Minireview

Pyruvate formate-lyase activating enzyme: elucidation of a novel mechanism for glycyl radical formation

Jeffrey M. Buis, Joan B. Broderick

Department of Chemistry, Michigan State University, East Lansing, MI 48824, United States

Received 3 August 2004, and in revised form 24 September 2004
Available online 2 November 2004

Abstract

Pyruvate formate lyase activating enzyme is a member of a novel superfamily of enzymes that utilize S-adenosylmethionine to initiate radical catalysis. This enzyme has been isolated with several different iron–sulfur clusters, but single turnover monitored by EPR has identified the [4Fe–4S]1+ cluster as the catalytically active cluster; this cluster is believed to be oxidized to the [4Fe–4S]2+ state during turnover. The [4Fe–4S] cluster is coordinated by a three-cysteine motif common to the radical/S-adenosylmethionine superfamily, suggesting the presence of a unique iron in the cluster. The unique iron site has been confirmed by Mössbauer and ENDOR spectroscopy experiments, which also provided the first evidence for direct coordination of S-adenosylmethionine to an iron–sulfur cluster, in this case the unique iron of the [4Fe–4S] cluster. Coordination of the unique iron anchors the S-adenosylmethionine in the active site, and allows for a close association between the sulfonium of S-adenosylmethionine and the cluster as observed by ENDOR spectroscopy. The evidence to date leads to a mechanistic proposal involving inner-sphere electron transfer from the cluster to the sulfonium of S-adenosylmethionine, followed by or concomitant with C–S bond homolysis to produce a 5′-deoxyadenosyl radical; this transient radical abstracts a hydrogen atom from G734 to activate pyruvate formate lyase.

Keywords: Pyruvate formate lyase; Radical SAM; S-Adenosylmethionine; Glycyl radical; Iron–sulfur cluster; EPR; ENDOR; Mössbauer

Iron–sulfur clusters are ubiquitous in nature, catalyzing a wide variety of reactions. These multi-functional cofactors can exist in multiple cluster types and redox states. The clusters have long been known to facilitate electron transfer in such crucial metabolic pathways as respiration and photosynthesis. More recently, they have been implicated in redox and non-redox catalysis in enzymes such as nitrogenase and aconitase, gene regulation in proteins such as the iron responsive protein, fumarate–nitrate reduction protein, and SoxR, and structural roles such as in the DNA repair protein MutY [1,2]. One of the most fascinating new discoveries has been the role of Fe–S clusters in initiating radical catalysis, a role found in the so-called radical SAM superfamily [3–9].

S-Adenosylmethionine (SAM, AdoMet)1 has long been established as a methylating agent in various biochemical pathways, but its recent emergence in radical catalysis is a surprising new role. Use of AdoMet as a cofactor or cosubstrate is one of the characteristic features of the radical SAM superfamily, as is the presence of a catalytically essential iron–sulfur cluster [4–9]. It is thought that these enzymes function by AdoMet cleavage via electron transfer from the Fe–S cluster to

---

1 Abbreviations used: AdoCbl, adenosylcobalamin; BioB, biotin synthase; LipA, lipoic acid synthase; PFL-AE, pyruvate formate lyase-activating enzyme; LAM, lysine-2,3-aminomutase; aRNR, anaerobic ribonucleotide reductase; PFL, pyruvate formate lyase; DTT, dithiothreitol; ENDOR, electron-double-nuclear-resonance.

0003-9861/S - see front matter © 2004 Elsevier Inc. All rights reserved.
generate a transient 5'-deoxyadenosyl radical and methionine. The adenosyl radical then acts to abstract a hydrogen atom from substrate and initiates further radical catalysis [4–11]. The only other known enzymes that act via an adenosyl radical are those utilizing adenosylcobalamin (AdoCbl), in which the Co–C bond is cleaved homolytically to initiate radical catalysis [12–14].

