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Characterization of SpnQ from the Spinosyn Biosynthetic Pathway of *Saccharopolyspora spinosa*: Mechanistic and Evolutionary Implications for C-3 Deoxygenation in Deoxysugar Biosynthesis

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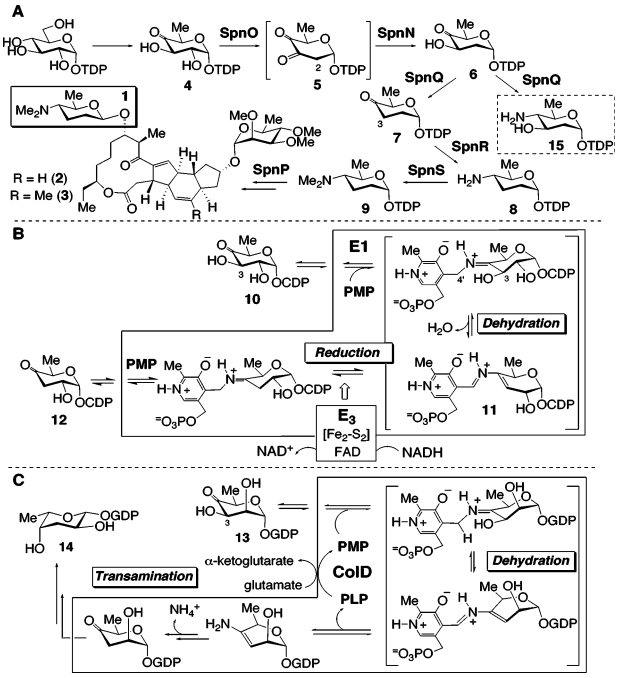
As part of our effort to characterize unusual sugar biosynthetic pathways and to collect genes useful for in vivo glycodiversification of secondary metabolites,[1] we investigated the biosynthesis of D-forosamine (**1**), a key structural component of the spinosyns, which are produced by *Saccharopolyspora spinosa*[2a] and *Saccharopolyspora pogona*.[2b] Spinosad, a mixture of Spinosyn A and D (**2**, **3**, Scheme 1A), is a potent yet environmentally benign insecticide.[2a] Structure−activity studies have shown that D-forosamine is essential for the insecticidal properties of the spinosyns.[3] There is also evidence that spinosad promotes wound healing in humans.[4]

The spinosyn (*spn*) biosynthetic gene cluster has been cloned and sequenced, and the genes *spnO*, *spnN*, *spnQ*, *spnR*, *spnS*, and *spnP* were proposed to be involved in forosamine biosynthesis and attachment.[2a] In a recent study, SpnR was shown to be a PLP-dependent aminotransferase catalyzing the conversion of **7** to **8**.[5] It is thus suggested that forosamine formation is initiated by C-2 deoxygenation, and likely proceeds via a sequence of (**4** → **5** → **6** → **7** → **8**, Scheme 1A). C-3 deoxygenation (**6** → **7**) is proposed to be mediated by SpnQ, which shows modest sequence identity to pyridoxamine 5′-monophosphate (PMP)-dependent hexose C-3 dehydrases E_1[6] and ColD[7] (49% and 26% identity, respectively). All three enzymes show moderate sequence identity to PLP-dependent aminotransferases.

Interestingly, E_1 and ColD have been shown to catalyze C-3 deoxygenation by related, yet distinct catalytic pathways. E_1, which has a [2Fe-2S] cluster in addition to PMP, catalyzes C-3 deoxygenation of **10** in the biosynthesis of D-ascarylose.[6,8] This reaction requires a specific [2Fe-2S]-flavoprotein reductase (E_3), which transfers two NADH-derived electron equivalents, via FAD and the [2Fe-2S] centers of E_3 and E_1,[9] to reduce the PMP-$\Delta^{3,4}$-glucoseen intermediate (**11**) formed in the active site of E_1 to give **12** and regenerate PMP (Scheme 1B).[10] ColD, which catalyzes the C-3 deoxygenation of **13** in the biosynthesis of L-colitose (**14**), on the other hand, lacks a [2Fe-2S] cluster, and does not require a reductase partner.[7] Instead, as shown in Scheme 1C, it behaves in an "aminotransferase-like" manner, regenerating the PMP cofactor using L-glutamate instead of employing a two-electron reduction as seen in the E_1/E_3 catalyzed deoxygenation. Thus, SpnQ could catalyze C-3 deoxygenation either in a similar manner to E_1 or to ColD. Because SpnQ shares higher sequence identity with E_1 than with ColD, and SpnQ also contains a [2Fe-2S] binding motif, we thought it would be like E_1, although we were puzzled by the apparent lack of an E_3 gene homologue in the *spn* cluster, normally seen closely linked to E_1 homologue-encoding genes.

