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A Consensus Mechanism for Radical SAM-Dependent Dehydrogenation? BtrN Contains Two [4Fe-4S] Clusters†

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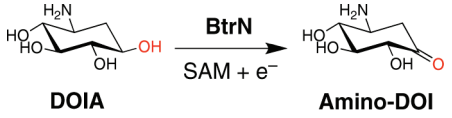
ABSTRACT: BtrN catalyzes the two-electron oxidation of the C3 secondary alcohol of 2-deoxy-*scyllo*-inosamine to the corresponding ketone and is a member of a subclass of radical *S*-adenosylmethionine (SAM) enzymes called radical SAM (RS) dehydrogenases. Like all RS enzymes, BtrN contains a [4Fe-4S] cluster that delivers an electron to SAM, inducing its cleavage to the common intermediate in RS reactions, the 5′-deoxyadenosyl 5′-radical. In this work, we show that BtrN contains an additional [4Fe-4S] cluster, thought to bind in contact with the substrate to facilitate loss of the second electron in the two-electron oxidation.

BtrN catalyzes the third step in the biosynthetic pathway of the 2-deoxystreptamine-containing aminoglycoside antibiotic butirosin B. The reaction involves a seemingly simple two-electron oxidation of the C3 secondary alcohol of 2-deoxy-*scyllo*-inosamine (DOIA)[1] to a ketone, affording amino-2-deoxy-*scyllo*-inosose (amino-DOI) (Scheme 1) (*1*). Interestingly, BtrN does not contain or employ any of the usual suspects that act as cofactors or cosubstrates in these types of oxidations, such as flavin-, pyridine-, or quinone-containing nucleotide metabolites. In fact, a thorough in vitro analysis of BtrN and its reaction indicates that the protein is a member of the radical *S*-adenosyl-L-methionine (SAM) superfamily, and that it catalyzes this two-electron oxidation via a radical mechanism (*1*, *2*).

Enzymes within the radical SAM (RS) superfamily use SAM as a precursor to a 5′-deoxyadenosyl 5′-radical (5′-dA•), which initiates turnover by abstracting a key hydrogen atom from the appropriate substrate (*3*, *4*). All known RS enzymes contain at least one [4Fe-4S] cluster ligated by three cysteinyl residues most often lying in a CxxxCxxC motif (*5*). SAM binds in a bidentate manner via its α-amino and α-carboxylate groups to the non-cysteinyl-coordinated Fe site of this cluster (*6*, *7*). In its reduced state, the $[4Fe\text{-}4S]^{+}$ cluster injects an electron into the sulfonium group of SAM, inducing its fragmentation into L-methionine and a 5′-dA• (*3*, *4*). Spectroscopic, biochemical, and analytical characterization of BtrN suggested that the protein contains one [4Fe-4S] cluster per polypeptide, and that the 5′-dA• initiates turnover by abstracting the hydrogen atom from C3 of the substrate (*1*, *2*). It was postulated that the remaining electron is returned to this $[4Fe\text{-}4S]^{2+}$ cluster concomitant with ketone formation to allow for subsequent rounds of SAM cleavage in the absence of additional reducing equivalents (*1*).

Scheme 1: Reaction Catalyzed by BtrN



DOIA **Amino-DOI**

The reaction catalyzed by BtrN is similar to those of the RS-dependent sulfatase maturation enzymes, which catalyze the oxidation of a seryl or cysteinyl residue to a formylglycyl (FGly) residue on a cognate protein (*8*−*11*). This FGly residue is then used as a cofactor in the hydrolysis of organosulfate monoesters (*12*). Characterization of AtsB, a RS-dependent sulfatase maturation enzyme, showed that it contains three [4Fe-4S] clusters, only one of which is housed in the CxxxCxxC motif (*10*). A working hypothesis was advanced in which one of the two remaining clusters is coordinated by the target seryl or cysteinyl residue of the substrate to facilitate loss of the second electron from a radical-containing substrate intermediate via inner-sphere electron transfer to afford the oxidized product (*10*). The finding that BtrN contains only one [4Fe-4S] cluster (*1*) would suggest that this mechanism is not operative, or that it is not universally conserved among RS dehydrogenases. However, a close inspection of the primary structure of the protein shows that it contains eight Cys residues, a quantity sufficient to coordinate two [4Fe-4S] clusters. In this work, we demonstrate using a combination of spectroscopic and analytical methods that BtrN harbors two [4Fe-4S] clusters and is therefore in accord with the mechanism that we proposed for RS-dependent dehydrogenation, specifically for the reaction catalyzed by AtsB (*10*). The results suggest that the presence of at least two [4Fe-4S] clusters is a general feature of RS dehydrogenases.