The discovery of the radical SAM superfamily started in the late 1960s and early 1970s with work carried out by Barker and co-workers on the isomerase lysine 2,3-aminomutase, which was shown to require AdoMet rather than the AdoCbl used by many similar isomerases [6,12,15,16]. Later, work on pyruvate formate lyase would show that it required a second enzyme for activity that was an AdoMet-dependent activase, which generated a glycyl radical [17,18]. Other radical-SAM enzymes that have been studied to date carry out diverse reactions. Biotin synthase (BioB) and lipoic acid synthase are representative members of this intriguing superfamily, is a monomeric protein which catalyzes the formation of the glycine rich region believed to bind AdoMet [3]. The pyruvate formate lyase (PFL), and pyruvate formate lyase (PFL) are involved in the creation of stable protein radicals [24–26]. Although only a relatively small number of enzymes in this superfamily have been characterized, a recent bioinformatics study has shown the possibility of over 600 different members, all of which contain the characteristic CX3CX2C cluster binding motif and the glycine rich region believed to bind AdoMet [3]. The pyruvate formate lyase-activating enzyme (PFL-AE), a representative member of this intriguing superfamily, is the focus of this review.

Pyruvate formate lyase catalyzes the conversion of pyruvate and coenzyme A to formate and acetyl CoA as seen in Fig. 1. This is a reversible reaction with turnover numbers of 770 s⁻¹ (forward) and 260 s⁻¹ (reverse) at 30 °C [27]. This reaction is the first committed step of anaerobic glucose metabolism and is important in *Escherichia coli* and other facultative anaerobes. PFL is a 170 kDa dimer that has no metal or cofactor present and requires post-translational activation by an activase called pyruvate formate lyase-activating enzyme (PFL-AE) [15,28]. PFL-AE activates PFL by generating a stable glycyl radical at Gly734; the surprising discovery of this radical provided the first example of a stable glycyl radical in an enzyme [29]. The active enzyme contains one radical spin per dimer and the EPR signal shows a prominent doublet with 55% of spin density located on the α carbon of G734 [29–31]. The radical is thought to be stabilized by the captodative effect and has a half-life of hours at 30 °C, however it is susceptible to rapid and irreversible oxygen inactivation resulting in 82 and 3 kDa fission products [31]. Knappe and co-workers [32] showed that it is the pro-S hydrogen which is abstracted from Gly734, and that the abstracted hydrogen ends up on 5'-deoxyadenosine, thereby implicating a 5'-deoxyadenosyl radical as the immediate H-atom abstracting species.

The structure of PFL shows a striking similarity to that of the aRNR; both have a 10 stranded α/β barrel with the protein’s active site glycine buried in the middle of the barrel [33–35]. In PFL, a cysteine residue, Cys419, is located close to Gly734 and its mutation yields inactive protein [36]. These studies along with further EPR studies using the biologically relevant pyruvate analog, mercaptopyruvate, suggested radical formation at Cys419 and Cys418 [37]. The glycyl radical is thus thought to generate a thyl radical via H atom abstraction in the active site, and the thyl radical is thought to be directly involved in C–C homolytic bond cleavage (Fig. 2) [36–38].

Pyruvate formate lyase-activating enzyme is a 28 kDa monomeric protein which catalyzes the formation of the Gly734 radical of PFL [17,26]. This protein’s activity is iron-dependent and has the CX3CX2C binding motif common among the radical SAM superfamily. It has been proposed that PFL-AE uses an Fe–S cluster to cleave AdoMet to form an adenosyl radical and that this radical goes on to abstract the pro-S hydrogen of the Gly734 residue of PFL, thus activating the enzyme as seen in Fig. 3 [4]. In our efforts to understand this unusual enzymatic reaction and to gain a greater insight into the mechanism of the radical SAM superfamily, we have focused primarily on the role of the Fe–S cluster and its interaction with AdoMet as discussed in the remainder of this review.

