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Electrochemical Resolution of the [4Fe-4S] Centers of the AdoMet Radical Enzyme BtrN: Evidence of Proton Coupling and an Unusual, Low-Potential Auxiliary Cluster

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Supporting Information

ABSTRACT: The *S*-adenosylmethionine (AdoMet) radical superfamily of enzymes includes over 113 500 unique members, each of which contains one indispensable iron−sulfur (FeS) cluster that is required to generate a 5′-deoxyadenosyl 5′-radical intermediate during catalysis. Enzymes within several subgroups of the superfamily, however, have been found to contain one or more additional FeS clusters. While these additional clusters are absolutely essential for enzyme activity, their exact roles in the function and/or mechanism of action of many of the enzymes are at best speculative, indicating a need to develop methods to characterize and study these clusters in more detail. Here, BtrN, an AdoMet radical dehydrogenase that catalyzes the two-electron oxidation of 2-deoxy-*scyllo*-inosamine to amino-dideoxy-*scyllo*-inosose, an intermediate in the biosynthesis of 2-deoxystreptamine antibiotics, is examined through direct electrochemistry, where the potential of both its AdoMet radical and auxiliary [4Fe-4S] clusters can be measured simultaneously. We find that the AdoMet radical cluster exhibits a midpoint potential of −510 mV, while the auxiliary cluster exhibits a midpoint potential of −765 mV, to our knowledge the lowest $[4Fe\text{-}4S]^{2+/+}$ potential to be determined to date. The impact of AdoMet binding and the pH dependence of catalysis are also quantitatively observed. These data show that direct electrochemical methods can be used to further elucidate the chemistry of the burgeoning AdoMet radical superfamily in the future.

The *S*-adenosylmethionine (AdoMet) radical enzyme superfamily catalyzes a diverse and stunning array of unusual biological transformations, including key steps in the biosynthesis and repair of DNA, the biosynthesis of antibiotics and other medicinally relevant natural products, and the biosynthesis of enzyme cofactors.[1−8] All enzymes in this superfamily contain at least one[9] [4Fe-4S] cluster in which three of its four iron ions are coordinated by cysteine residues typically found in a CxxxCxxC motif. The fourth iron ion has available coordination sites to bind to the α-amino and α-carboxylate groups of AdoMet in a bidentate manner. AdoMet is then reductively cleaved to generate a 5′-deoxyadenosyl 5′-radical (5′-dA$^{\bullet}$) and methionine,[10,11] where the 5′-dA$^{\bullet}$ is responsible for initiating subsequent chemistry, most often by abstracting a substrate hydrogen atom. While the discovery of novel AdoMet radical enzyme reactions is occurring at a rapid pace,[12−15] a major bottleneck in elucidating the mechanistic details of many of the reactions is the inability to assess and rationalize the redox potentials of the iron−sulfur (FeS) clusters and other cofactors/substrates of the enzymes systematically.[2] Further complexity arises as emerging families of AdoMet radical enzymes contain either one or two additional FeS clusters housed in so-called TWITCH or SPASM domains,[3] where the roles played by the additional clusters, including electron transfer, are unclear.[12,14,16−18] Here we report the direct measurement of the redox potentials of an AdoMet radical enzyme possessing multiple FeS clusters for the first time, as well as elucidate the impact of ligand-binding and protonation.

To achieve the electrochemical resolution of the FeS clusters of an AdoMet radical enzyme, we chose to examine an AdoMet radical dehydrogenase, which catalyzes the two-electron oxidation of a substrate via two sequential one-electron oxidations. Our model AdoMet radical dehydrogenase is BtrN from *Bacillus circulans* (Figure 1), a 28.5 kDa protein that catalyzes the third step in the biosynthesis of the antibiotic, butirosin B. The reaction of BtrN (Figure 1A), is the two electron oxidation of the C3 hydroxyl group of 2-deoxy-*scyllo*-inosamine (DOIA) to a ketone, affording 3-amino-2,3-dideoxy-*scyllo*-inosose (amino-DOI). BtrN contains two [4Fe-4S] clusters. One is ligated by three cysteines residing in the canonical CxxxCxxC motif, while the auxiliary cluster is ligated by cysteines residing in a TWITCH domain, a newly discovered structural motif used by some AdoMet radical enzymes.[3,14] X-ray crystallographic studies on BtrN indicate that the auxiliary cluster is ligated by four cysteine residues and is 15.8 Å away from the nearest ion of the AdoMet radical cluster and 9.6 Å away from C3 of the substrate, the site of the radical intermediate. While the function and redox properties of the auxiliary cluster have been unknown to date, its location close to the surface of the protein suggests that it might interact with an exogenous electron acceptor upon being reduced by a radical intermediate. Yet, given that the putative substrate radical is at a similar distance (8.6 Å) to the AdoMet radical

