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ENDOR Spectroscopy Shows That Guanine N1 Binds to [4Fe−4S] Cluster II of the *S*-Adenosylmethionine-Dependent Enzyme MoaA: Mechanistic Implications

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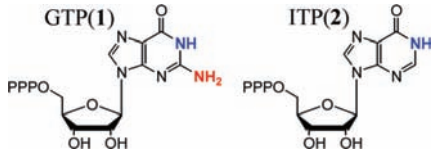
The enzymes MoaA and MoaC catalyze the first step in the biosynthesis of the biologically important molybdenum cofactor (Moco), the conversion of guanosine 5′-triphosphate (5′-GTP) (**1**) to precursor Z, an oxygen-sensitive tetrahydropyranopterin with a cyclic phosphate.[1] MoaA has been identified as an *S*-adenosylmethionine (SAM)-dependent (Radical SAM) enzyme and features two catalytically important, oxygen-sensitive $[4Fe-4S]^{2+,+}$ clusters, each ligated by only three cysteine residues.[2] The N-terminal [4Fe−4S] cluster (cluster I), present in all Radical SAM proteins, binds SAM at the unique Fe site (Fe4) and carries out the reductive cleavage of SAM to generate the 5′-deoxyadenosyl radical, which subsequently initiates the transformation of substrate **1** via a radical reaction. X-ray crystallography[3] revealed that the triphosphate moiety of **1** is well-anchored to MoaA by multiple interactions, including 12 hydrogen bonds. In contrast, the electron density for the ribose and base were not well-defined. The purine ring N1 and amino N2 nitrogens appear to lie at distances of 2.8 and 2.4 Å from Fe4 of cluster II, which are too long for bonding, so the role (if any) of interactions with the cluster has remained unresolved.[3]

Chart 1



We here report that a comparison of continuous-wave (CW)[4] and pulsed[5] ^{14,15}N ENDOR data from **1**(^{14,15}N) and **2**(^{14}N) (Chart 1) bound to MoaA surprisingly shows that [4Fe−4S] cluster II positions its substrate by binding N1 of the purine ring to the unique Fe4. We further propose that this binding induces tautomerization of the base.

We initiated these studies by examining the C24S/C28S/C31S MoaA triple mutant, which does not contain the catalytic SAM-binding cluster I,[3] to eliminate possible interference by this cluster in the EPR/ENDOR spectra. We subsequently confirmed that cluster II of the wild-type enzyme shows ENDOR spectra identical to those of the mutant, eliminating the possibility that the mutation affects the cluster II−substrate interaction. In the absence of substrate, 35 GHz CW EPR spectra of dithionite-reduced $[Fe_4-S_4]^+$ cluster II at 2 K exhibit an axial signal from a state with $S = {}^1/_2$, $g_{\|} = 2.062$, and $g_{\perp} = 1.911$ (see Figure S1 in the Supporting Information); in addition, there is a barely observable signal from the cluster in a high-spin state with $g_1 = 8.80$ and $g_2 = 4.20$. Binding of substrate **1** induces a slight change in the g values of the $S = {}^1/_2$ cluster (2.063, 1.897) and an increase in the relative concentration of the $S = {}^3/_2$ state (Figure S1).

Figure 1 shows 35 GHz CW ENDOR spectra at 2 K collected at g_2 for samples with ^{14}N- and ^{15}N-labeled **1**. The spectrum for bound **1**(^{14}N) shows the ν_+ branch of a ^{14}N($I = 1$) signal; it arises from overlapping signals from multiple orientations, each offset from the ^{14}N Larmor frequency [$\nu(^{14}\text{N}) = 4.04$ MHz] by half the orientation-dependent hyperfine coupling and further split into a doublet by the orientation-dependent quadrupole interaction.

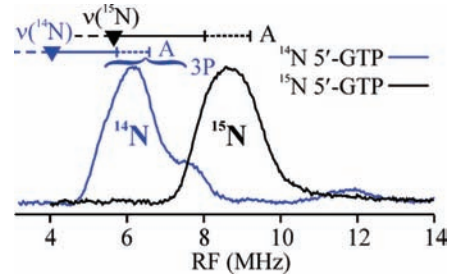


Figure 1. ENDOR spectra at g_2 (35 GHz, CW) of **1**(^{14,15}N)−MoaA triple mutant at 2 K. Conditions: 4.0 G; 100 kHz modulation; 0.4 MHz/s RF sweep low-to-high with 100 kHz noise (^{15}N), 0.5 MHz/s (^{14}N); frequency 34.752 GHz (^{15}N), 34.109 GHz (^{14}N).