**The iron–sulfur cluster of PFL-AE**

PFL-AE was first isolated by Knappe and co-workers [17] from non-overexpressing *E. coli* and exhibited an absorption spectrum characteristic of a covalently bond cofactor. This PFL-AE was dependent on the presence

![Fig. 1. The reaction of pyruvate to formate as catalyzed by PFL.](image-url)
of exogenous iron for activity, although the role of the iron or form in which it was used was unclear. PFL-AE was later isolated from overexpressing *E. coli*, however the overexpressed protein was present in inclusion bodies and thus required denaturation with guanidine HCl prior to purification [39]. The purified, refolded protein had no metal present, but addition of iron produced some enzyme activity. Although the refolded PFL-AE could bind other metals such as Cu(II) and Cd(II), these were found to yield no activity and to inhibit activity in the presence of iron. Even after iron binding, the refolded protein had a low specific activity of 1.9 U/mg versus 25 U/mg for the protein isolated from non-overexpressing *E. coli* [39].

---

**Fig. 2.** Proposed mechanism of pyruvate formate lyase. The Gly734 radical abstracts hydrogen from Cys419 and causes radical formation at Cys418. This radical interacts with pyruvate to facilitate C–C bond homolysis yielding formate. Adapted from [35].

**Fig. 3.** The reaction catalyzed by pyruvate formate lyase activating enzyme. PFL-AE facilitates AdoMet cleavage to methionine and 5'-deoxyadenosine, producing a glyyl radical at Gly734 and activating PFL.
Although it was clear from these early studies that the presence of iron was required for activity, it was not known what the state of the iron was. In subsequent work carried out on protein purified under crude anaerobic conditions without denaturation, EPR and resonance Raman spectroscopy demonstrated for the first time the presence of iron–sulfur clusters in PFL-AE. The as-isolated protein contained a mixture of [2Fe–2S]^{2+} and [4Fe–4S]^{2+} clusters, which were converted to [4Fe–4S]^{2+} clusters upon reduction [26]. The addition of AdoMet was required for reduction of the cluster to the [4Fe–4S]^{1+} state. The activity of this native PFL-AE (5.4 U/mg) also proved to be lower than that reported by Knappe and co-workers [17,26]. Subsequent to the identification of an iron–sulfur cluster in PFL-AE, Knappe and co-workers [18] demonstrated the ability to reconstitute a [4Fe–4S] cluster in purified apo-PFL-AE. They also identified the CX_{3}CX_{2}C binding motif as the coordinating site of the iron–sulfur cluster by site-directed mutagenesis of each of the six cysteines in PFL-AE, followed by reconstitution. Each of the cysteine mutants of the CX_{3}CX_{2}C motif showed no activity, providing support for this conserved motif as the binding site of the Fe–S cluster [18]. For both the purified “native” PFL-AE and the reconstituted apo-PFL-AE, it was demonstrated that exogenous iron was not needed for protein activity, thereby showing that the iron previously required for PFL-AE activity was needed only to reconstitute the catalytically essential iron–sulfur cluster [18,26].

Since crude anaerobic conditions allowed the first isolation of PFL-AE containing an iron–sulfur cluster, subsequent purifications were done under strictly anaerobic conditions, which dramatically improved not only cluster content but also enzyme specific activity [40]. Mössbauer spectroscopy of PFL-AE purified in this way revealed a mixture of cluster states, including [2Fe–2S]^{2+}, [3Fe–4S]^{1+}, and [4Fe–4S]^{2+} clusters [41]. Upon reduction, the clusters were converted into a mixture of the [4Fe–4S]^{2+} and [4Fe–4S]^{1+} states; in contrast to earlier work, AdoMet was not necessary for reduction to the [4Fe–4S]^{1+} state [40,41]. Taken together, this work demonstrated that PFL-AE was an iron–sulfur cluster protein with the cluster binding at the CX_{3}CX_{2}C site.

**Identification of the active cluster**

While the work just described confirmed that PFL-AE possessed an Fe–S cluster, it was not yet known which of the several observed cluster states was the active form. To identify the active state cluster, a series of EPR experiments under controlled reduction conditions was carried out [42]. It was found that PFL-AE could be reduced by 5-deazariboflavin via photoreduction. This photoreduction procedure allowed for quantitative reduction to the [4Fe–4S]^{1+} state, as well as rapid and easy removal of exogenous reductant by removing the light source. Deazariboflavin-mediated photoreduction allowed us to monitor single turnover of the enzyme from well-defined oxidation states, in the absence of complications due to reductant-promoted cluster rearrangements. It was thought that the [4Fe–4S]^{1+} was the active cluster state in the radical SAM enzymes, as earlier work on LAM and aRNR had shown the presence of a [4Fe–4S]^{1+} in active protein samples [43,44]. Therefore, PFL-AE was photo-reduced using 5-deazariboflavin at various illumination times to control the amount of reduction and thereby control the amount of [4Fe–4S]^{1+}. A 10-fold excess of AdoMet was added so that the cluster would be the limiting reagent. The samples were kept in the dark after illumination to eliminate the exogenous source of electrons, and PFL was then added to one set of samples, with an identical set kept as controls.

