

Deconvoluting the Reduction Potentials for the Three [4Fe-4S] Clusters in an AdoMet Radical SCIFF Maturase

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S Supporting Information

ABSTRACT: Enzymes in the *S*-adenosyl-L-methionine (AdoMet) radical enzyme superfamily are metalloenzymes that catalyze a wide variety of complex radical-mediated transformations with the aid of a [4Fe-4S] cluster, which is required for activation of AdoMet to generate the 5'-deoxyadenosyl radical to initiate the catalytic cycle. In addition to this cluster, some enzymes share an additional domain, the SPASM domain, that houses auxiliary FeS clusters whose functional significance is not clearly understood. The AdoMet radical enzyme Tte1186, which catalyzes a thioether cross-link in a cysteine rich peptide (SCIFF), has two auxiliary [4Fe-4S] clusters within a SPASM domain that are required for enzymatic activity but not for the generation of the 5'-deoxyadenosyl radical intermediate. Here we demonstrate the ability to measure independently the midpoint potentials of each of the three [4Fe-4S] clusters by employing Tte1186 variants for which only the first, second, or AdoMet binding cluster is bound. This allows, for the first time, assignment of reduction potentials for all clusters in an AdoMet radical enzyme with a SPASM domain. Our results show that the clusters have midpoint potentials that are within 100 mV of each other, suggesting that their electrochemical properties are not greatly influenced by the presence of the nearby clusters.

The *S*-adenosyl-L-methionine (AdoMet) radical enzyme superfamily has >300000 distinct members that carry out a plethora of diverse chemical transformations.¹ Most of the enzymes in this superfamily possess a characteristic CxxxCxxC motif housing a site-differentiated [4Fe-4S] cluster [reaction cluster (RC)] that in turn binds AdoMet at a unique iron. In the +1 oxidation state, the RC reductively cleaves AdoMet to form the 5'-deoxyadenosyl radical intermediate (5'-dA[•]).^{2,3} The highly reactive 5'-dA[•] is capable of performing a diverse array of chemical reactions, commonly initiating catalysis via H atom abstraction. While only a single [4Fe-4S] cluster is necessary to generate this radical intermediate, which serves as the unifying first step among the AdoMet radical superfamily, many of these enzymes have additional FeS clusters whose functions have been largely elusive, with the exception of BioB and LipA, where the auxiliary cluster is sacrificed as a source of S.^{4,5}

Previous studies of the redox properties of AdoMet radical enzymes (AREs) containing two [4Fe-4S] clusters have shown

diverse electrochemical properties. For the dehydrogenase enzyme BtrN, the auxiliary cluster has the lowest determined reduction potential for a [4Fe-4S]^{2+/+} cluster, nearly 300 mV lower than that of the AdoMet binding cluster.⁶ Conversely, for methylthiotransferases (MTTases), the auxiliary cluster has an initial reduction potential that is in fact higher than that of the AdoMet cluster, which is in turn decreased by installation of a thiomethyl group.⁷ While initial studies of the redox properties have been performed for AdoMet radical enzymes with a single auxiliary cluster, those with multiple auxiliary clusters have yet to be characterized in a similar fashion.

It is challenging to predict the properties of the auxiliary clusters (ACs) of the AdoMet radical superfamily: while the RC resides in either a full or partial TIM barrel fold,⁸ additional FeS clusters can be found in various N- or C-terminal domain extensions of cryptic function.⁹ One of these domains is known as the SPASM domain, a C-terminal extension with a characteristic seven-cysteine motif of CX₉₋₁₅GX₄C-X_nCX₂CX₅CX₃C-X_nC that is known to typically bind two [4Fe-4S] clusters, which can be observed in the crystal structure of CteB, which is a thioether cross-linking SPASM domain-containing protein (Figure 1).¹⁰⁻¹³ The

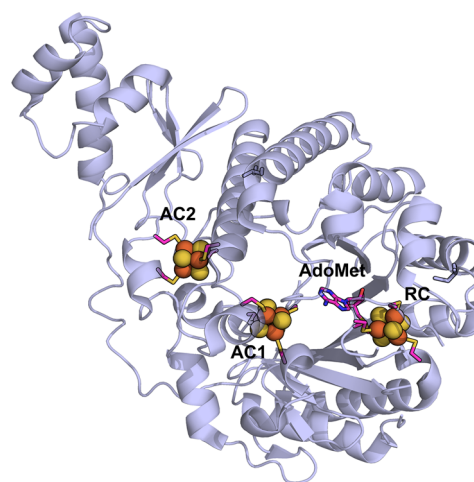


Figure 1. Crystal structure of SPASM domain-containing AdoMet radical enzyme CteB (Protein Data Bank entry SWGG) with AdoMet bound, illustrating the relative placement of the three FeS clusters.

