

## Coordination of Adenosylmethionine to a Unique Iron Site of the [4Fe-4S] of Pyruvate Formate-Lyase Activating Enzyme: A Mössbauer Spectroscopic Study

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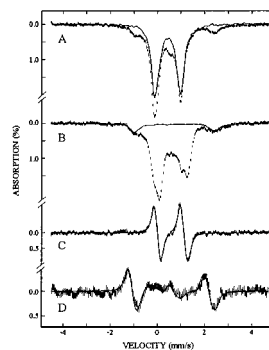
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Iron–sulfur clusters have long been recognized as important electron-transfer centers in biological systems. More recently, numerous alternative roles for Fe–S clusters have been revealed,<sup>1</sup> including a key role in catalyzing *S*-adenosylmethionine (AdoMet or SAM)-mediated radical reactions.<sup>2,3</sup> The so-called “radical-SAM superfamily” of enzymes now includes hundreds of probable members,<sup>4</sup> all of which presumably use an Fe–S cluster and AdoMet to catalyze radical reactions. The remarkably diverse range of reactions catalyzed by the Fe–S/AdoMet enzymes includes generation of catalytically essential protein radicals,<sup>5,6</sup> isomerization reactions,<sup>7</sup> sulfur insertion,<sup>8,9</sup> and DNA repair.<sup>10</sup> Despite this diversity of function, evidence suggests that the Fe–S/AdoMet enzymes operate by common initial steps involving the generation of an AdoMet-derived adenosyl radical intermediate.<sup>12</sup> How this cofactor ensemble interacts to generate an adenosyl radical intermediate remains a central unresolved question for this class of enzymes. The three cysteine CX<sub>3</sub>CX<sub>2</sub>C cluster binding motif common to all members of the superfamily suggests a unique Fe site in the [4Fe-4S] cluster, which has been presumed to interact with AdoMet to effect reductive cleavage and radical generation.<sup>2,3</sup> Here we provide the first direct spectroscopic evidence for such a unique iron site in the [4Fe-4S] cluster of one of the radical-SAM enzymes, pyruvate formate-lyase activating enzyme (PFL-AE), and we demonstrate that the unique site plays a key role in catalysis by coordinating AdoMet.

PFL-AE generates the catalytically essential glycy radical of PFL.<sup>5</sup> Anaerobically isolated PFL-AE contains a mixture of cluster forms, including cuboidal [3Fe-4S]<sup>+</sup> as the major cluster form, with linear [3Fe-4S]<sup>+</sup>, [4Fe-4S]<sup>2+</sup>, and [2Fe-2S]<sup>2+</sup> present as minor components.<sup>11,12</sup> Reduction of PFL-AE converts all cluster forms to [4Fe-4S] clusters,<sup>12</sup> which we have shown to be the catalytically relevant cluster of PFL-AE.<sup>13</sup> The variety of cluster forms supported in PFL-AE, together with their conversion to [4Fe-4S] clusters under reducing conditions, suggested a similarity to aconitase, the only other enzyme reported to contain all these cluster forms.<sup>14</sup> In particular, the ready accessibility of a [3Fe-4S]<sup>+</sup> cluster suggested that PFL-AE, like aconitase, contains a catalytically relevant unique iron site in the [4Fe-4S] cluster.

A dual-iron-isotope (<sup>56</sup>Fe/<sup>57</sup>Fe) approach was used to demonstrate the existence of a unique Fe site in the [4Fe-4S] of PFL-AE and its interaction with AdoMet by Mössbauer spectroscopy. Anaerobically purified PFL-AE (98% <sup>56</sup>Fe) was exposed to air to generate the paramagnetic [3Fe-4S]<sup>+</sup> form, and gel-filtered to remove released iron. <sup>57</sup>Fe<sup>II</sup> was added to reconstitute the [4Fe-4S] cluster, and DTT was added as a reductant.<sup>15</sup> The reconstituted enzyme was found to be EPR silent, indicating that the [4Fe-4S] is in the diamagnetic 2+ oxidation state.



**Figure 1.** Mössbauer spectra of <sup>56</sup>Fe PFL-AE reconstituted with <sup>57</sup>Fe and DTT in the absence (A) and presence (B) of AdoMet. The data (hashed marks) were recorded at 4.2 K in a magnetic field of 50 mT applied parallel to the  $\gamma$  rays. The solid line in A is the experimental spectrum of [4Fe-4S]<sup>2+</sup> clusters in PFL-AE normalized to 70% of the total Fe absorption of A. The solid line in B is the spectrum of a control sample containing only the reconstitution ingredients and AdoMet but without PFL-AE and is normalized to 15% of the total Fe absorption of B. A difference spectrum of B minus A is shown in C. Spectrum D is a difference spectrum of the spectra of samples A and B recorded in a parallel field of 8 T. The solid lines in C and D are difference spectra of theoretical simulations of the unique Fe site with and without AdoMet using the parameters given in the text and assuming diamagnetism.