The samples were characterized by EPR spectroscopy as shown in Fig. 4 [42]. EPR spectra of the controls show an increasing amount of [4Fe–4S]^{1+} with increasing illumination time. EPR spectra of the PFL-added samples show an increasing amount of glycol radical signal but
no signal corresponding to the [4Fe–4S]^{1+}. Spin quantitation of these EPR signals revealed a 1 to 1 correspondence between the amount of [4Fe–4S]^{1+} in the controls and the amount of glycy radical in the PFL-added samples over the entire range of illumination times [42]. The correspondence of the glycy radical signal in the “+PFL” samples with that of the [4Fe–4S]^{1+} signal in the “−PFL” samples demonstrated that the [4Fe–4S]^{1+} was being oxidized to an EPR-silent state during reaction, and that it was generating the glycy radical in PFL. This could occur by electron transfer from the cluster to AdoMet to precipitate adenosyl radical formation and subsequent abstraction of hydrogen from G734 of PFL. This experiment demonstrated conclusively for the first time that the [4Fe–4S]^{1+} was the active cluster of PFL-AE, and by extrapolation, likely the active cluster for all enzymes in the radical SAM superfamily.

A unique iron site in PFL-AE

Similar to aconitase [45], the proteins of the radical SAM superfamily have only three conserved cysteine residues to coordinate the irons of the [4Fe–4S] cluster. Another ligand must therefore be coordinating the remaining fourth iron position, making it unique among the irons in the [4Fe–4S] cluster. Because the unique iron site appears to be conserved among the radical SAM superfamily, as evidenced by the conserved CX_{3}CX_{2}C motif, it was theorized that like aconitase, the unique iron site plays a pivotal role in catalysis.

To confirm the presence of a unique iron in PFL-AE, Mössbauer spectroscopy samples were prepared using a dual 56/57Fe isotope approach [46]. Natural abundance (56Fe) PFL-AE was grown and purified under anaerobic conditions to produce a sample containing mainly [4Fe–4S]^{2+}. This PFL-AE was then exposed to air to produce a [3Fe–4S]^{1+} cluster, and gel filtered to remove the released iron. The oxidation procedure was likely to result in loss of iron from the unique site only, as this non-cysteine-coordinated site was expected to be more labile than the other three sites. The cluster was then reconstituted by adding 57Fe and dithiothreitol (DTT) to produce the EPR silent [4Fe–4S]^{2+} cluster, and gel filtered to remove the released iron. The oxidation procedure was likely to result in loss of iron from the unique site only, as this non-cysteine-coordinated site was expected to be more labile than the other three sites. The cluster was then reconstituted by adding 57Fe and dithiothreitol (DTT) to produce the EPR silent [4Fe–4S]^{2+} cluster, presumably with 57Fe in the unique site. Mössbauer spectra were taken of the 56/57Fe PFL-AE in both the presence and the absence of a 10-fold excess of AdoMet (Fig. 5) [46]. The data indicate that 57Fe was successfully incorporated into the [3^{56}Fe–4S]^{1+} at the unique site to form the [3^{56}Fe(57Fe)–4S]^{2+}, with a quadrupole doublet characteristic of a typical [4Fe–4S]^{2+} cluster. In the presence of AdoMet a new quadrupole doublet formed while the original doublet decreased in intensity (Fig. 5). The presence of a new doublet indicates the formation of a new 57Fe species, and the large isomer shift is consistent with an increase in iron coordination or the binding of more ionic ligands, both of which suggest the coordination of AdoMet to the unique iron of the cluster [46].