BtrN containing a C-terminal hexahistidine tag was purified by immobilized metal affinity chromatography under anaerobic conditions as described in Supporting Information. It displays a brown color, and its UV−vis spectrum is consistent with that of an iron−sulfur (Fe−S) protein (Figure S1 and S8 of Supporting Information) and similar to that previously published (*2*). Analysis of the protein for Fe and S^{2-} reveals that it contains 4.8 ± 0.4 equiv of the former and 6.1 ± 0.3 equiv of the latter per polypeptide (Table S3 of Supporting Information). The 4.2-K/53-mT Mössbauer spectrum of as-isolated (AI), hexahistidine-tagged,

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[1]Abbreviations: AI, as-isolated; 5′-dA, 5′-deoxyadenosine; 5′-dA•, 5′-deoxyadenosyl 5′-radical; amino-DOI, 2-deoxy-*scyllo*-inosose; DOIA, 2-deoxy-*scyllo*-inosamine; DOS, 2-deoxystreptamine; RCN, reconstituted; RS, radical SAM; SAM, *S*-adenosyl-L-methionine; wt, wild-type.

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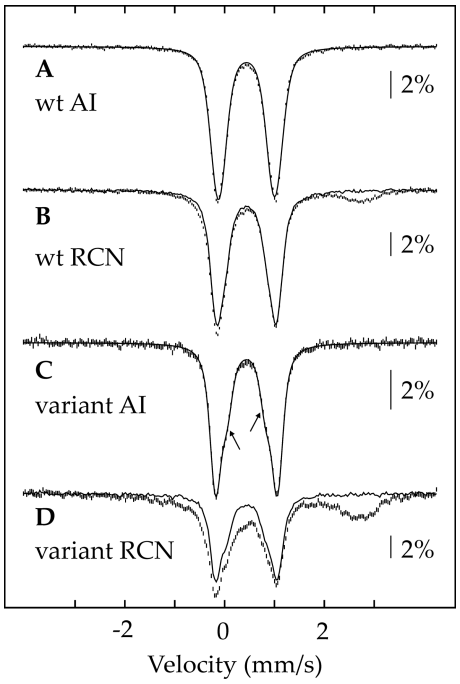


FIGURE 1: 4.2-K/53-mT Mössbauer spectra of wt BtrN (A and B) and C16A/C20A/C23A BtrN (C and D). Spectra A and C are for the as-isolated forms, and spectra B and D are for the reconstituted forms. The solid lines in spectra A and C are quadrupole doublet simulations with parameters quoted in the text. The solid lines in spectra B and D are the spectra of the corresponding AI forms, scaled to 87 and 57% of the total intensity, respectively.

wild-type (wt) BtrN is shown in Figure 1A as vertical bars; 98% of its intensity can be simulated to a quadrupole doublet with parameters [isomer shift (δ) of 0.44 mm/s and quadrupole splitting parameter (ΔE_Q) of 1.13 mm/s] typical of $[4Fe\text{-}4S]^{2+}$ clusters. In addition, the electron paramagnetic resonance (EPR) spectrum of this sample did not reveal any signals. These observations, in combination with Fe and protein analyses (4.8 Fe ions per BtrN), demonstrate that AI wt BtrN harbors 1.2 $[4Fe\text{-}4S]^{2+}$ clusters. When AI wt BtrN is further reconstituted with iron and sulfide (RCN wt BtrN), it is found to contain 7.7 ± 0.1 equiv of the former and 8.8 ± 0.8 equiv of the latter per polypeptide (Table S3 of Supporting Information). The corresponding Mössbauer spectrum (Figure 1B, vertical bars) reveals a large fraction of total Fe (87%) that is identical to the spectrum of AI wt BtrN (solid line in Figure 1B). Given that RCN wt BtrN contains 7.7 Fe ions per polypeptide, analysis by Mössbauer spectroscopy indicates the presence of 1.7 $[4Fe\text{-}4S]^{2+}$ clusters per polypeptide. Thus, reconstitution increases the amount of $[4Fe\text{-}4S]^{2+}$ clusters significantly (1.4 fold), suggesting that BtrN harbors two [4Fe-4S] clusters.

