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The Elusive 5'-Deoxyadenosyl Radical: Captured and Characterized by Electron Paramagnetic Resonance and Electron Nuclear Double Resonance Spectroscopies

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Abstract

The 5'-deoxyadenosyl radical (5'-dAdo[•]) abstracts a substrate H atom as the first step in radical-based transformations catalyzed by adenosylcobalamin-dependent and radical *S*-adenosyl-L-methionine (RS) enzymes. Notwithstanding its central biological role, 5'-dAdo[•] has eluded characterization despite efforts spanning more than a half-century. Here, we report generation of 5'-dAdo[•] in a RS enzyme active site at 12 K using a novel approach involving cryogenic photoinduced electron transfer from the [4Fe-4S]⁺ cluster to the coordinated *S*-adenosylmethionine (SAM) to induce homolytic S–C5' bond cleavage. We unequivocally reveal the structure of this long-sought radical species through the use of electron paramagnetic resonance (EPR) and electron nuclear double resonance (ENDOR) spectroscopies with isotopic labeling, complemented by density-functional computations: a planar C5' (2p π) radical (~70% spin occupancy); the C5'(H)₂ plane is rotated by ~37° (experiment)/39° (DFT) relative to the C5'–C4'–(C4'–H) plane, placing a C5'–H antiperiplanar to the ribose-ring oxygen, which helps stabilize the radical against elimination of the 4'–H. The agreement between ϕ from experiment and in vacuo DFT indicates that the conformation is intrinsic to 5-dAdo[•] itself, and not determined by its environment.

Graphical Abstract

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Supporting Information

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Additional methods; X-band EPR spectra; simulations of X-band FT-EPR spectra; hyperfine tensors of 5'-dAdo[•]; DFT calculations; comparison of hyperfine couplings calculated with B3LYP and BP86 functionals; DFT calculated energy; Q-band EPR spectra and simulations; CW stochastic ¹³C ENDOR; XPR spectra (PDF)

The authors declare no competing financial interest.

INTRODUCTION

The discovery by Barker et al. in 1958 of a cobalamin involved in catalyzing an isomerization reaction,¹ and its subsequent identification as adenosylcobalamin (coenzyme B₁₂ (Figure 1)) by Lenhert and Hodgkin in 1961,² set the stage for decades of study focused on the biochemical reactions of this remarkable organometallic cofactor. We now know that coenzyme B₁₂ serves as a reversible radical generator, undergoing Co–C bond homolysis to give a 5′-deoxyadenosyl radical (5′-dAdo•) that initiates chemistry by H atom abstraction from a substrate.³ The involvement of 5′-dAdo• has been shown primarily through label transfer studies, for example, by observing the incorporation of substrate deuterons into adenosylcobalamin during catalysis.^{4–6} Important insights into its behavior have been attained through kinetic and spectroscopic investigations in which the Co–C5′ bond of B₁₂ is photolytically cleaved, after which the 5′-dAdo• radical can either reform that bond or proceed to react with substrate.^{7,8} However, strenuous efforts over many years⁹ to trap and fully characterize the 5′-dAdo• formed from B₁₂ have been unavailing: the radical has simply been too reactive to trap.

The significance of the 5′-dAdo• radical in biology broadened dramatically with the recognition that *S*-adenosyl-L-methionine (SAM) plays a role analogous to that of coenzyme B₁₂ in initiating radical reactions.^{10,11} This role for SAM was first suggested for lysine 2,3-aminomutase^{12,13} and pyruvate formate-lyase activating enzyme (PFL-AE),^{14,15} but it is now implicated throughout the vast and diverse radical SAM (RS) superfamily.^{16–19} Until recently, the accepted mechanism for the RS enzymes involved SAM coordination to the unique iron of the [4Fe–4S] cluster in the active site; electron transfer from the reduced [4Fe–4S]⁺ cluster to SAM promotes reductive cleavage of SAM to give methionine and 5′-dAdo•, which initiates chemistry via substrate H atom abstraction.^{16,20–22} The recent discovery of the catalytically competent organometallic intermediate **Q** formed during the RS reaction provides a new twist on the mechanism by implicating a coenzyme B₁₂-like organometallic intermediate with an Fe–C5′ bond that undergoes homolytic bond cleavage to release 5′-dAdo•.^{20,23,24}