To characterize SpnQ and to differentiate between the two mechanistic possibilities, the *spnQ* gene was cloned and the resulting construct was expressed in *E. coli* BL21(DE3) cells.[11] SpnQ was purified to near homogeneity using DEAE, hydroxy-apatite, and Sephacryl S-200 chromatography.[11] The as-purified SpnQ is a red-brown homodimeric protein and exhibits absorption features characteristic for PMP and an iron-sulfur cluster.[11]

Scheme 1



The predicted substrate of SpnQ, **6**, was prepared from **4** using SpnO and SpnN in the presence of NADPH.[12] To test whether SpnQ catalyzes C-3 deoxygenation in an E_1-like manner, SpnQ (30 μM) was incubated with 1 mM **6**, 60 μM E_3, 2 mM NADH, and 0.3 mM PMP in 50 mM potassium phosphate buffer (pH 7.5). HPLC analysis of the reaction mixture gave no indication of turnover of **6** under these conditions. Neither reconstitution of the iron-sulfur cluster nor use of the alternate electron-transfer mediators, FAD and FMN, made SpnQ catalytically active. These observations suggest that either SpnQ functions analogously to ColD, employing L-glutamate as a cosubstrate, or SpnQ behaves like E_1, but E_3 is not a suitable electron donor for SpnQ.

To test the first possibility, we assayed SpnQ (10 μM) in the presence of 1 mM **6**, 3 mM L-glutamate, and 250 μM PLP in 50 mM potassium phosphate buffer (pH 7.5). HPLC analysis of the incubation mixture revealed the time-dependent formation of a new product (52% conversion after 16 h at 37 °C), which was identified by MS and HPLC analysis[11] as the C-4 aminosugar **15** (Scheme 1A).[5] SpnQ is apparently capable of functioning nominally as a C-4 aminotransferase, catalyzing the conversion of **6** → **15** under these conditions. The inability of SpnQ to act as a C-3 dehydrase using L-glutamate, as occurs in the ColD reaction, suggests that a

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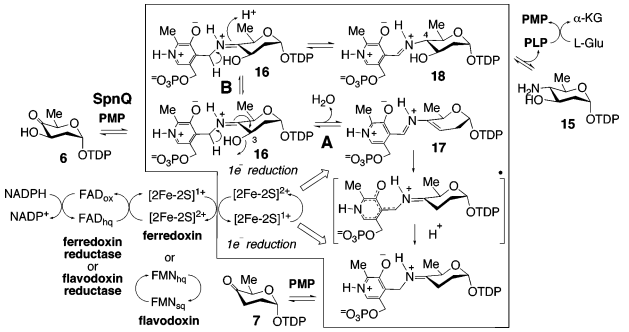
Scheme 2



two-electron reduction may be a requirement to drive the SpnQ-catalyzed C-3 deoxygenation. Hence, we examined the second possibility that a second electron donor might be needed.

Reasoning that sodium dithionite could likely serve as an electron source in lieu of a physiological reductase, incubation of a mixture of 30 μM SpnQ, 0.6 mM **6**, 250 μM PMP, and 0.6 mM sodium dithionite in 50 mM potassium phosphate buffer (pH 7.5) under anaerobic conditions was performed. To our delight, a new product was detected by HPLC analysis of the incubation mixture (70% yield after 16 h at 25 °C). This compound was later isolated from a large-scale incubation and was spectroscopically identified as **7**.[11] Observation of dithionite-dependent turnover strongly implicated **17** as an intermediate formed by C−O bond cleavage at C-3 of **16**, in SpnQ catalysis (Scheme 2, path A). Reduction by dithionite of **17**, likely in a stepwise two electron-transfer, led to the C-3 deoxygenated product. The [2Fe-2S] center of SpnQ is an indispensable part of the electron-transfer conduit, since SpnQ depleted of the iron-sulfur center was incapable of catalyzing C-3 deoxygenation.