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SPASM domain was named for its founding members Alba, PqqE, anSME, and MftC, which are involved in the maturation of subtilisin A, pyrroloquinoline quinone, anaerobic sulfatase, and mycofactacin, respectively. To date, all characterized members of this family carry out post-translational modifications. With the exception of anSME, all of these enzymes modify short ribosomally synthesized peptides that are termed ribosomally synthesized and post-translationally modified peptides (RiPPs).¹⁴ The biological and structural diversity in RiPPs, which is introduced by the corresponding maturases, has garnered significant attention.^{15,16} In many genomes, RiPP precursor peptides cluster with genes encoding other enzymes, such as AdoMet radical enzymes, which can make various modifications to the peptide. The co-clustering of the AdoMet radical enzyme and the peptide makes it relatively easy to identify these RiPP maturases bioinformatically.

By employing bioinformatics, the ARE Tte1186 from *Caldanaerobacter subterraneus* was previously identified¹⁶ and shown to modify a peptide, Tte1186a, encoded in the same gene cluster.¹⁷ Tte1186 creates a cross-link on Tte1186a, which has six cysteines in forty-five (SCIFF) residues, and is presumed to contain a sulfur–carbon thioether linkage. Tte1186 has been shown biochemically and spectroscopically to harbor three [4Fe-4S] clusters, all of which are required for activity. Only the canonical RC cluster is capable of binding and activating AdoMet to form 5'-dA[•]. The other two FeS clusters, auxiliary cluster 1 (AC1) and auxiliary cluster 2 (AC2), are located in the cysteine rich SPASM domain. AC1 and AC2 are required for the formation of the thioether cross-link found in the Tte1186 product but not for formation of the 5'-dA[•] intermediate. Previous work has clearly shown that the removal of the two auxiliary clusters does not impact the ability of the RC cluster to form the 5'-dA[•] intermediate, which has not always been shown to be the case in other AdoMet radical enzymes.^{17,18} In an effort to better understand the role the FeS clusters play in the SPASM domain, the reduction potentials of the three [4Fe-4S] clusters from the enzyme Tte1186 were characterized using protein film voltammetry (PFV). PFV allows for the direct measurement of the reduction potentials for each of the FeS clusters within the AdoMet radical enzymes without the need for external mediators.¹⁹

The wild-type Tte1186 enzyme film was generated by directly depositing the enzyme (see the [Supporting Information](#); 4 μ L, >0.5 mM) on an edge-plane graphite (EPG) electrode modified with multiwalled carbon nanotubes (MWCNTs)²⁰ and allowing it to incubate at room temperature for \sim 4 min before gently rinsing off excess protein. Upon generation of the stable protein film directly adsorbed to the graphite electrode surface, the reduction potentials of the three [4Fe-4S] clusters were measured using cyclic voltammetry (CV) at 4 $^{\circ}$ C in a multicomponent buffer at pH 7.5 with a scan rate of 50 mV/s. A single feature in the oxidative and reductive wave centered at -540 mV versus SHE could be observed in the cyclic voltammogram resulting from wild-type Tte1186 (Figure 2), suggesting that the three FeS clusters have reduction potentials very close to one another thus appearing as an envelope signal. Three one-electron transfers with reduction potentials of -490 , -540 , and -585 mV versus SHE were successfully fit to the wild-type envelope signal using the software QSoas with the number of electrons transferred all being equal to approximately unity for each subfunction and the surface coverage of all three redox active species being equal.²¹ Therefore, each reduction potential in turn represents

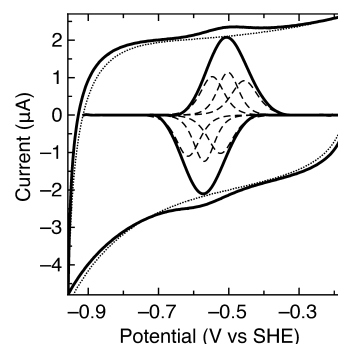


Figure 2. Cyclic voltammogram of wild-type Tte1186 on an EPG electrode modified with MWCNT and deconvoluted to three one-electron transfers using QSoas.

the reduction potentials of the three individual [4Fe-4S] clusters. These results were further supported by characterization of the Tte1186 protein with various FeS cluster knockouts described below.