Insights into the characteristics of the 5′-dAdo• radical intermediate in RS enzymes have been achieved via the SAM analog 3′,4′-anhydro-*S*-adenosylmethionine (anSAM), which generates an allylically stabilized 5′-dAdo• analog that is amenable to characterization,^{25,26}

but the 5'-dAdo $\cdot$ radical is so extraordinarily reactive that so far attempts to generate and characterize this radical itself have not been productive. For example, photolysis of coenzyme B₁₂ in frozen solution appears to produce 5'-dAdo $\cdot$, but spin-spin interactions between the Co(II; $S = 1/2$) corrinoid and the $S = 1/2$ radical result in a broad, poorly defined electron paramagnetic resonance (EPR) signal that is not amenable to analysis.²⁷ A sharp fluid-solution FT-EPR spectrum acquired upon photolysis of B₁₂ and thought to arise from 5'-dAdo $\cdot$ is instead the result of rapid radical rearrangement rather than 5'-dAdo $\cdot$ itself (see SI Text and Figure S2).²⁸ An organic radical that accumulates during the reaction of the RS enzyme spore photoproduct lyase with SAM at 40 °C also has been proposed to be 5'-dAdo $\cdot$,²⁹ but we show in the Supporting Information that this radical too is not 5'-dAdo $\cdot$ (see SI Text, Figure S3, and Table S1).

Here, after more than half a century of efforts by numerous investigators to trap and characterize the biologically central 5'-dAdo $\cdot$ radical, we demonstrate the formation of 5'-dAdo $\cdot$ in a RS enzyme active site by using a novel approach involving photoinitiated electron transfer from the reduced [4Fe-4S]⁺ cluster to the coordinated SAM. The resulting 5'-dAdo $\cdot$ is definitively identified through the use of isotopically labeled SAM combined with EPR and electron nuclear double resonance (ENDOR) spectroscopy, with its structure analyzed using density functional theory (DFT) computation.

EXPERIMENTAL METHODS

Materials.

[Methyl-¹³C]-L-methionine, ¹⁵N-L-methionine, and 2,8-D₂-1',2',3',4',5',5''-D₆-adenosine 5'-triphosphate salt solution were purchased from Cambridge Isotope Laboratories, Inc. [¹³C₁₀, ¹⁵N₅]-Adenosine 5'-triphosphate sodium salt solution was purchased from Sigma, and 3,3,4,4-L-methionine-*d*₄ was obtained from CDN Isotopes.

Protein and SAM Preparation.

PFL-AE, unlabeled SAM, and labeled SAMs were prepared as previously reported.³⁰

Photolysis.

PFL-AE was prepared for photolysis by reducing the enzyme and adding labeled or unlabeled SAM. Photoreduced PFL-AE was prepared as previously described³⁰ with minor alterations. PFL-AE (0.55 mM), dithiothreitol (DTT, 1.0 mM), and deazariboflavin (200 μ M, dissolved in DMSO) were combined in buffer (50 mM Tris, 100 mM KCl, pH 7.5) in an anaerobic COY chamber. The sample was irradiated by a 500 W halogen lamp for 1 h while the samples remained in an ice water bath. Labeled or unlabeled SAM (5.5 mM) was then added. The samples were then transferred to EPR tubes and frozen in liquid nitrogen inside the COY chamber.

Dithionite reductions were performed similarly as follows: PFL-AE (0.55 mM), DTT (1.0 mM), and sodium dithionite (3.0 mM) were combined in an anaerobic COY chamber and allowed to sit 3 min before SAM (5.5 mM) was added. The samples were flash frozen as described above and stored in liquid nitrogen.

Photolysis was carried out in situ using a 450 nm Thorlabs diode laser. The time course of 12 K photolysis of (PFL-AE¹⁺+SAM) complex was monitored during intracavity photolysis in an X-band EPR spectrometer. The in situ photolysis of the (PFL-AE¹⁺+SAM) complex prerduced to the [4Fe-4S]⁺ state using either deazariboflavin or dithionite gives identical EPR spectra, Figure S1. The in situ photolyzed samples were subjected to X- and Q-band CW EPR and Q-band ENDOR measurements as described below.

EPR and ENDOR Measurements.