The state of the added <sup>57</sup>Fe after cluster reconstitution was monitored by Mössbauer spectroscopy. A 4.2 K Mössbauer spectrum (Figure 1A, hashed marks) of the reconstituted enzyme shows that 70% of the added <sup>57</sup>Fe appears as a quadrupole doublet typical of a [4Fe-4S]<sup>2+</sup> in PFL-AE<sup>12</sup> (solid line, Figure 1A). We estimate the amount of <sup>57</sup>Fe incorporated in [4Fe-4S] clusters (365  $\mu$ M) is comparable to the amount of [3Fe-4S]<sup>+</sup> (380  $\mu$ M, from EPR) present prior to addition of <sup>57</sup>Fe and DTT.<sup>16</sup> Taken together, these data strongly suggest that under the reconstitution conditions the [3Fe-4S]<sup>+</sup> cluster is converted into a [4Fe-4S]<sup>2+</sup> cluster by selective incorporation of the <sup>57</sup>Fe into the fourth, unique Fe site, a process also observed in aconitase.<sup>17</sup> Addition of AdoMet (10 equiv) to the reconstituted enzyme yields the Mössbauer spectrum shown in Figure 1B. The quadrupole doublet arising from the unique Fe site has reduced in intensity while a new quadrupole doublet appears, indicating that a portion of the unique Fe site has converted into a new Fe species.<sup>18</sup> This conversion is best illustrated by a difference spectrum between A and B to eliminate contributions from Fe species that are common to both samples (Figure 1C, B minus A, hashed marks). The amount of the unique Fe site that converts to the new Fe species appears as a doublet pointing upward and accounts for 32% of the total <sup>57</sup>Fe in the sample, while the new Fe species accounting for the same portion appears as a doublet pointing downward. In the absence of AdoMet, as expected, the quad-

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rupole splitting ( $\Delta E_Q = 1.12$  mm/s) and isomer shift ( $\delta = 0.42$  mm/s) are typical for Fe sites in a  $[4\text{Fe-4S}]^{2+}$  cluster. In the presence of AdoMet, however, the parameters ( $\Delta E_Q = 1.15$  mm/s,  $\delta = 0.72$  mm/s) are distinct, with the unusually large isomer shift signaling an increase of coordination number and/or binding of more ionic ligands, suggesting coordination of the substrate AdoMet to the unique Fe site. The solid line shown in Figure 1C is a theoretical difference spectrum of the two doublets mentioned above.

To show that the doublets in A and B originate from Fe associated with PFL-AE and not from Fe in solution, we obtained the Mössbauer spectrum of a sample containing  $^{57}\text{Fe}^{\text{II}}$ , DTT, and AdoMet but without PFL-AE. The spectrum, shown as a solid line in Figure 1B and normalized to 15% of the Fe absorption, exhibits a broad quadrupole doublet with parameters ( $\Delta E_Q = 3.38$  mm/s,  $\delta = 0.73$  mm/s) typical of a tetracoordinate  $\text{Fe}^{\text{II}}\text{S}_4$  complex. The quadrupole doublets assigned to the unique Fe are not observed in the control sample.

To demonstrate that both quadrupole doublets are due to an Fe site that is part of a diamagnetic  $[4\text{Fe-4S}]^{2+}$  cluster, we have recorded the spectra of the reconstituted PFL-AE with and without AdoMet in a parallel applied field of 8 T. A difference spectrum of the two 8-T spectra is shown in Figure 1D. The solid line overlaid with the experimental data is a theoretical difference spectrum using the parameters obtained for the two doublets mentioned above and assuming diamagnetism. The excellent agreement between theory and experiment establishes unambiguously that the two doublets are indeed arising from an Fe site associated with a diamagnetic system. This observation also indicates that upon binding of the substrate, the unique Fe site remains exchange coupled to the other 3 Fe atoms in the cluster.