Fortunately, various permutations of cluster variants of Tte1186 were generated and characterized previously by biochemical assays and EPR spectroscopy to confirm the cluster content.¹⁷ The Tte1186 enzymes with only the AC1 cluster (Δ RC/ Δ AC2), only the AC2 cluster (Δ RC/ Δ AC1), only the RC cluster (Δ AC1/ Δ AC2), and only the RC and AC1 clusters (Δ AC2) were all characterized in the same manner as described for the wild-type enzyme, forming a protein film by directly depositing the protein on and EPG electrode modified with MWCNTs, with the exception of the RC only variant whose film was formed by soaking the electrode in a diluted protein solution at room temperature for 2 h.

Importantly, these cluster variants allowed each FeS cluster to be assessed independently, consequently allowing the assignment of the reduction potentials to a particular [4Fe-4S] cluster within the enzyme. A single redox active feature can be observed for the cyclic voltammograms of each of the four single cluster variants (Figure 3). The RC cluster gave a reduction potential of -475 mV; the AC1 cluster showed a reduction potential of -525 mV, and the AC2 cluster gave a reduction potential of -560 mV versus SHE all showing an upshift in potential (\sim 10–25 mV) compared to those determined by the wild-type enzyme. The RC/AC1 variant showed reduction potentials of -505 and -570 mV versus SHE, showing a slight downshift (\sim 20 mV) in potential from those determined by the wild-type enzyme. These differences in reduction potentials are possibly due to the absence of the other FeS clusters resulting in a change in the surrounding cluster environment. Upon comparison of the CVs resulting from each of the single-cluster variants, the reduction potentials of the individual clusters fit well when compared to the envelope signal generated by the wild-type enzyme (Figure 4). The consistent upshift in potential observed for the single-cluster variants may be caused by the loss of positive charges coming from the other two FeS clusters. Lacking the additional positive charges, the oxidized state of the cluster ($[4\text{Fe-4S}]^{2+}$) becomes more stabilized compared to that experienced by the wild type in the presence of two additional positively charged residues; therefore, the reduction potential shifts positively.²² From these data, it is therefore possible to assign the reduction potentials obtained from the single-cluster variants to the reduction potentials obtained by fitting the

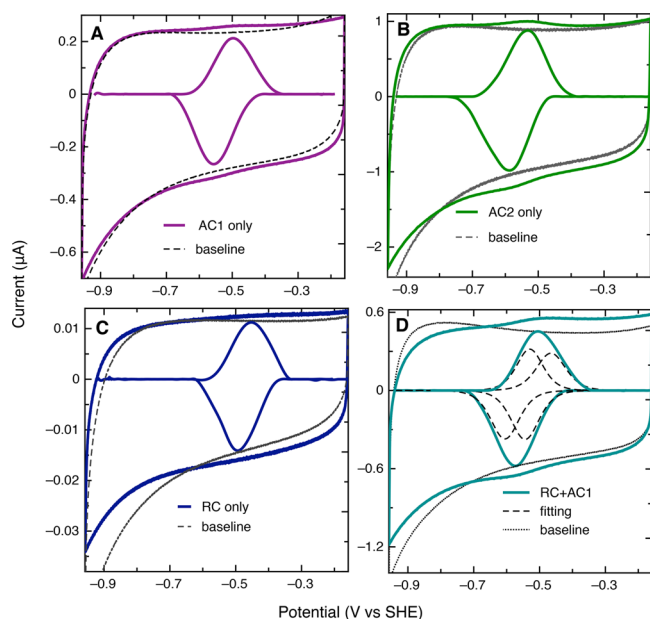


Figure 3. Cyclic voltammograms measured at 50 mV/s, 4 °C, and pH 7.5 of (A) only AC1, (B) only AC2, (C) only the RC, and (D) the RC and AC1 together.

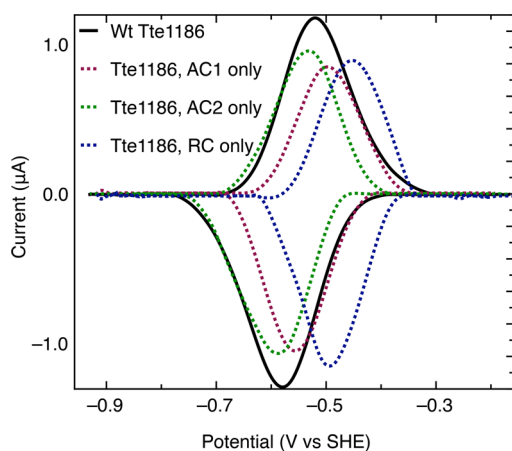


Figure 4. Cyclic voltammograms of the wild type, only RC/AC1, only AC1, and only AC2 subtracted for baseline capacitance and overlaid.

wild-type envelope signal with the RC cluster at -490 mV, the AC1 cluster at -540 mV, and the AC2 cluster at -585 mV versus SHE.