X-band continuous wave (CW) EPR spectroscopy was conducted on a Bruker ESP 300 spectrometer equipped with an Oxford Instruments ESR 910, while Q-band CW EPR spectroscopy was conducted on a Bruker EMX spectrometer equipped with an Oxford Instruments Mercury iTC continuous helium flow cryostat. Typical experimental parameters were at 12 and 40 K, 9.38 or 34.0 GHz, and 10 G modulation amplitude. EPR simulations were performed with the EasySpin^{5,2,23} program operating in Matlab.³¹

35 GHz CW and pulse ENDOR spectroscopic data were collected on spectrometers, described previously,^{32–34} that are equipped with liquid helium immersion dewars for measurements at 2 K. The CW measurements employed 100 kHz field modulation and dispersion-mode detection under rapid passage conditions. ¹H CW ENDOR spectra employed bandwidth broadening of the RF to 100 kHz to improve signal-to-noise.³⁵ ¹H CW ENDOR spectra were collected using the stochastic-field modulation detected ENDOR sequence³⁶ to improve ENDOR line shape. For a single molecular orientation and for nuclei with a nuclear spin of $I = 1/2$ (¹³C), the ENDOR transitions for the $m_s = \pm 1/2$ electron manifolds are observed to be first order as shown in the equation:

$$\nu_{\pm} = \left| \nu_n \pm \frac{A}{2} \right| \quad (1)$$

where ν_n is the nuclear Larmor frequency and A is the orientation-dependent hyperfine coupling. When $\nu_n > A/2$, the pattern is a doublet split by A and centered at ν_n ; when $\nu_n < A/2$, the pattern is a doublet centered at $A/2$ and split by $2\nu_n$.

DFT Calculations.

All DFT computations of 5'-dAdo[•] were performed in ORCA 4.0.1.³⁷ Initial coordinates for the 5'-dAdo fragment were taken from two structures: PFL-AE (2.77 Å resolution) with SAM bound to the [4Fe-4S] cluster (PDB 3CB8)³⁸ and a 1.15 Å resolution crystal structure of a SAM-dependent methyltransferase (PDB 3DLC). All protein residues, water, and other molecules were removed, and the adenine was replaced with a methyl group. Hydrogens were added appropriately to the structure with the unbiased starting geometry of the 5'-CH₂ hydrogens being in a pseudotetrahedral conformation. Both geometry optimizations and single-point calculations used the spin unrestricted B3LYP^{39–41} hybrid functional and the Ahlrichs' valence triple- ξ with a polarization function basis set.⁴² The molecular orbitals were visualized as Gaussian cubes and an isosurface of 0.08 au in Pymol. Hyperfine and g tensors were calculated by the coupled-perturbed self-consistent field (SCF) approach as implemented in ORCA 4.0 using the B3LYP hybrid functional and EPR-III basis^{43,44} in

combination with the accurate spin-orbit coupling operator [RI-SOMF(1X)].⁴⁵ Calculations using the BP86³⁹ functional were carried out in parallel.

The optimized structures and all energetic and spectroscopic output parameters for 5'-dAdo· are identical for the two starting points. The results presented are those from the high-resolution structure. Similar results are obtained for the two functionals, as shown in Table S2.

RESULTS

Photolysis of PFL-AE/[4Fe-4S]¹⁺/SAM.

The present study was indirectly prompted by the idea that the Fe-C5' bond of the Ω ^{23,24} intermediate in RS enzymes might be cleaved photolytically, similar to the Co-C5' bond of coenzyme B₁₂. If this were to occur, then photoinitiated homolysis of Ω would produce the diamagnetic [4Fe-4S]²⁺ cluster and the sought-after 5'-dAdo· radical. However, because sulfoniums have been reported to be photochemically reactive,^{46,47} we first carried out control experiments in which SAM and PFL-AE/[4Fe-4S]¹⁺ were combined and photolyzed in the absence of substrate PFL, circumstances in which SAM is not enzymatically cleaved and Ω does not form. Much to our surprise, irradiation of such samples in the EPR cavity at 450 nm and 12 K results in rapid conversion of the SAM-bound [4Fe-4S]¹⁺ state of PFL-AE (Figure 2A) to one with a new free-radical signal that is partially saturated and thus poorly resolved at 12 K, Figure 2B, but becomes well-resolved at 40 K (Figure 3A). A time course of the photolysis shows the loss of the [4Fe-4S]¹⁺ signal directly correlates with the appearance of the new radical signal (Figure 2C).

The data is consistent with photoinduced electron transfer (ET) from the [4Fe-4S]¹⁺ cluster to SAM,⁴⁸ providing the EPR-silent [4Fe-4S]²⁺ cluster and a SAM-derived organic cryo-trapped radical to study; such photoinduced ET has not previously been reported for RS enzymes. On the basis of known RS enzyme chemistry, the most likely identity of the new radical species would be the 5'-dAdo· radical, resulting from reductive cleavage of SAM. However, other forms for the trapped radical are possible.⁴⁹ Rearrangement to form a 4' radical or even a cyclo-adenosine radical must be considered, and if the highly reactive 5'-dAdo· is formed, it might have attacked a nearby protein residue to generate a protein radical, even at 12 K. We therefore pursued detailed studies that unequivocally identify the structure of this radical species.