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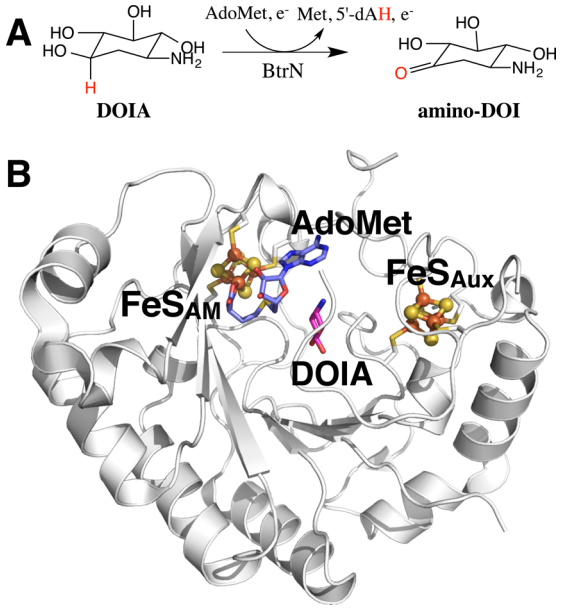

Figure 1. (A) Reaction scheme and (B) structure of BtrN, showing the disposition of the active-site [4Fe-4S] cluster with AdoMet bound in slate (FeS_{AM}), approximately 16 Å removed from the auxiliary [4Fe-4S] cluster (FeS_{Aux}). Substrate DOIA is shown bound between the clusters, in pink. Constructed in PyMol, using 4M7T.pdb.

cluster, it is unclear to which cluster the electron is transferred. Further, electron paramagnetic resonance (EPR) studies of BtrN indicate that the AdoMet radical [4Fe-4S] cluster is readily reduced to the +1 oxidation state upon incubation of the protein with dithionite, while the auxiliary cluster does not become reduced under similar conditions.[19]

In comparing BtrN to other AdoMet radical enzymes, we note that prior efforts to determine the redox potentials of the AdoMet radical [4Fe-4S] cluster of biotin synthase,[8] lysine 2,3-aminomutase (LAM),[20,21] and MiaB[22] have resulted in midpoint potentials (E_m) of −479 to −505 mV, yet such potentials are far removed from estimates of the AdoMet potential of −1.8 V.[23] To address these open questions regarding BtrN redox chemistry and the AdoMet radical superfamily, we have used protein film electrochemistry (PFE) to resolve the multiple redox potentials of an AdoMet radical enzyme for the first time. We find that the auxiliary cluster exhibits the lowest redox potential determined for a [4Fe-4S]$^{2+/+}$ couple to date.

PFE of BtrN non-covalently adsorbed upon pyrolytic graphite edge electrodes yields two peaks in the cyclic voltammogram: at pH 8, one set of voltammetric features is centered at −765 mV and the other at −510 mV (Figure 2A). To assign the peaks to their corresponding clusters, PFE was performed using the Cys-16,20,23-Ala triple variant of BtrN, which eliminates the AdoMet radical cluster. Figure 2B shows the resulting voltammogram, which displays only one peak at −765 mV. These results are in agreement with a previous EPR study, which showed that the AdoMet radical cluster could be reduced but the auxiliary cluster was too low in potential to be reduced by dithionite or titanium citrate.[17] The midpoint potential of −510 mV for the AdoMet radical cluster is in fairly close agreement to the value of −484 mV reported for LAM with cysteine bound to the open coordination site of the cluster.[20] For the cleavage of AdoMet, the AdoMet radical cluster must first be reduced; therefore, the higher reduction

