD4 in order to facilitate the net C3'-deoxygenation of the pseudodisaccharide substrates in the biosynthesis of apramycin (**2**) and tobramycin (**3**). LivW and LivY are expected to play analogous roles in the lividomycin (**5**) biosynthetic pathway. The dehydration reaction catalyzed by AprD4 bears a close resemblance to the deamination catalyzed of DesII, which is also a radical SAM enzyme catalyzing the conversion of TDP-4,6-dideoxy-4-amino-D-glucose to TDP-4,6-dideoxy-3-keto-D-glucose.^{40,43–49} However, the latter is unable to catalyze dehydration upon direct substitution of the eliminated amine with a hydroxyl and instead acts as a dehydrogenase.⁴⁵ This makes the comparative study of the AprD4 and DesII of particular interest for understanding how enzymes are able to control the fate of their radical intermediates during turnover.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Mark Ruszczycky for his help with manuscript preparation and critical comments. We would also thank Dr. Reid McCarty for his help with the early experiments.

References

1. Magnet S, Blanchard J. Chem Rev. 2005; 105:477–498. [PubMed: 15700953]
2. Moazed D, Noller HF. Nature. 1987; 327:389–396. [PubMed: 2953976]
3. Becker B, Cooper MA. ACS Chem Biol. 2013; 8:105–115. [PubMed: 23110460]
4. O'Connor S, Lam LKT, Jones ND, Chaney MO. J Org Chem. 1976; 41:2087–2092. [PubMed: 932851]
5. Stark WM, Hoehn MM, Knox NG. Antimicrob Agents Chemother (Bethesda). 1967; 7:314–323. [PubMed: 5596154]
6. Koch KF, Davis FA, Rhoades JA. J Antibiot. 1973; 12:745–751. [PubMed: 4792386]
7. Mori T, Kyotani Y, Watanabe I, Oda T. J Antibiot (Tokyo). 1972; 25:149–150. [PubMed: 5034809]
8. Kudo F, Eguchi T. Methods Enzymol. 2009; 459:493–519. [PubMed: 19362652]
9. Llewellyn NM, Spencer JB. Nat Prod Rep. 2006; 23:864–874. [PubMed: 17119636]
10. Kudo F, Eguchi T. J Antibiot. 2009; 62:471–481. [PubMed: 19644520]
11. Park JW, Hong JSJ, Parajuli N, Jung WS, Park SR, Lim SK, Sohng JK, Yoon YJ. Proc Natl Acad Sci USA. 2008; 105:8399–8404. [PubMed: 18550838]