The radical as trapped in the active site of PFL-AE is stable at 77 K and below but is lost upon annealing for 1 min at 150 K. Figure 3A shows that the EPR spectrum of the radical species under nonsaturating conditions (40 K) can be well simulated by incorporating ¹H hyperfine interactions appropriate for trapped 5'-dAdo·, Figure 3A. The simulation³¹ includes anisotropic hyperfine couplings to two near-equivalent α -type ¹H on 5'-C plus coupling to one near-isotropic β -type ¹H on 4'-C; the hyperfine tensors are reported in Table 1. The two resulting α -type ¹H hyperfine tensor components, confirmed and refined as described below, have values roughly in the ratio of ~ 1/2/3, as expected for a trigonal-planar, C α -¹H carbon radical, and indeed quantitatively agree quite well with those of the C α -¹H radical of ·CH(COOH)₂ formed by irradiation of malonic acid (Table 1). In addition,

it is well-established^{50–52} that a β -type ^1H , such as the ^1H -4'-C of 5'-dAdo $\cdot$, gives a large, near-isotropic coupling, as observed here (Table 1). The Q-band EPR spectrum (Figure 3F) is equally well simulated with these parameters and has the additional benefit of enhancing the influence of the small free-radical g-anisotropy, which helped refine both the g and hyperfine tensors (Table 1). Of particular note as an “internal check”, the intermediate hyperfine component of an α -proton must lie parallel to the $2p\pi$ orbital, which is in turn parallel to $g_3 \approx g_e$ ^{50–54} and, thus, must be parallel for the two α -protons of 5'-dAdo $\cdot$. This was required for the optimized simulations of the radical spectra, particularly the Q-band spectrum (Table 1), even though the tensors were determined without imposing this as a prior constraint.

Confirmation of Radical Species Using SAM Isotopologs.

To confirm this new radical species as the 5'-dAdo $\cdot$ radical and to refine its ^1H hyperfine interaction parameters, we carried out photolysis experiments utilizing isotopically labeled SAMs.³⁰ Assignment of the α -type couplings to the two 5'-C α protons is confirmed by the collapse of both the X-band (Figure 3B) and Q-band (Figure S5) spectra of the radical generated with [adenosyl-5',5''-D₂]-SAM to a doublet resulting from the near-isotropic splitting from the 4'-C β proton. This splitting in turn is lost in both the X-band and Q-band spectra of the radical generated with the perdeuterated-Ado SAM isotopologue ([adenosyl-2,8-D₂-1',2',3',4',5',5''-D₆]-SAM) (Figures 3C and S5). Together, these measurements show that the radical formed by photolysis is indeed the long-sought 5'-dAdo $\cdot$.

This identification is further confirmed, and the characterization of 5'-dAdo $\cdot$ is strengthened and enriched by the examination of the radical formed with ^{13}C isotopologues. When prepared with [adenosyl- $^{13}\text{C}_{10}$, $^{15}\text{N}_5$]-SAM (Figure 3D), the radical X-band EPR spectrum shows additional splittings from two ^{13}C , one with the anisotropic hyperfine tensor expected^{50–52} for the 5'- $^{13}\text{C}\alpha$ “sp²” carbon and one with the smaller, nearly isotropic coupling expected for the 4'- $^{13}\text{C}\beta$ carbon (Table 1). The assignment of the two couplings to their respective carbons is confirmed by the EPR spectrum of radical prepared from singly labeled 5'- ^{13}C SAM (Figure 3E), which shows the splittings with the anisotropic tensor assigned to 5'- $^{13}\text{C}\alpha$ (Table 1). Note that the maximum ^{13}C coupling for a spin in a carbon $2p\pi$ orbital must also lie along $g_3 = g_e$,^{50–52} as found experimentally for 5'- ^{13}C , without prior constraint (Table 1).

The Q-band ENDOR spectra for 5'- ^{13}C -SAM, Figure 3G, shows a peak centered at a frequency corresponding to approximately $A_3/2$ of 5'- ^{13}C (Table 1). The Q-band ENDOR spectra from the radical prepared from [adenosyl- $^{13}\text{C}_{10}$, $^{15}\text{N}_5$]-SAM (Figure 3G) show not only the 5'- ^{13}C signal but also a signal from the near-isotropically coupled 3'- ^{13}C centered at a frequency determined by essentially half its isotropic coupling (Table 1), as well as a doublet with a much weaker coupling ($A = 0.8$ MHz) assigned to 1'- ^{13}C and/or 2'- ^{13}C ; the signal from 4'- ^{13}C is hidden under the ^1H ENDOR response from weakly coupled protons (Figure S6). These ^{13}C measurements unambiguously confirm the photochemically generated radical is indeed 5'-dAdo $\cdot$.