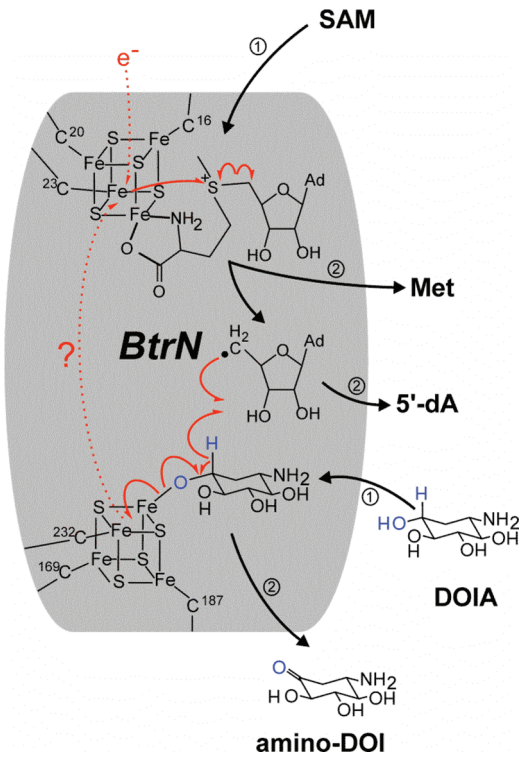
To quantify cluster content more rigorously, a triple variant, in which the cysteinyl residues of the canonical radical SAM motif (C16, C20, and C23) were replaced with alanyl residues, was constructed and analyzed. The AI triple variant contains 3.7 ± 0.1 Fe ions and 4.6 ± 0.3 S^{2-} ions per polypeptide, numbers slightly lower than those observed for the wt protein (Table S3 of Supporting Information). The 4.2-K/53-mT Mössbauer spectrum of the AI triple variant (Figure 1C) is similar, albeit not identical, to that of AI wt BtrN, because it contains pronounced shoulders, which are indicated by the arrows. The spectrum can be analyzed with two quadrupole doublets [δ_1 = 0.44 mm/s, and $\Delta E_{Q,1}$ = 1.24 mm/s (70% of the total intensity); δ_2 = 0.44 mm/s, and $\Delta E_{Q,2}$ = 0.78 mm/s (27% of the total intensity)]. In conjunction with the finding of 3.7 Fe ions per polypeptide, we conclude that the AI BtrN C16A/C20A/C23A triple variant harbors 0.9 $[4Fe\text{-}4S]^{2+}$ cluster. Reconstitution of this protein with additional iron and sulfide results in uptake of additional iron (6.2 Fe ions per polypeptide), but the associated 4.2-K/53-mT Mössbauer spectrum (Figure 1D, vertical bars) demonstrates that only ~57% of the total intensity is attributable to $[4Fe\text{-}4S]^{2+}$ clusters (solid line), which corresponds to a stoichiometry of 0.9 $[4Fe\text{-}4S]^{2+}$ cluster per triple variant. The remainder of the spectrum is attributed to adventitiously bound iron, because an identical EPR sample did not reveal the presence of [Fe-S] clusters with $S = {}^1/_2$ ground states. Collectively, these results indicate that BtrN harbors two [4Fe-4S] clusters.

The model proposed for radical SAM dehydrogenation indicates that at least two [4Fe-4S] clusters are coordinated by only three Cys ligands, which would present open coordination sites for binding of SAM to one cluster and the appropriate substrate (DOIA in the case of BtrN) to the other. To determine which cysteines in BtrN act as ligands to this second [Fe-S] cluster, single C → A variants of the five cysteinyl residues outside of the CxxxCxxC motif were constructed with the intention of assessing the ability of each to catalyze the reaction. Three of the variants (C169A, C187A, and C232A) were produced as insoluble aggregates and were not analyzed further. The two remaining variants (C69A and C235A) were soluble and displayed UV−vis spectra (Figures S3 and S4 of Supporting Information) and quantities of Fe and S^{2-} (Table S3 of Supporting Information) that are consistent with the presence of [Fe-S] clusters.

As shown in Table S3 of Supporting Information, wt RCN BtrN displays significant SAM reductase activity (3.7 min^{-1}), while the formation of DOS occurs with a rate constant of 0.22 min^{-1}, similar to that reported by Yokoyama et al. (0.2 min^{-1}) for assays in which the production of DOS was monitored (*1*). As expected, neither SAM reductase activity nor DOIA oxidation was detected for the RCN C16A/C20A/C23A triple variant. The BtrN RCN C69A variant behaved like wt BtrN, while the RCN C235A variant displayed SAM reductase and substrate turnover activities that were 7 and 9%, respectively, of those displayed by wt hexahistidine-tagged BtrN. A subsequent independent isolation and recharacterization of wt BtrN produced similar results (Figure S8 of Supporting Information). The AI and RCN proteins exhibited rate constants for formation of DOS of 0.34 and 0.45 min^{-1}, respectively, showing that reconstitution enhances activity.