The data presented here support the conclusion that the unique iron site of the  $[4\text{Fe-4S}]$  is coordinated by AdoMet in the enzyme–substrate complex. The observation that AdoMet coordinates to the  $[4\text{Fe-4S}]^{2+}$  cluster prior to the injection of the reducing equivalent required for catalysis further indicates that this coordination is a necessary prerequisite to adenosyl radical generation. The  $\text{CX}_3\text{-CX}_2\text{C}$  cluster-binding motif conserved among Fe–S/AdoMet enzymes suggests that a site-differentiated  $[4\text{Fe-4S}]$  cluster, as shown here for PFL-AE, may also be conserved throughout this superfamily.

AdoMet has multiple potential coordinating atoms, including the amino and carboxylate groups, the ribose hydroxyls, and the sulfonium center. Interaction of the sulfonium with an Fe site of the  $[4\text{Fe-4S}]$  has been suggested as the initial step in the formation of the adenosyl radical for the radical-SAM superfamily member lysine 2,3-aminomutase (LAM). A Se X-ray absorption spectroscopic (XAS) study of LAM showed an Fe–Se interaction of 2.7 Å between Se–Met (the cleavage product of Se–AdoMet) and an Fe of the cluster.<sup>19</sup> The large Mössbauer isomer shift observed in the present work for the AdoMet-bound unique Fe site, however, disfavors sulfur coordination. This discrepancy may reflect the different roles played by AdoMet in the two enzymes. In PFL-AE, AdoMet is a substrate that is irreversibly cleaved, yielding an adenosyl radical that generates the catalytically active glycy radical on PFL. In contrast, AdoMet acts as a cofactor in LAM, and is reversibly cleaved to yield the catalytically active adenosyl radical. Also, it should be pointed out that in the LAM XAS study, the Fe–Se interaction observed represents an interaction of the cluster with cleaved products, while the unique Fe–AdoMet interaction reported here represents interaction prior to substrate cleavage.

In the case of aconitase, coordination of substrate to the unique Fe site also results in an increase of the isomer shift (from 0.44 mm/s for the unbound state to 0.84 and 0.89 mm/s for the substrate-

bound states). This increase in isomer shift, when correlated with X-ray crystallographic results, reflects binding of both a carboxylate and a hydroxyl group to the Fe–S cluster.<sup>17</sup> By analogy to aconitase, coordination of the amino and carboxylate groups, or the ribose hydroxyls of AdoMet to the unique site in PFL-AE would be consistent with the observed increase in isomer shift. We currently favor these possibilities, since there are precedents from model chemistry for these binding modes for transition metals.<sup>20–22</sup> Furthermore, these binding modes could position AdoMet in a conformation where the sulfonium or 5'-C is in close proximity to the  $[4\text{Fe-4S}]$  cluster. Adenosyl radical generation may then proceed by nucleophilic attack of a coordinated cysteinate or  $\mu_3$ -sulfide at the 5'-C or via direct interaction of the cluster with the sulfonium. The data presented here are insufficient to unambiguously identify the coordinating group(s). Further experiments are underway to clarify the situation.