While these results identify a unique iron site in the [4Fe–4S] of PFL-AE, and provide evidence for the coordination of AdoMet to the unique iron, they do not identify the specific interaction of the cluster and AdoMet. For lysine 2,3-aminomutase in the presence of selenomethionine and 5′-deoxyadenosine, selenium X-ray absorption studies showed the close association (2.7 Å) between the selenium and an iron of the cluster, however no such interaction between Se-AdoMet and Fe was found [47]. In PFL-AE, in contrast, the large isomer shift in the Mössbauer spectrum of the new (+AdoMet) iron species disfavors the interaction of AdoMet with the iron via the sulfonium. These results were further supported by selenium X-ray absorption spectroscopic data for PFL-AE complexed with Se-AdoMet or Se-methionine and 5′-deoxyadenosine, in which there was no observed close contact between the sulfonium and the iron of the [4Fe–4S] cluster [48]. These results suggest...
that AdoMet may interact with the iron–sulfur cluster of LAM in subtly differently ways than it interacts with the cluster in PFL-AE. Although at first surprising given the expectation of similar mechanisms among members of the superfamily, this difference in interaction may correlate with the difference in use of AdoMet as a cofactor (LAM) or as a substrate (PFL-AE).

Coordination of the unique iron by AdoMet

Mössbauer spectroscopy provided evidence for a unique iron in the [4Fe–4S] cluster of PFL-AE, and also for a direct interaction of this unique iron with AdoMet [46]. To better define this interaction, electron-double-nuclear-resonance (ENDOR) spectroscopy experiments were pursued [49,50]. ENDOR can probe the interactions of the electron spins of the paramagnetic cluster with the nuclear spins of surrounding atoms, yielding detailed information on coordination and distance [51]. The use of this method provides valuable insight into the structural interaction between AdoMet and the iron–sulfur cluster of PFL-AE, as well as potential cleavage mechanisms.

Sample preparation for ENDOR spectroscopy required the synthesis of isotopically labeled AdoMet, for example, incorporating $^2$H or $^{13}$C at the methyl position [49,50]. The labeled AdoMet was added to [4Fe–4S]$^{1+}$-PFL-AE and then frozen for examination by ENDOR spectroscopy. Alternatively the labeled AdoMet was added to the diamagnetic [4Fe–4S]$^{2+}$-PFL-AE, which was then frozen and subjected to $\gamma$-irradiation to reduce the cluster. The latter method (cryoreduction) generates the ENDOR-detectable [4Fe–4S]$^{1+}$ state trapped in the geometry of the [4Fe–4S]$^{2+}$ state, thereby allowing us to probe the diamagnetic $^{2+}$ state geometry by ENDOR spectroscopy.

ENDOR spectra of PFL-AE in the presence of $^2$H AdoMet reveal substantial hyperfine coupling between the cluster and the deuterons of the methyl group for both the $1^+$ and $2^+$ (cryoreduced) cluster states (Fig. 6) [49]. By modeling the interaction of the deuterons and the cluster, a distance between the two of 3–5 Å was determined. Similar results were found when methyl $^{13}$C AdoMet was used, and modeling provides a distance of between 4 and 5 Å (Fig. 7) [49]. The observed coupling to the $^{13}$C requires an overlap between the orbitals of the AdoMet and those of the cluster. These incipient covalent interactions are most likely of dative character due to overlap of the sulfonium and a bridging sulfide of the cluster, and likely provide a pathway for inner-sphere electron transfer from the cluster to AdoMet [49]. As Noodleman and Case [52] demonstrated by density functional theory, most of the increased electron density in a cluster changing from the $2^+$ to $1^+$ state resides on the S and not the Fe, which suggests the possibility of sulfur centered, rather than iron-centered, redox chemistry. For PFL-AE, sulfur-centered redox chemistry would be in line with the above interaction of the sulfide and sulfonium. Based both upon these ENDOR spectroscopy results and the Mössbauer spectroscopy results described previously, a model was proposed in which the carboxylate group of AdoMet coordinates the unique iron in a bidentate fashion with the sulfide and sulfonium in close proximity to one another [49].