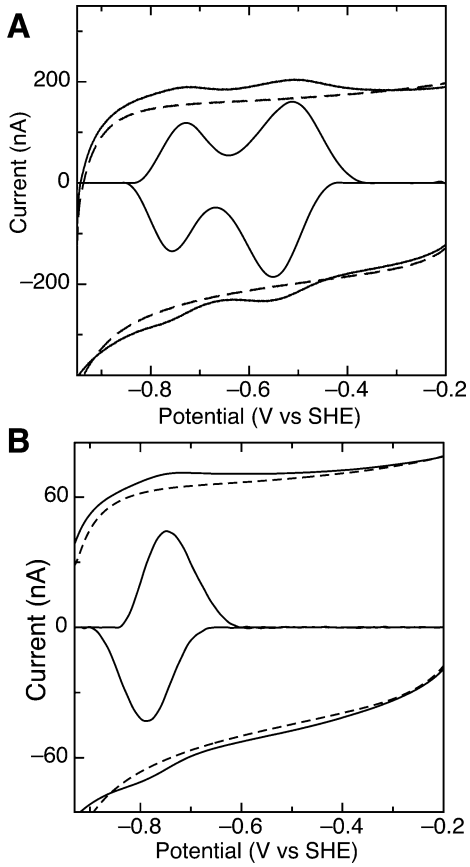

Figure 2. (A) PFE response of BtrN at a pyrolytic graphite electrode at pH 8, with the background electrode response (dashed) and corrected for the non-Farradaic response (inset). (B) Similar PFE analysis of the BtrN triple variant devoid of the AdoMet radical FeS cluster.

potential of the AdoMet radical cluster ensures that it will be preferentially reduced.

The potential of the auxiliary cluster is unusually low for the +2/+1 couple of a [4Fe-4S] cluster. While we can now systematically investigate the impact of the protein environment upon auxiliary cluster potential, we can note that the auxiliary cluster is surrounded by many bulky, hydrophobic residues, which may prevent solvent accessibility to the cluster and therefore, aid in achieving the low potential.[24] Additionally, there is an arginine residue (Arg226) that is oriented toward a sulfur atom of the cluster (Supporting Information, Figure S1), which may assist in stabilizing the negative charge on the auxiliary cluster following reduction.

In terms of the potential for proton-coupling for redox reactions at the FeS clusters of BtrN, previous studies on LAM and biotin synthase both suggested that the reduction potential of the AdoMet cluster is pH invariant.[20,21] In the present study, the midpoint potential of the auxiliary and AdoMet radical clusters of BtrN were monitored over a pH range of 4 to 10. Figure 3 displays the observed values of E_m for both clusters. The AdoMet radical cluster displays a pH dependence consistent with that expected for a one-proton/one-electron process (−56 mV/pH unit at 10 °C),[25] with pK_{ox} = 6.3 and pK_{red} = 8.9, indicating that there is a proton transfer coupled to the electron transfer. This is the first report to associate a proton transfer with the electron transfer of the AdoMet radical cluster. With respect to the possible protonation sites in close

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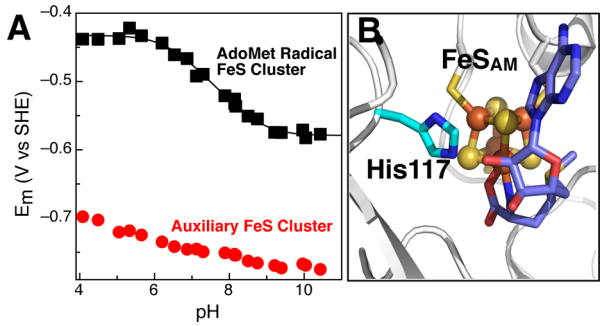

Figure 3. (A) pH dependence of reduction potentials of BtrN FeS clusters. The higher potential AdoMet radical cluster (black squares) shows clear $1H^+$:$1e^-$ stoichiometry. The auxiliary cluster (red circles) shows a nominal pH dependence. Fitting of the AdoMet radical cluster pH dependence is described in the Supporting Information. (B) Environment of the AdoMet radical cluster with AdoMet bound, in slate, depicting the conserved His117 residue in cyan.

proximity to the AdoMet radical cluster, histidine (His117) is within hydrogen-bonding distance of a sulfide of the AdoMet cluster (Figure 3B), and is widely conserved in BtrN orthologs or substituted by a Tyr residue. We are now in the position to further assess the impact of proton transfers at the active site across the AdoMet radical enzyme superfamily. In contrast, the auxiliary cluster displays an overall modest −12 mV/pH unit dependence, much less than the expected value for a one-proton/one-electron process, suggesting that the redox couple of the auxiliary FeS cluster is not coupled to protons directly.