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## References

- Beinert, H. *J. Biol. Inorg. Chem.* **2000**, *5*, 2–15.
- Cheek, J.; Broderick, J. B. *J. Biol. Inorg. Chem.* **2001**, *6*, 209–226.
- Frey, P. A. *Annu. Rev. Biochem.* **2001**, *70*, 121–148.
- Sofia, H. H.; Chen, G.; Hetzler, B. G.; Reyes-Spindola, J. F.; Miller, N. E. *Nucleic Acids Res.* **2001**, *29*, 1097–1106.
- (a) Knappe, J.; Elbert, S.; Frey, M.; Wagner, A. F. V. *Biochem. Soc. Trans.* **1993**, *21*, 731–734. (b) Wong, K. K.; Kozarich, J. W. *Met. Ions Biol. Syst.* **1994**, *30*, 279–313. (c) Broderick, J. B.; Duderstadt, R. W.; Fernandez, D. C.; Wojtuszewski, K.; Henshaw, T. F.; Johnson, M. K. *J. Am. Chem. Soc.* **1997**, *119*, 7396–7397.
- (a) Ollagnier, S.; Mulliez, E.; Schmidt, P. P.; Eliasson, R.; Gaillard, J.; Deronzier, C.; Bergman, T.; Gräslund, A.; Reichard, P.; Fontecave, M. *J. Biol. Chem.* **1997**, *272*, 24216–24223. (b) Ollagnier, S.; Meier, C.; Mulliez, E.; Gaillard, J.; Schünemann, V.; Trautwein, A. X.; Mattioli, T.; Lutz, M.; Fontecave, M. *J. Am. Chem. Soc.* **1999**, *121*, 6344–6350.
- (a) Lieder, K.; Booker, S.; Ruzicka, F. J.; Beinert, H.; Reed, G. H.; Frey, P. A. *Biochemistry* **1998**, *37*, 2578–2585. (b) Petrovich, R. M.; Ruzicka, F. J.; Reed, G. H.; Frey, P. A. *Biochemistry* **1992**, *31*, 10774–10781.
- (a) Sanyal, I.; Cohen, G.; Flint, D. H. *Biochemistry* **1994**, *33*, 3625–3631. (b) Duin, E. C.; Lafferty, M. E.; Crouse, B. R.; Allen, R. M.; Sanyal, I.; Flint, D. H.; Johnson, M. K. *Biochemistry* **1997**, *36*, 11811–11820.
- (a) Busby, R. W.; Schelvis, J. P. M.; Yu, D. S.; Babcock, G. T.; Marletta, M. A. *J. Am. Chem. Soc.* **1999**, *121*, 4706–4707. (b) Ollagnier-de Choudens, S.; Fontecave, M. *FEBS Lett.* **1999**, *453*, 25–28.
- Rebeil, R.; Sun, Y.; Choo-back, L.; Pedraza-Reyes, M.; Kinsland, C.; Begley, T. P.; Nicholson, W. L. *J. Bacteriol.* **1998**, *180*, 4879–4885.
- Broderick, J. B.; Henshaw, T. F.; Cheek, J.; Wojtuszewski, K.; Trojan, M. R.; McGhan, R.; Smith, S. R.; Kopf, A.; Kibbey, M.; Broderick, W. E. *Biochem. Biophys. Res. Commun.* **2000**, *269*, 451–456.
- Krebs, C.; Henshaw, T. F.; Cheek, J.; Huynh, B. H.; Broderick, J. B. *J. Am. Chem. Soc.* **2000**, *122*, 12497–12506.
- Henshaw, T. F.; Cheek, J.; Broderick, J. B. *J. Am. Chem. Soc.* **2000**, *122*, 8331–8332.
- Kennedy, M. C.; Kent, T. A.; Emptage, M.; Merkle, H.; Beinert, H.; Münck, E. *J. Biol. Chem.* **1984**, *259*, 14463–14471.
- PFL-AE was purified as described previously<sup>11</sup> except that 1 mM DTT was included in all buffers. Inclusion of DTT has been found to result in  $[4\text{Fe-4S}]^{2+}$  as the primary cluster form.  $[3\text{Fe-4S}]^+$  was generated by exposure of concentrated PFL-AE to air for 30 min on ice, followed by gel filtration (50 mM Tris, 200 mM NaCl, pH 8.5) through a 5 mL HiTrap (Pharmacia) desalting column. The amount of  $[3\text{Fe-4S}]^+$  formed was quantified with use of EPR spectroscopy. Reconstitution with  $^{57}\text{Fe}$  was accomplished by addition of 1.4 equiv (relative to  $[3\text{Fe-4S}]^+$ ) of  $^{57}\text{FeSO}_4$  to the  $[3\text{Fe-4S}]^+$ , under an inert atmosphere, followed by addition of DTT (10 mM).
- Similar reconstitution experiments have been repeated twice and in each case the amount of  $^{57}\text{Fe}$  incorporated into the  $[4\text{Fe-4S}]$  is comparable to that of  $[3\text{Fe-4S}]^+$  quantified by EPR.
- Beinert, H.; Kennedy, M. C.; Stout, C. D. *Chem. Rev.* **1996**, *96*, 2335–2373 and references therein.
- Reconstitution of air-exposed  $^{57}\text{Fe}$  PFL-AE with  $^{56}\text{Fe}$  yields a Mössbauer spectrum ( $\Delta E_Q = 1.07$  mm/s and  $\delta = 0.45$  mm/s) that is similar to that of the  $[4\text{Fe-4S}]^{2+}$  but insensitive to the presence of AdoMet.
- Cosper, N. J.; Booker, S. J.; Ruzicka, F.; Frey, P. A.; Scott, R. A. *Biochemistry* **2000**, *39*, 15668–15673.
- Burger, J.; Klufers, P. Z. *Anorg. Allg. Chem.* **1996**, *622*, 1740–1748.
- Angus-Dunne, S. J.; Batchelor, R. J.; Tracey, A. S.; Einstein, F. W. B. *J. Am. Chem. Soc.* **1995**, *117*, 5292–5296.
- Evans, C. A.; Guevremont, R.; Rabenstein, D. L. *Metal Ions Biol. Syst.* **1979**, *9*, 41–75.

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