Structure of 5'-dAdo•

Beyond the identification of 5'-dAdo•, the observed hyperfine interactions teach us about its electronic and geometric structure. The two $^1\text{H}\alpha$ and the $5'-^{13}\text{C}\alpha$ coupling tensors are in good agreement with those for the odd electron in the $2p\pi$ orbital of the planar (sp^2) carbon-centered radical of X irradiated malonic acid (Table 1).⁵⁴ The match, notably in the isotropic couplings, indicates that the spin density in the $2p\pi$ orbital on $\text{C}5'$ of 5'-dAdo• (ρ_π) is comparable to that in malonic acid, $\rho_\pi \sim 0.7$. Of particular note, the spin-polarization-induced isotropic coupling of the $2p\pi$ spin of planar $^{13}\text{C}5'$ would have been sharply increased by a pseudotetrahedral “doming” distortion at $\text{C}5'$, which would introduce $2s$ character, with its large isotropic coupling,⁵¹ into the odd-electron orbital. Thus, we infer that the $\text{H}_2\text{-C}5'\text{-C}4'$ fragment is essentially planar. The isotropic coupling of a proton β to a carbon $2p\pi$ electron spin, such as $^1\text{H-C}4'$, is known to obey the relationship, $a_{\text{iso}} \approx \rho_\pi B \cos^2 \varphi$ MHz, where B reflects the transmission of spin to ^1H through hyper-conjugation, $\rho_\pi B \approx 140$ MHz, and φ is the dihedral angle between the $2p\pi$ orbital and the $\text{C}\beta\text{-H}$ bond,⁵¹ corresponding to the angle between the $[4'\text{CH-}4'\text{C-}5'\text{C}]$ plane and of the $2p\pi$ orbital (normal to the plane formed by $\text{C}5'$ and its two H atoms). Applying this relationship to the measured $^1\text{H-C}4'$ isotropic coupling (Table 1) yields a value of $\varphi \approx 37^\circ$.

These conclusions from the experimental finding are confirmed and extended by DFT computations³⁷ for 5'-dAdo• (Table S2 and Figure S4). First, the computations yield an energy-minimized structure that reproduces the observed hyperfine couplings extremely well, Table 1, with a value for the spin density in the $\text{C}5'$ $2p\pi$ orbital of $\rho_\pi = 0.7$, in agreement with the experimental analysis. The computations further generate the radical's structure (Figure 4, top), which exhibits both a rigorously planar geometry at $\text{C}5'$ and a dihedral “twist” at the $\text{C}5'\text{-C}4'$ bond, as inferred above. The dihedral (twist) angle in the energy-minimized geometry $\varphi \approx 39^\circ$ (Figure 4, bottom) is quite close to that indicated by the experimental analysis above and creates a structure with a $\text{C}5'\text{-H}$ antiperiplanar to the oxygen of the ribose ring, which helps stabilize the radical against elimination of $4'\text{-H}$, as suggested long ago.⁵⁷ The agreement between φ determined experimentally for 5'-dAdo• in the active site of a frozen enzyme and φ from DFT of the radical in vacuo indicates that the dihedral angle is determined by interactions within the radical itself, not by those with its environment.

5'-dAdo• in the Active Site.

Expanding our focus, the use of additional SAM isotopologues gives a sense of the relationship of the SAM fragments (5'-dAdo• and Met) subsequent to homolysis. When 5'-dAdo• is prepared from [3,3,4,4-methionine- D_4]-SAM, subtle changes in the shape of the EPR spectrum (Figure S7) indicate that the apparent EPR line width of the natural-abundance spectrum includes the effects of weak hyperfine couplings to protons of the methionine side chain. In contrast, there are no changes in the EPR spectrum when prepared from CD_3 -methyl or ^{13}C -methyl methionine SAM, and no weakly coupled ^{13}C ENDOR signal is introduced by the ^{13}C substitution (Figure S7), indicating the radical site is remote from the methyl group. These observations indicate that, upon SAM $\text{S-C}5'$ homolytic bond cleavage, the $\text{C}5'$ radical has shifted toward the methionine C3 and C4 and away from the methyl, as illustrated schematically in Figure 5. Such a movement leaves the spin-bearing

C5' in proximity to the methionine side chain, allowing unresolved hyperfine couplings to those hydrogens to influence the EPR spectrum, yet removed enough from the methionine ^{13}C -methyl so as to leave no detectable couplings to that nucleus in either EPR or ENDOR spectra.