The ^{14}N feature is absent in the spectrum of enzyme with bound **1**(^{15}N) and is replaced by the ν_+ branch for ^{15}N; the shift in frequency matches the ratio of nuclear g values for $^{14/15}$N and the shape is simpler because ^{15}N ($I = {}^1/_2$) has no quadruple splitting. The ^{15}N hyperfine coupling, $A(^{15}\text{N}) \approx 6.1$ MHz, is equivalent to $A(^{15}\text{N}) = 5.8$ MHz observed at $g_{\perp}$ for the amino nitrogen of SAM coordinated to an Fe ion of the $[4Fe-4S]^+$ cluster of PFL−AE.[6] Together, these observations prove that at least one nitrogen of **1** indeed binds to Fe4 of MoaA cluster II.

To determine the $^{14/15}$N hyperfine coupling tensors and the number of distinct nitrogen nuclei contributing to the signal, a complete two-dimensional (2D) field−frequency pattern consisting of ENDOR spectra collected at magnetic field values across the MoaA EPR envelope was collected for both samples. Both patterns were well-simulated[7,8] by that for a single interacting nitrogen nucleus (Figures S2−S4) whose substantial isotropic coupling, $a_{iso}(^{14}\text{N}) = 3.6$ MHz, requires the presence of a typical coordinate-covalent bond between a single N of **1** and the Fe4 ion of cluster II.

But which nitrogen of **1** is bound, N1 of the purine ring or the exocyclic N2 amino moiety? In lieu of time-consuming selective ^{15}N labeling, we addressed this question through use of the substrate analogue inosine 5′-triphosphate (5′-ITP) (**2**), which is identical to substrate **1** except that it lacks the exocyclic amino group. Earlier in vitro binding studies with purine nucleotides other than **1** revealed

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40% relative affinity for adenosine 5′-triphosphate (5′-ATP), 60% for **2**, and 100% for xanthosine 5′-triphosphate (5′-XTP).[3] **2** not only binds but also behaves similarly to **1** in increasing the rate of the reductive cleavage of the cosubstrate SAM, the first step in MoaA catalysis, by a factor of ~10 and significantly inhibits precursor Z biosynthesis while ATP and XTP do not (Figure S5). Together, these observations indicate that **2** binds in the active site similarly to **1**, while 5′-XTP does not. This conclusion implies that if the amino group of **1**(^{14}N) binds to cluster II, then this signal should be lost in the complex with **2**(^{14}N); however, if purine ring nitrogen N1 binds, the signal should persist, although it may be altered somewhat by small differences in the binding orientations of **1** and **2**.

The CW EPR spectrum of **2**−MoaA (Figure S1; $g_{\|} = 2.057$ and $g_{\perp} = 1.883$) again differs from that of substrate-free MoaA, and its g values are similar to those of **1**−MoaA, as expected if **2** binds in a similar fashion to **1**. The mode of binding **2** to cluster II was probed by ^{14}N ENDOR measurements. As illustrated by the g_2 spectra in Figure 2 (also see Figures S2 and S6), the ^{14}N ENDOR signals of **2**(^{14}N)−MoaA are very similar to those of **1**(^{14}N)−MoaA. As for **1**(^{14}N), the 2D pattern for **2**(^{14}N) can be simulated with a single ^{14}N; the coupling parameters are similar for the two complexes, with essentially the same isotropic hyperfine interaction, a_{iso}(^{14}N) = 3.5 MHz for **2** (Figure S6). The two substrates thus have an equivalent hyperfine-coupled ^{14}N bound to Fe4 of cluster II.

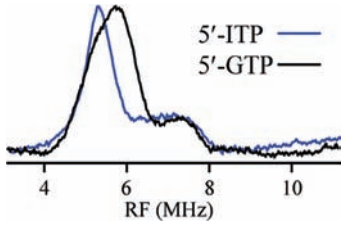

Figure 2. ENDOR spectrum at g_2 (35 GHz) of **1**(^{14}N)− and **2**(^{14}N)−MoaA triple mutant at 2 K. Conditions: 4.0 G; 100 kHz modulation; 0.4 MHz/s RF sweep high-to-low with 100 kHz noise (GTP), 0.5 MHz/s (ITP); frequency 35.026 GHz (GTP), 35.005 GHz (ITP).