12. Dairi T, Ohta T, Hashimoto E, Hasegawa M. *Mol Gen Genet*. 1992; 236:39–48. [PubMed: 1494349]
13. Kondo S, Hotta K. *J Infect Chemother*. 1999; 5:1–9. [PubMed: 11810483]
14. Piepersberg, W.; Aboshanab, KM.; Schmidt-Beissner, H.; Wehmer, UF. *Aminoglycoside Antibiotics*. Arya, DP., editor. Wiley-VCH; Weinheim: 2007. p. 15–118.
15. Hirayama T, Tamegai H, Kudo F, Kojima K, Kakinuma K, Eguchi T. *J Antibiot*. 2006; 59:358–361. [PubMed: 16915821]
16. Oda T, Mori T, Ito H, Kunieda T, Munakata K. *J Antibiot*. 1971; 24:333–338. [PubMed: 5091208]
17. Mori T, Ichiyana R, Kondo H, Tokunaga K, Oda T, Munakata K. *J Antibiot*. 1971; 24:339–346. [PubMed: 5091209]
18. Park SR, Park JW, Ban YH, Sohng JK, Yoon YH. *Nat Prod Rep*. 2013; 30:11–20. [PubMed: 23179168]
19. Park JW, Park SY, Nepal KK, Han aR, Ban YH, Yoo YJ, Kim EJ, Kim eM, Kim D, Sohng JK, Yoon YJ. *Nat Chem Biol*. 2011; 7:843–852. [PubMed: 21983602]
20. Ni X, Li D, Yang L, Huang T, Li H, Xia H. *Appl Microbiol Biotechnol*. 2011; 89:723–731. [PubMed: 20936279]
21. Boderick JB, Duffins BT, Duschene KS, Shepard EM. *Chem Rev*. 2014; 114:4229–4317. [PubMed: 24476342]
22. Frey PA. *Acc Chem Res*. 2014; 47:540–549. [PubMed: 24308628]
23. Frey PA, Hegeman AD, Ruzicka FJ. *Crit Rev Biochem Mol Biol*. 2008; 43:63–88. [PubMed: 18307109]
24. Sofia HJ, Chen G, Hetzler BH, Reyes-Spindola JF, Miller NE. *Nucleic Acids Res*. 2001; 29:1097–1106. [PubMed: 11222759]
25. Kamat SS, Williams HJ, Rauchel FM. *Nature*. 2011; 480:50–573.
26. McGlynn SE, Boyd ES, Shepard EM, Lange RK, Gerlach R, Broderick JB, Peters JW. *J Bacteriol*. 2010; 192:595–598. [PubMed: 19897660]
27. Dowling DP, Bruender NA, Young AP, McCarty RM, Bandarian V, Drennan CL. *Nat Chem Biol*. 2014; 10:106–112. [PubMed: 24362703]
28. Zhang Y, Zhu X, Torelli AT, Lee M, Dzikovski B, Koralewski RM, Wang E, Freed J, Krebs C, Ealick SE, Lin H. *Nature*. 2010; 465:891–896. [PubMed: 20559380]
29. Chatterjee A, Li Y, Zhang Y, Grove TL, Lee M, Krebs C, Booker SJ, Begley TP, Ealick SE. *Nat Chem Biol*. 2008; 4:758–765. [PubMed: 18953358]
30. Fish WW. *Methods Enzymol*. 1988; 158:357–364. [PubMed: 3374387]
31. Beinert H. *Anal Biochem*. 1983; 131:373–378. [PubMed: 6614472]
32. Ugulava NB, Gibney BR, Jarrett JT. *Biochemistry*. 2000; 39:5206–5214. [PubMed: 10819988]
33. Ollagnier S, Meier C, Mulliez E, Gaillard J, Schuenemann V, Trautwein A, Mattioli T, Lutz M, Fontecave M. *J Am Chem Soc*. 1999; 121:6344–6350.
34. Vehovec T, Obreza A. *J Chromagr A*. 2010; 1217:1549–1556.
35. Buckel W. *Angew Chem Int Ed*. 2009; 48:6779–6787.
36. Stubbe J, van der Donk WA. *Chem Biol*. 1995; 2:793–801. [PubMed: 8807812]
37. Bandarian, V.; Reed, GH. *Chemistry and Biochemistry of B₁₂*. Banerjee, R., editor. John Wiley & Sons; New York: 1999. p. 811–833.
38. Toraya T. *Chem Rev*. 2003; 103:2095–2127. [PubMed: 12797825]
39. Toraya T. *Arch Biochem Biophys*. 2014; 544:40–57. [PubMed: 24269950]
40. Ko Y, Rusczycky MW, Choi S-h, Liu H-w. *Angew Chem Int Ed*. 2015; 54:860–863.
41. (a) O'Brien JR, Raynaud C, Croux C, Girbal L, Soucaille P, Lanzilotta WN. *Biochemistry*. 2004; 43:4635–4645. [PubMed: 15096031] (b) Raynaud C, Sarcabal P, Meynial-Salles I, Croux C, Soucaille P. *Proc Natl Acad Sci USA*. 2003; 100:5010–5015. [PubMed: 12704244]
42. Demick JM, Lanzilotta WN. *Biochemistry*. 2011; 50:440–442. [PubMed: 21182298]
43. Lin G-M, Choi S-H, Rusczycky MW, Liu H-w. *J Am Chem Soc*. 2015; 137:4954–4967.
44. Rusczycky MW, Choi S-h, Liu H-w. *J Am Chem Soc*. 2010; 132:2359–2369. [PubMed: 20121093]

45. Szu PH, Rusczycky MW, Choi S-h, Yan F, Liu H-w. J Am Chem Soc. 2009; 131:14030–14042. [PubMed: 19746907]
46. Rusczycky MW, Choi S-h, Mansoorabadi SO, Liu H-w. J Am Chem Soc. 2011; 133:7292–7295. [PubMed: 21513273]
47. Rusczycky MW, Ogasawara Y, Liu H-w. Biochim Biophys Acta. 2012; 1824:1231–1244. [PubMed: 22172915]
48. Rusczycky MW, Choi S-h, Liu H-w. Proc Natl Acad Sci USA. 2013; 110:2088–2093. [PubMed: 23329328]
49. Rusczycky MW, Liu H-w. Israel J Chem. 2015; 54:315–324.

Figure 1.
Selected examples of aminoglycosides

Figure 2.

HPLC traces of AprD4 reaction mixtures: A) production of 5'-dAdo (**19**) observed by UV detection at 260 nm, and B) consumption of paromamine (**11**) observed by Corona CAD detection (see text and Supporting Information for details). Trace (a) corresponds to 5'-dAdo (**19**) and paromamine (**11**) standards in A and B, respectively. The remaining traces in both A and B are from reaction mixtures containing 10 μ M AprD4, 1 mM paromamine, 2 mM SAM, 10 mM DTT, 2 mM dithionite, in 50 mM NH_4HCO_3 (pH 7.8) buffer with the following modifications: (b) no changes, (c) no AprD4, (d) no paromamine, (e) nonreconstituted AprD4, (f) no SAM, (g) with 10 μ M AprD3 and 1 mM NADPH and (h) with 10 μ M AprD3 and no NADPH.

Figure 3. Electrospray ionization (ESI) mass spectra of the AprD4 reaction with paromamine (**11**) (A), together with the addition of (B) phenylhydrazine, (C) NaBH₄ and (D) NaBD₄ as described in the text. (E) ESI mass spectrum of the AprD4/AprD3 co-incubation with paromamine and NADPH.

Scheme 1.
AprD4, AprD3 and derivatization reactions

Scheme 2.

Proposed mechanisms for the AprD4-catalyzed dehydration of paromamine (**11**).