DISCUSSION

The 5'-dAdo $\cdot$ radical is the central species responsible for H atom abstraction from the substrate in both coenzyme B₁₂ and the large RS superfamily of enzymes, but until now, it had eluded characterization. In the present work, we generated the 5'-dAdo $\cdot$ radical in PFL-AE by cryogenic photoinduced electron transfer (ET) from the [4Fe-4S]¹⁺ to SAM, causing reductive cleavage of SAM to generate the 5'-dAdo $\cdot$ radical, and methionine chelated to the diamagnetic [4Fe-4S]²⁺ cluster. Because the cluster is diamagnetic, there are no spin-spin interactions that would interfere with the characterization of the photogenerated radical, and its assignment as 5'-dAdo $\cdot$ has been unequivocally established through the use of ^{13}C and ^2H labels.

Our early ENDOR studies showed that, in the absence of PFL substrate, SAM is coordinated to the reduced PFL-AE [4Fe-4S]¹⁺ cluster through the amino acid group of SAM with the sulfonium in close contact to the cluster (<4 Å).^{58,59} Thus, this photoinduced ET is analogous to the inner-sphere ET thought to occur during enzymatic catalysis,^{58,60} with key roles being played by configurational interaction between donor and acceptor orbitals on the sulfonium and the PFL-AE/[4Fe-4S]¹⁺ cluster,⁶¹ conformational and electronic influences of substrate binding,⁶² and the mechanistic requirement of Ω formation.^{20,23,24} Why, then, is 5'-dAdo $\cdot$, rather than Ω , produced in the current experiments? One possible answer is that Ω formation might require the conformational consequences of the presence of substrate. Unfortunately, we have not yet been able to test this hypothesis because adding substrate to the PFL-AE/[4Fe-4S]¹⁺/SAM complex leads to immediate substrate turnover.⁶⁰ Even when PFL-AE/[4Fe-4S]¹⁺, SAM, and substrate are combined in rapid-freeze quench experiments with short deadtimes, Ω is formed and 5'-dAdo $\cdot$ is not observed. In other words, the enzymatic turnover system appears to be designed to avoid the production of free dAdo $\cdot$ and rather to form the more stable 5'-dAdo $\cdot$ precursor Ω . As we have previously postulated,²⁵ the highly reactive 5'-dAdo $\cdot$ is never free under normal catalytic conditions.

An additional consideration is the nature of the process of electron transfer and SAM cleavage itself. During catalysis, RS enzymes must overcome a large potential barrier for reductive cleavage of SAM, due to the mismatch in reduction potentials between RS [4Fe-4S]¹⁺ clusters (-400 to -600 mV)⁶³⁻⁶⁷ and SAM (estimated as $\sim$ -1.8 V).^{68,69} The binding of SAM and substrate in the active site is thought to perturb both potentials, lowering the barrier to reductive SAM cleavage.⁶² RS catalysis via formation of Ω likely also lowers the barrier to this cleavage through the stabilization contributed by the Fe-C5' bond formation, thus providing a favorable alternative pathway to direct SAM cleavage by simple ET to form 5'-dAdo $\cdot$.^{20,23,24}

The photoinduced ET reported here instead uses photon energy to overcome the barrier to direct the formation of 5'-dAdo $\cdot$. While understanding the precise nature of this process will

require further study, prior studies of photoreduction by metal centers⁷⁰ suggest that in the present study the photon excites the cluster to a ligand-to-metal charge transfer (LMCT) excited state, which quickly relaxes to a *relatively* long-lived $[4\text{Fe-4S}]^{1+}$ ligand field excited state that is a potent and effective electron donor. In short, the energy of the incident photon, as captured in an ET-reactive excited state, is sufficient to enable the direct formation of $5'$ -dAdo $\cdot$ by S-C5' homolysis without the formation of Ω .