The finding that C → A substitutions at three of the five cysteinyl residues outside of the CxxxCxxC motif (C169, C187, and C232) afford insoluble proteins, while C → A substitutions at the remaining two afford active proteins, suggests that the second [Fe-S] cluster that we detect is ligated by C169, C187, and C232. Moreover, this finding is consistent with a model in which an open coordination site on the second cluster allows the substrate C3 hydroxyl group to coordinate to the cluster (Scheme 2). This cluster coordination should facilitate loss of the proton of the C3 hydroxyl group, hydrogen atom abstraction at C3 by the 5′-dA•, and inner-sphere electron transfer from the ensuing substrate radical to the $[4Fe\text{-}4S]^{2+}$ cluster to afford the product amino-DOI. It is conceivable that the electron transferred upon generation of amino-DOI to the second [Fe-S] cluster is subsequently transferred to the RS [4Fe-4S] cluster, which would allow the latter to cleave SAM reductively and generate another 5′-dA• for

Scheme 2: Proposed Mechanism for BtrN[a]



[a]Binding of (co)substrate and dissociation of (co)products are labeled with ① and ②, respectively. Electron flow is indicated by red arrows.

the next reaction cycle, which was originally proposed by Yokoyama et al. (*1*).

Evidence of the role of the second cluster as an electron acceptor is provided by EPR analysis of the reaction. When RCN BtrN is reduced by treatment with dithionite, it displays a strong axial EPR spectrum having the following **g**-tensor values: $g_{\|} = 2.04$ and $g_{\perp} = 1.92$, similar to those of other radical SAM proteins, as well as those previously published (Figure S5 of Supporting Information, red trace) (*2*). The addition of SAM perturbs the spectrum, resulting in significant broadening, (Figure S5 of Supporting Information, black trace). Remarkably, under turnover conditions, a completely different axial EPR spectrum is observed, exhibiting the following **g**-tensor values: $g_{\|} = 1.83$ and $g_{\perp} = 1.99$ (Figure S6 of Supporting Information, black trace). This spectrum is unlike any other observed for radical SAM proteins; although it is axial, $g_{\|}$ is now smaller than $g_{\perp}$. This spectrum is only generated as a result of turnover; neither dithionite nor titanium citrate was able to support reduction of the second cluster. As shown in Figure S7 of Supporting Information (black trace), upon treatment of the RCN C16A/C20A/C23A triple variant with dithionite, the protein remained EPR-silent. These observations suggest that the second [4Fe-4S] cluster exhibits a very low redox potential or is inaccessible to the reductant. A second cluster with a very low redox potential would be consistent with the mechanism shown in Scheme 2, which necessitates that the radical SAM [4Fe-4S] cluster be preferentially reduced to prevent blockage of turnover because of the lack of an electron acceptor. Furthermore, the ensuing ketyl radical would be expected to exhibit a redox potential on the order of −2.2 V, observed for the ketyl radical of cyclohexanone (*2*, *13*).

It must be mentioned that these EPR findings differ slightly from those of Yokoyama et al., who reported that the broad spectrum exhibiting **g**-tensor values of $g_{\|} = 1.83$ and $g_{\perp} = 1.99$ derives from the reduced enzyme−SAM complex (*2*). In this same study, they also reported that their reconstituted enzyme contained 4.0 ± 0.1 iron ions per monomer subsequent to treatment with EDTA and gel filtration, leading them to report that the enzyme contained one [4Fe-4S] cluster. Interestingly, they further reported that the EDTA-treated enzyme was just as active as the reconstituted enzyme that was not treated with EDTA. At present, we have no explanation for these contrasting results; however, it is clear from the spectroscopic and biochemical studies reported herein that BtrN contains *two* [4Fe-4S] clusters rather than one and that the model originally proposed for the RS enzyme AtsB may in fact be a common mechanistic feature of RS dehydrogenases.

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SUPPORTING INFORMATION AVAILABLE

Materials and methods, UV−visible spectra of wild-type and variant forms of BtrN, relevant EPR spectra, and sequence of the optimized *btrN* gene. This material is available free of charge via the Internet at http://pubs.acs.org.

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