The results of this study support the hypothesis that the auxiliary clusters may be used to store electrons following the one-electron oxidation of a peptide radical. The two auxiliary clusters are quite close together in potential, quite unlike the extreme potential difference between the AC and RC observed in BtrN. The AC redox potentials observed in Tte1186 would allow for such an intermediate to undergo reversible, internal electron transfer, which is in keeping with the previously proposed mechanism for Tte1186.¹⁷ Similarly, it had been suggested that two auxiliary clusters in anSME play the role of transporting electrons from the active site to the surface of the protein.^{9,11,18} Also, pH-dependent analyses indicate that all three clusters do not participate in proton-coupled electron transfer (PCET) (Figure S2).

Relative perturbations of the clusters' potentials also yield useful insights into their roles in catalysis. All of the clusters

bound by Tte1186 do not appear to communicate with each other extensively, given that their individual reduction potentials show no sense of cooperativity, and they are minimally affected by the loss of one or both of the other clusters. However, the reduction potentials of the clusters can be perturbed in the presence of SAM and even more significantly in the presence of the Tte1186 substrate. When the RC cluster variant was incubated with either SAM or SAM and the substrate, the same upward shift in potential ($+40$ mV) was observed, suggesting that this shift was caused by the presence of SAM and not the substrate (Figure S3), which is similar to the results observed by Frey et al. with LAM.³ These results can also be compared to those for the AC1 only variant that exhibited nearly the same upshift in potential in the presence of the substrate or the substrate and SAM ($+60$ mV), suggesting this change results from the presence of the substrate. These upward shifts in potential caused by the presence of SAM and/or the substrate can also be observed in the wild-type envelope signal (Figure S3 and Table S3). These results indicate the auxiliary clusters have potentials that would be appropriate for accepting electrons during substrate oxidation, thus supporting the previously proposed mechanism for why AC1 and AC2 clusters are required for generating the product thioether linkage.¹⁷ Lastly, because voltammetry shows the RC cluster as having the highest reduction potential, the data suggest that the RC cluster will in fact be the first to be reduced possibly by either a ferredoxin or a flavodoxin along with the two auxiliary clusters.

While further studies are needed to address the entire reaction mechanism, these results definitively indicate that FeS clusters in the SCIFF maturase function differently when compared to AREs that lack a third FeS cluster.^{6,7,19} Despite sharing a partial SPASM domain, known as a Twitch domain, the auxiliary cluster of BtrN exhibits a much lower reduction potential (-765 mV vs SHE),⁶ suggesting that the auxiliary clusters in Tte1186 cannot be playing the same role as observed for this seemingly similar ARE. Likewise, what is observed for Tte1186 is also distinct from what is postulated for the methylthiotransferase enzymes, MiaB and RimO, where the reduction potential of the auxiliary cluster is in fact higher than that of the RC cluster and also exhibits a low-potential state corresponding to a transient methylated cluster.⁷ Given the differences observed here for the SCIFF maturase system, these data suggest that the role played by the two SPASM auxiliaries is thus far unique among those of the superfamily of AREs. Their proximity in potential suggests that they may act as a redox wire to transport electrons accepted from the substrate.

The reduction potentials of the clusters appear to act in an additive fashion to generate the envelope signal observed in the wild-type enzyme. This finding is the very first example of the ability to determine and assign specific roles to the specific FeS clusters in an AdoMet radical enzyme containing a SPASM domain. The full characterization of the redox properties of the AdoMet binding FeS cluster along with the two auxiliary FeS clusters provides greater insight into the roles of these clusters in AdoMet radical enzyme catalysis.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b00846.

A full description of the methods used and additional electrochemical data (PDF)

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

AdoMet, S-adenosylmethionine; ARE, AdoMet radical enzyme; dAdo[•], 5'-deoxyadenosyl radical; RC, reaction cluster; SPASM, subtilisin A, pyrroloquinoline quinone, anaerobic sulfatase, mycofactocin; RiPPs, ribosomally synthesized and post-translationally modified peptides; sactipeptide, sulfur-to- α -carbon thioether cross-linked peptide; SCIFF, six cysteines in forty-five; AC1, auxiliary cluster 1; AC2, auxiliary cluster 2; EPG, edge-plane graphite; MWCNTs, multiwalled carbon nanotubes; CV, cyclic voltammetry; EPR, electron paramagnetic resonance; FeS, iron-sulfur.

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