CONCLUSION

In summary, after more than half a century of attempts, the primary-carbon $5'$ -dAdo $\cdot$ radical has at last been captured, and its electronic and geometric structure has been characterized by multifrequency EPR, Q-band ENDOR, and DFT computation. The capture without radical rearrangement was accomplished via cryogenic (12 K), intra-EPR-cavity, photo-induced ET from the PFL-AE $[4\text{Fe-4S}]^{1+}$ to cluster-bound SAM, triggering reductive cleavage of SAM to generate the $5'$ -dAdo $\cdot$ cryo-trapped radical along with the diamagnetic $[4\text{Fe-4S}]^{2+}$ cluster. The facile and efficient nature of this photo-induced ET, not previously reported for any radical SAM enzyme, raises the intriguing question of whether photo-initiated reactions might play an as-yet unrecognized role in RS enzymes or other catalytic systems involving iron-sulfur clusters. Perhaps the most surprising finding about $5'$ -dAdo $\cdot$ itself is the absence of surprises: its remarkable reactivity accompanies properties that are almost precisely as foundational studies of organic radicals long ago would have predicted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Adenosylcobalamin/coenzyme B₁₂ (left) and *S*-adenosylmethionine bound to a [4Fe-4S] cluster in the active site of RS enzymes (modeled at right) both serve as precursors to the 5'-dAdo• radical intermediate (center).

Figure 2.

X-band EPR spectra of the $([\text{4Fe-4S}]^+ + \text{SAM})$ PFL-AE complex (A) before and (B) after photolysis at 12 K with 450 nm LED for 1 h to produce the $5'\text{-dAdo}\cdot$ radical with near-complete loss of the initial complex signal. Conversion is quantitative; residual cluster signal in spectrum B is from the enzyme out of the laser beam. (C) Time course for decay of $([\text{4Fe-4S}]^+ + \text{SAM})$ (■) upon photolysis monitored at 3600 G and increase of $5'\text{-dAdo}\cdot$ (●) monitored at 3360 G. The two progress curves are fit to stretched-exponential (as the result of light scattering within the “snow” samples) decay, $I = \exp(-[t/\tau]^n)$, and rise, $I = 1 - \exp(-[t/\tau]^n)$, functions with the same parameters, $\tau = 11(1)$ min and $n = 0.43(2)$. EPR conditions: microwave frequency, 9.38 GHz; modulation, 10 G; T , 12 K.

Figure 3.

A–E, X-band EPR spectra (black) and simulations (red) of 5'-dAdo•. 5'-dAdo• generated with (A) natural abundant SAM; (B) [adenosyl-5',5''-D₂]-SAM; (C) [adenosyl-2,8-D₂-1',2',3',4',5',5''-D₆]-SAM; (D) 5'-dAdo• with [adenosyl-¹³C₁₀,¹⁵N₅]-SAM; E, 5'-dAdo• with [adenosyl-5'-¹³C]-SAM. Features associated with the minor photolysis products are revealed in spectra of isotopologues (B, C) and indicated by (*). F, Q-band EPR, 5'-dAdo• generated with natural abundant SAM; the additional line width at low field is attributable to g/A strain. Simulations: Generated with EasySpin³¹ using a 5'-dAdo• model with parameters listed in Table 1. EPR conditions: microwave frequency, 9.38 GHz (A–C and G) and 34 GHz (D–F); modulation, 10 G; T, 40 K. Simulations: generated with Easyspin³¹ using a 5'-dAdo• model with parameters listed in Table 1. G, Q-band CW ¹³C ENDOR of 5'-dAdo•. From 11 to 15 MHz, generated with [adenosyl-¹³C₁₀,¹⁵N₅]-SAM where (▼) represents ¹³C Larmor frequency and the “goalpost” connecting the doublet from 1'-¹³C and/or 2'-¹³C, split by A = 0.8 MHz (see eq 1). From 27 to 170 MHz, generated with [adenosyl-¹³C₁₀,¹⁵N₅]-SAM where (●) represents A/2 and only the high-frequency members of the doublets for 3'-¹³C and 5'-¹³C are seen, as indicated, separated from their respective A/2 by ¹³C Larmor frequency. Spectrum between 70 and 149 MHz also seen when generated from [adenosyl-5'-¹³C]-SAM. It is overlaid on the signal from ¹H of C4' and 5' in the spectrum with natural-abundance SAM (gray line). CW ENDOR conditions: microwave frequency, 34.8 GHz; modulation, 2 G; T, 2 K.

Figure 4.

DFT models of 5'-dAdo-. Top: Perspective view of the optimized structure. Adenosine is represented by a violet sphere; the isosurface plot of the calculated HOMO (yellow) uses an isodensity of 0.08 au and shows the direction of g_3 normal to the $C5'H_2$ plane. Bottom: left, conformer with a dihedral "twist" at the $C5'-C4'$ bond, $\varphi = 0$; right, optimized structure geometry $\varphi = 39.3^\circ$.