Demonstration that SpnQ can catalyze C-3 deoxygenation of **6** using dithionite provided convincing support for its proposed function. However, the identity of the authentic SpnQ reductase remained unknown. Examination of a number of gene clusters containing SpnQ homologues failed to yield any gene products with homology to E_3 or other reductases of unknown function. This suggests that SpnQ and its homologues, unlike E_1, may not require a specific reductase partner, instead employing a generic cellular reductase for their reactions. Subsequent assays of SpnQ (30 μM) using reductase pairs ferredoxin/ferredoxin reductase (30 μM each) or flavodoxin/flavodoxin reductase (30 μM each) as electron donors in the presence of 4 mM NADPH, 150 μM PMP, and 0.7 mM **6** in 50 mM potassium phosphate buffer (pH 7.5) both resulted in efficient conversion of **6** to **7** (90% conversion after 2 h at 25 °C). Characterization of the product from a large-scale reaction by NMR spectroscopy confirmed it as **7**.[11]

Ferredoxin/ferredoxin reductase and flavodoxin/flavodoxin reductase are ubiquitous electron-transfer enzymes in the general cellular pool that deliver electron equivalents from NADPH to low potential one-electron acceptors.[13] In the SpnQ case, a similar electron relay path as in the E_1−E_3 reaction may be operative (see Scheme 2, path A). It should be noted that when E_1 was assayed in the presence of its natural substrate (**10**), ferredoxin/ferredoxin reductase, and NADPH, it was 5-fold less effective at C-3 deoxygenation than when assayed using E_3 under standard conditions (18% versus 100% conversion after 1.5 h at 25 °C). These results suggest that while E_1 has evolved to work with E_3, a cellular reductase, rather than a specific reductase as in the case of E_1, is likely the in vivo partner of SpnQ in catalyzing the C-3 deoxygenation reaction.

Interestingly, after observing SpnQ-catalyzed conversion of **6** to **15** using PLP and L-glutamate, we also assayed E_1 using its natural substrate **10** in the presence of L-glutamate and a catalytic amount of PLP and observed that it catalyzes C-3 deoxygenation rather than C-4 aminotransfer under these conditions, although 6-fold less efficiently than with E_3 and NADH (16% versus 100% conversion after 1.5 h at 25 °C). Thus, in the absence of reductase and using L-glutamate and their natural substrates, E_1 and SpnQ follow different reaction pathways. This is intriguing in that, although E_1 and SpnQ seem to catalyze mechanistically identical reactions in the presence of their respective reductase partners, SpnQ, and not E_1 or ColD, has retained an ancestral aminotransferase activity (Scheme 2, path B, **6** → **16** → **18** → **15**), whereas E_1 behaves like ColD, catalyzing the reductase-independent C-3 deoxygenation (Scheme 1C).

Overall, these results fully demonstrate the role of SpnQ in the biosynthesis of D-forosamine. The mechanism of the SpnQ-catalyzed reaction is clearly different from that of the ColD reaction, but closely resembles that of the E_1/E_3 reaction. The C-3 deoxygenation steps in the biosynthesis of other TDP-2,3,6-trideoxysugars, whose gene clusters seem to universally lack an E_3-like reductase gene, are likely to be catalyzed by SpnQ homologues employing reductases from the general cellular pool. In addition, SpnQ can act as a transaminase when the electron-transfer path is blocked. Interestingly, under the same conditions, both E_1 and ColD catalyze C-3 deoxygenation, pointing to a unique evolutionary pathway for SpnQ and other closely related TDP-2,6-deoxysugar 3-dehydrases. Future structural studies of E_1, ColD, and SpnQ will be invaluable in deciphering the subtle differences among the active site geometries of these enzymes which result in their unique reaction pathways and may provide further insight into the evolutionary paths by which each evolved.

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Supporting Information Available: Details of SpnQ purification, assay and HPLC conditions, and spectral data of **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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