This model proved to need a slight adjustment when studies of the PFL-AE cluster in the presence of (carboxylate-$^{17}$O)-AdoMet, (amino-$^{14/15}$N)-AdoMet, and (carboxylate-$^{13}$C)-AdoMet were performed [50]. ENDOR spectroscopy of PFL-AE in complex with (carboxylate-$^{17}$O)-AdoMet showed a hyperfine splitting of 12.2 MHz (Fig. 8), indicating a direct coordination between a carboxyl oxygen and the unique iron, similar to what was previously observed withaconitase [53,54]. PFL-AE in the presence of (carboxylate-$^{13}$C)-AdoMet showed a hyperfine coupling constant of 0.71 MHz, also similar to that of aconitase [53,54]. The $^{14}$N ENDOR data of PFL-AE complexed with natural abundance AdoMet showed a poorly resolved feature extending to 8 MHz; this feature is replaced by a better resolved signal at 5.8 MHz for the sample enriched with $^{15}$N at the amino nitrogen.

Fig. 6. ENDOR spectra of PFL-AE with methyl-D$_3$ AdoMet. Samples (A and D) are photoreduced, sample (B) is cryoreduced, and (C and E) are simulations. Spectra were recorded at Q-band using a Mims pulse sequence. For other parameters see [47].
This latter splitting is similar to that found for the amino groups of the Rieske [2Fe–2S] cluster, and points to a direct coordination of the amino group of AdoMet to the unique iron [55]. Taken together, these ENDOR results provide the basis for the revised model of AdoMet interacting with the Fe–S cluster (Fig. 9) [50]. This model proposes an anchoring role for the unique iron of PFL-AE, in which it positions AdoMet by coordination of the amino acid end, while a close association between the sulfide and sulfonium leads to the possibility of inner sphere electron transfer from the cluster to AdoMet to facilitate bond cleavage [50]. A key feature of the AdoMet–[4Fe–4S] cluster interaction, the coordination of the unique iron by the amino and carboxylate groups of AdoMet, has now been observed in X-ray crystal structures of two other members of the radical SAM superfamily, BioB and HemN, suggesting that this unusual and unprecedented AdoMet-cluster interaction is a common feature of the radical SAM enzymes [23,56].

A proposed mechanism for PFL-AE

In conclusion, PFL-AE has been shown to employ a novel radical mechanism that utilizes an Fe–S cluster and AdoMet to facilitate generation of a putative adenosyl radical (Fig. 10). This adenosyl radical carries out H-atom abstraction at G734 of PFL, thereby activating it...
for catalysis. While purified in a number of states, single turnover EPR experiments have shown the active cluster of PFL-AE to be [4Fe–4S]1+, which is oxidized to [4Fe–4S]2+ during turnover [42]. Mössbauer spectroscopy in combination with ENDOR spectroscopy have shown that the Fe–S cluster in PFL-AE has a unique iron site in the [4Fe–4S] cluster which is used to coordinate an amino nitrogen and carboxyl oxygen to anchor AdoMet in the active site [46,49,50]. This anchoring allows for the potential inner-sphere electron transfer from the bridging sulfide to the sulfonium of AdoMet causes C=S bond homolysis, which produces a 5′-deoxyadenosyl radical and methionine. The adenosyl radical abstracts a hydrogen from Gly734 of PFL and 5′-deoxyadenosine and methionine are replaced with another AdoMet. The source of the electron is proposed to be a reduced flavodoxin.

Fig. 10. Proposed mechanism for the activation of PFL by PFL-AE and AdoMet. Inner-sphere electron transfer from a bridging sulfide of the [4Fe–4S]1+ cluster to the sulfonium of AdoMet causes C=S bond homolysis, which produces a 5′-deoxyadenosyl radical and methionine. The adenosyl radical abstracts a hydrogen from Gly734 of PFL and 5′-deoxyadenosine and methionine are replaced with another AdoMet. The source of the electron is proposed to be a reduced flavodoxin.

Acknowledgment

Work in our laboratory on pyruvate formate lyase activating enzyme is supported by a grant from the NIH (GM 54608).

References