Figure 5. Cartoon illustrating the proposed movement of 5'-dAdo· upon S-C(5') bond cleavage, as inferred from EPR and ENDOR data, based on PDB ID 3CB8;³⁸ [-C5'(H)₂] is shown as a purple ball.

Table 1.

Hyperfine Tensors (MHz) of 5'-dAdo $\cdot$ from the Experiment^a Plus DFT-Computed Values in Parentheses,^b with Reference Values for Radicals Generated from Malonic Acid^c

5'-dAdo $\cdot$	A ₁	A ₂	A ₃
5'-C- ¹ H α_a	-15(-28)	-105(-104)	-60(-63)
5'-C- ¹ H α_b	-20(-25)	-95(-101)	-60(-61)
4'-C- ¹ H β	+80(+90)	+80(+90)	+110(+105)
5'- ¹³ C α	+10(+2)	+10(+3)	+230(+240)
4'- ¹³ C	60(+69)	80(+86)	60(+68)
3'- ¹³ C	40(-36)	40(-33)	50(-37)
1'- or 2'- ¹³ C	0.8	0.8	3
$\cdot\text{CH}(\text{COOH})_2^c$	A ₁	A ₂	A ₃
¹ H α_a	-91	-29	-58
¹³ C α	+22.8	+42.2	+212.7
$\cdot\text{CH}_2(\text{COOH})^c$	A ₁	A ₂	A ₃
¹ H α_a	-30	-55	-91
¹ H α_b	-37	-59	-92

^aThe signs ($\pm$) of experimental 5'-dAdo $\cdot$ tensors are assigned according to ref 52; the hyperfine signs for 1'- and/or 2'-¹³C, 3'-¹³C, and 4'-¹³C are not determined. The simulations employ $g = [2.0075, 2.0015, 2.000]$ ($g_{\text{ave}} = 2.003$) and tabulated hyperfine couplings. The A₃ components for ¹H α_a and ¹H α_b are parallel to g_3 and normal to the C5'H₂ plane (see Figure 3) as seen in refs 54 and 55. For ¹H α_a , A₁|| g_1 ; simulation required the ¹H β tensor be rotated relative to that of ¹H α_a by an angle approaching $\alpha \sim 120^\circ$ expected for sp² hybridized C5' and found by DFT optimization, but because of the small g-anisotropy, this angle could be allowed to vary over the range $90^\circ \leq \alpha \leq 120^\circ$. A₃ for 4'-C-¹H β makes an angle of $\sim 40^\circ$ with respect to g_3 . The EPR simulations tightly constrain the values of the tensor components; for strongly coupled nuclei (all but 1'- and/or 2'-¹³C), numerous simulations indicate that one tensor component value may vary by as much as ± 5 MHz, but the sum of multiple variations must also be no more than roughly ± 5 MHz. Additional EasySpin simulation parameters: EPR line width, 36 MHz (X-band); 45 MHz (Q-band). Hyperfine strain parameters employed to account for unresolved hyperfine coupling: [30, 10, 10] MHz for A-C, E, and F; [30, 10, 40] MHz for D. The simulation for D is the sum of 80% ¹³C 5'-dAdo $\cdot$ and 20% ¹²C, and for E, it is the sum of 85% ¹³C 5'-dAdo $\cdot$ and 15% ¹²C.

^bThe hyperfine tensors in parentheses are from the DFT calculation with B3LYP/G (Supporting Information). The energy-minimized structure (Figure 4) has equivalent C5'-H bonds and ¹H hyperfine tensors, whereas the experiment gives slightly different tensors for the two. We attribute the observed inequivalence to a slight desymmetrization due to interactions in the enzyme active site that are absent in the computation.

^c¹H and ¹³C tensors for $\cdot\text{CH}(\text{COOH})_2$ formed by irradiation of malonic acid, taken from McConnell et al.⁵⁵ and Cole and Heller,⁵⁶ respectively.

¹H tensors for $\cdot\text{CH}_2(\text{COOH})$ ($g = [2.0042, 2.0034, 2.0020]$, $g_{\text{ave}} = 2.0032$) formed by irradiation of malonic acid; see ref 54. The somewhat greater inequivalence between the hyperfine tensors for two α -type ¹H of 5'-dAdo $\cdot$ and $\cdot\text{CH}_2(\text{COOH})$ likely reflects the influence of contacts with the surrounding residues, as does the observation that the two radicals have the same g_{ave} yet differ in their in-plane g-values, g_1 and g_2 .