The impact of AdoMet binding upon the redox potentials of the FeS clusters of BtrN is readily achieved through protein electrochemistry as well. In the presence of AdoMet, the redox couples still behave as simple electron-transfer processes, as indicated by reversible cyclic voltammetry signals. Figure 4A

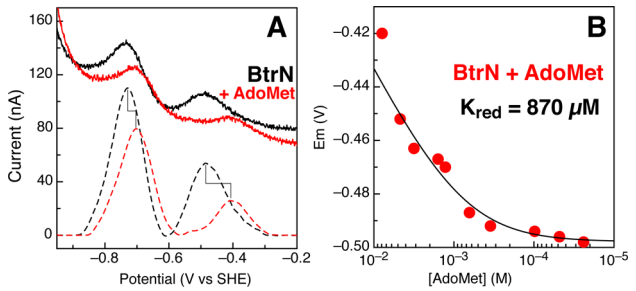

Figure 4. (A) Square wave voltammetry demonstrating the impact of AdoMet binding to BtrN FeS clusters where the raw data (solid) and baseline-corrected data (dashed lines) are shown for BtrN in the absence (black) and presence (red) of 7.9 mM AdoMet. (B) Changes observed for the reduction of the AdoMet radical cluster as a function of the AdoMet concentration (circles). These data were fit (line) using eq 2 (Supporting Information), and were collected with frequency = 10 Hz, E_{amp} = 20 mV, pH 8, and 10 °C.

shows the impact of AdoMet binding by square wave voltammetry, which improves the sensitivity of the detection. Upon addition of AdoMet to the cell solution, the reduction potential of the AdoMet radical cluster shifts by +80 mV to a more positive potential (Figure 4A). Here, complete saturation of the AdoMet radical cluster could not be achieved before the protein film became destabilized. However, an association constant of AdoMet and the reduced form of BtrN can be observed with K_{red}^{AdoMet} = 870 μM. K_{ox}^{AdoMet} is estimated to be >10 mM. Thus, binding of AdoMet is tighter to the reduced state. Further, an overall positive shift in redox potential is consistent with the trend observed with LAM (+50 mV)[20] and is likely to ensure that the cluster can be reduced *in vivo* by biological reducing systems.[23] Intriguingly, the auxiliary cluster also shifts to a more positive potential, though only by ∼+20 mV. When considering the impact of products, the commercial unavailability of amino-DOI challenges a similar analysis. However, in the presence of the products of the cleavage reaction (5′-dA and methionine), and including the substrate (DOIA, as a surrogate for the true product), the AdoMet radical cluster potential displays a negative shift of −25 mV to lower potential, while the potential of the auxiliary cluster is unchanged (Supporting Information, Figure S2). Thus, the AdoMet radical cluster becomes yet harder to reduce until products are removed. This flexibility in terms of potential further demonstrates the ability of the AdoMet radical cluster to modulate its potential throughout the catalytic cycle.

Here we have directly measured the midpoint potentials of the auxiliary and AdoMet radical clusters of BtrN using direct electrochemistry. These data shed light on a potential mechanism for BtrN and other AdoMet radical dehydrogenases (shown in Supporting Information, Scheme S1), suggesting a potential role for the auxiliary FeS cluster: when AdoMet and DOIA are bound, reduction of the active site cluster in a pH dependent fashion allows for reductive cleavage of AdoMet, generating 5′-dA$^\bullet$, which can then abstract a hydrogen atom from the DOIA substrate forming DOIA$^\bullet$. The DOIA$^\bullet$ radical has an estimated potential of −1.6 V, indicating that it could easily reduce either the auxiliary cluster or the AdoMet radical cluster itself. In the former case, the reduced auxiliary cluster would in turn re-reduce the active site cluster, following loss of the amino-DOI product, and 5′-dA and Met co-products (Figure S1). While the reduction of the active site cluster by the auxiliary cluster would require electron transfer over a distance of ∼16 Å, we have shown that the driving force for that process would be enhanced by AdoMet binding for a second round of catalysis. Should DOIA$^\bullet$ reduce the active site itself, the role of auxiliary cluster is less clear, though it may serve a protective role for turnovers where either 5′-dA$^\bullet$ or DOIA$^\bullet$ cannot complete a catalytic cycle. A final mechanistic possibility is that DOIA$^\bullet$ may reduce the auxiliary cluster, which in turn passes the electron to an exogenous redox partner. In such a case, we would anticipate that in the presence of DOIA and AdoMet, a reversible oxidative catalytic current could be observed in a PFE voltammetric analysis due to DOIA oxidation; however, we have not found such limiting currents here.

Given the possibility to achieve electrochemistry on members of the AdoMet radical superfamily, the long-term possibilities are exciting. Here the two potentials and the pH dependencies of the BtrN [4Fe-4S] clusters have been resolved, paving the way for future comparisons of AdoMet radical superfamily members bearing multiple FeS clusters.

■ ASSOCIATED CONTENT

Supporting Information

Complete description of the materials and methods used, equations used for fitting the pH dependencies, the environment of the auxiliary cluster, and a proposed reaction mechanism. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b03384.

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Notes
The authors declare no competing financial interest.

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