The persistence of the substrate-derived ^{14}N ENDOR signal upon replacement of **1** with **2** establishes that this signal does not come from the amino group of **1**. In conjunction with the X-ray results, it proves that the signals of **1** and **2** both come from the purine ring nitrogen N1; the binding of any other ring nitrogen of **2** to cluster II would require a massive reorientation of **2** relative to that of **1**. Since the triphosphate of **1** is so tightly anchored[3] and **2** clearly binds in the same general fashion as **1**, we conclude that **1** (or **2**) is positioned for catalysis through binding of the purine ring N1 to the unique Fe4 of cluster II.

Examination of the X-ray crystal structure of **1**−MoaA suggests that when N1 binds, at least two other nitrogen atoms, N3 and the amino nitrogen, should be close enough to Fe4 to exhibit observable ^{15}N dipolar hyperfine coupling to the cluster spin. Pulsed Mims ENDOR spectra of **1**(^{15}N)−MoaA reveal two ^{15}N doublets centered at the ^{15}N Larmor frequency, with respective maximum splittings of A(^{15}N) = 0.5 and 0.2 MHz (Figure S7). Overlap of the peaks, combined with the distortions from the Mims suppression "hole" at the Larmor frequency,[9] precludes a precise determination of the hyperfine tensors for the two nitrogens. However, simulations of the spectra establish the maximum and minimum dipolar components for each, from which we can set rough limits on the possible distances between the nitrogens and cluster Fe4 (Supporting Information). This analysis places one nitrogen between 2.6 and 5.1 Å from the Fe and the other at a distance greater than 3.6 Å.[10]

The bond between Fe4 of cluster II and N1 of **1**, along with the anchoring of the triphosphate of **1** by the enzyme, strongly constrain the possible orientations of both the ribose and purine of bound **1**. If we fix the triphosphate moiety as in the X-ray structure, only minor movements and rotations of the base and ribose are required to generate a structure of bound **1** consistent with the ENDOR data (Figure 3). In this model the Fe4−N1 distance is 1.94 Å, with the other two weakly coupled nitrogens at ENDOR-compatible distances of 3.0 Å (N2) and 4.2 Å (N3). These differences from the X-ray structure may reflect the fact that the latter was obtained after soaking crystals with **1**, SAM, and sodium dithionite, conditions that had initiated catalysis and might have altered the binding mode. Alternatively, the ENDOR experiments were performed on an unconstrained enzyme−substrate complex formed in solution and subsequently frozen, whereas packing effects in the crystal might limit conformational changes needed to accommodate the substrate.

The proposed structure of Figure 3 incorporates an Fe4−N1 bond that lies in the plane of the purine ring and thus implies that the purine binds as the enol tautomer, not the normally favored keto form. In support of this idea, S4 of cluster II and O6 of the putative guanine hydroxyl group are separated by an H-bonding distance (2.4 Å), although this was not imposed as a constraint while generating the structure. We surmise that such binding-induced tautomerization may play a mechanistic role by modulating the purine reactivity and/or stabilizing reaction intermediates.

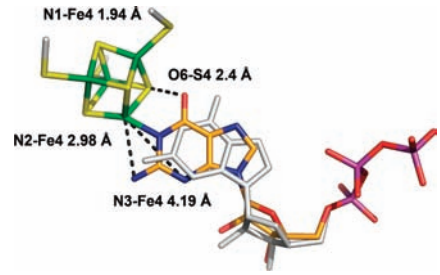

Figure 3. Proposed model for 5′-GTP binding (C, orange; N, blue; O, red; P, purple) to the Fe4 ion of cluster II (S, yellow; Fe, green). The 5′-GTP model derived by X-ray crystallography (PDB entry 2FB3) is shown in white.

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Supporting Information Available: Procedures for simulation of ENDOR spectra and in vitro activities. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) If one of these nitrogen signals is the amino nitrogen, then it should not be visible in the **2**(^{14}N) sample. However, the ^{14}N signals that correspond to the weakly coupled ^{15}N peaks of **1**(^{15}N) proved too difficult to detect, so this could not be checked.

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