The [4Fe-4S] 1 Cluster of Pyruvate Formate-Lyase Activating Enzyme Generates the Glycyl Radical on Pyruvate Formate-Lyase: EPR-Detected Single Turnover

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Pyruvate formate-lyase activating enzyme (PFL-AE), which generates the catalytically essential glycyl radical on PFL (Scheme 1), 1,2 is a representative member of an emerging group of enzymes that utilize iron−sulfur clusters and 5-adenosylmethionine (AdoMet) as required cofactors in radical generation. This group includes related activating enzymes such as the anaerobic ribonucleotide reductase activating enzyme (aRNR-AE) from E. coli,2 as well as biotin synthase,3,4 lipoc acid synthase,5,6 and lysine aminomutase (LAM).7 Though diverse in function, these enzymes have been proposed to have in common key mechanistic features including the generation of an intermediate 5'-deoxyadenosyl radical that initiates catalysis by hydrogen atom abstraction. Isotopic labeling has provided indirect evidence for such a mechanism for PFL-AE and LAM.8,9 Recently, elegant work by Frey and co-workers has provided direct spectroscopic evidence for an allotypic analogue of the 5'-deoxyadenosyl radical for LAM.10

A central question surrounding this group of enzymes is the mechanism by which the iron−sulfur clusters participate in generation of the 5'-deoxyadenosyl radical intermediate. A variety of iron−sulfur clusters, including [2Fe-2S], [3Fe-4S], and [4Fe-4S], have been identified in these AdoMet-dependent glycyl radical enzymes.2,7-11 It has been difficult, however, to identify unequivocally the catalytically relevant cluster. A [4Fe-4S] 1+ has been implicated as the active cluster for aRNR,12 and LAM containing a [4Fe-4S] 1+ EPR signal has been shown to be catalytically active.13 We report here that for PFL-AE under conditions of limiting reductant, each [4Fe-4S] 1+ cluster is capable of generating a single glycyl radical on PFL. Our results provide the first direct quantitative spectroscopic evidence that the [4Fe-4S] 1+ of PFL-AE is the catalytically relevant cluster, and that this cluster provides the electron necessary for AdoMet-dependent glycyl radical generation. PFL-AE isolated under anaerobic conditions contains primarily [3Fe-4S] 2+ clusters, as identified by UV−vis, EPR, and resonance Raman spectroscopies.14 The [3Fe-4S] 2+ cluster accounts for ~62% of the total iron, and is characterized by an axial EPR signal centered at g = 2.03.15 Upon reduction with dithionite, EPR spectra indicate that ~20% of the reduced [4Fe-4S] 1+ is generated, with the remainder of the clusters being in an EPR-silent state with UV−vis spectral properties characteristic of [4Fe-4S] 1+ clusters.14 We have now found that illumination in the presence of 5-deazariboflavin allows nearly quantitative reduction to the [4Fe-4S] 1+ state in a time-dependent manner, as indicated by spin quantitation of the resulting EPR signals (Figure 1A).15

As indicated by Scheme 1, single turnover conditions for PFL-AE can be achieved by limiting the amount of reductant. The clean conversion to [4Fe-4S] 1+ provided by photoreduction has allowed us to carry out single turnover experiments for glycyl radical production, since removing illumination eliminates the exogenous reductant. PFL-AE was photoreduced for various times, after which a 10-fold excess of AdoMet was added and the sample was wrapped in aluminum foil to prevent further reduction.15 Each sample was then split into two halves and equimolar PFL was added to one-half in the dark. EPR spectra were recorded to detect formation of [4Fe-4S] 1+ and glycyl radical in these samples. Figure 1A shows, from bottom to top, the 12 K EPR spectra of PFL-AE photoreduced in the presence of 5-deazariboflavin for 0, 1, 2, 5, 10, and 30 min.16 Quantitation of these EPR signals results in 0, 2.8(±0.5), 17(±2), 28(±3), 41(±4), and 54(±5) μM spins, respectively.17 The nearly complete conversion to [4Fe-4S] 1+ is not the result of an increase in [4Fe-4S] 1+ via exchange with the bulk PFL-AE, but rather a direct consequence of the single turnover conditions for PFL-AE.

(14) Henshaw, T. F.; Cheek, J.; Broderick, J. B. Unpublished observations.

(15) Although no exogenous iron is added during reduction, the [4Fe-4S] clusters are presumably assembled by cova lential reduction of the [3Fe-4S] clusters. The cluster composition reported herein is native PFL-AE different from our previous preparations14 due to modifications in the growth/expressions/pretreatment conditions. It should be noted, however, that the spectroscopic properties of the reduced enzyme are the same in both reports. Knapp and co-workers have reconstituted apo-PFL-AE and report Fe/S-protein stoichiometries similar to those of our native PFL-AE (2.5−3 Fe and 5 per protein), but with primarily [4Fe-4S] clusters as prepared.12 PFL-AE is monomeric,12 therefore the stoichiometry is interpreted as partial occupation of a single [4Fe-4S] site per monomer. Dithionite reduction of Knappe's reconstituted PFL-AE generates ~40% [4Fe-4S] 1+, and the EPR g-values of this cluster, both in the presence and absence of AdoMet, are distinct from those reported here for native PFL-AE, including slight differences in the native vs reconstituted clusters.12 The specific activity of our PFL-AE is similar to that reported by Knappe (31% vs 46%) for assays in the absence of added iron.

(16) PFL-AE was purified as described previously12 except that 1 mM DTT was included in all buffers. Samples were prepared in an anaerobic chamber at 0°C using 200 μM PFL-AE (2.65 Fe/monomer) in 50 mM Hepes, 50 mM Tris (pH 7.4). 5-Deazariboflavin was added in the dark to a final concentration of 100 μM. The samples were illuminated by a 500 W xenon lamp for the indicated times, followed by addition of AdoMet to 2 mM. In the dark, each sample was split into two, and to one an equal volume of PFL solution (200 μM PFL, 20 mM oxamate (allosteric effector), 100 μM 5-deazariboflavin) was added. Samples were stored frozen in the dark until EPR spectra were recorded.

(17) The [3Fe-4S] 2+ EPR signal observed for PFL-AE as isolated12 is not observed for the 0 min illumination sample due to fortuitous reduction resulting from exposure to the ambient lighting in the anaerobic chamber during sample preparation (~10 min). A 0 min illumination sample prepared in more complete darkness did show the S = 5/2 EPR signal characteristic of a [3Fe-4S] 1+ cluster (not shown).

Scheme 1

Figure 1A

[Diagram showing EPR spectra and quantitation of EPR signals for PFL-AE photoreduced in the presence of 5-deazariboflavin for various times.]

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axial EPR signals shown in Figure 1A are characteristic of a [4Fe-4S]^{1+} cluster, and are essentially identical to the EPR signal previously reported for dithionite-reduced PFL-AE in the presence of AdoMet.11,19 After 60 min of illumination, 85% of the cluster in PFL-AE is in the reduced [4Fe-4S]^{1+} state.15 Saturation of cluster reduction is indicated by the illumination time course shown in Figure 2.

EPR spectra (60 K) for samples with PFL added are shown in Figure 1B. Increasing amounts of a multiplet EPR signal characteristic of the PFL glycyl radical20 are observed with increasing time. Spin quantitation of the glycyl radical observed and the amount of [4Fe-4S]^{1+} cluster present prior to addition of PFL, as shown in Figure 2. The glycyl radical spin quantitations are 3.6(±0.5), 16(±2), 28(±3), 36(±4), and 52(±5) μM for 1, 2, 5, 10, and 30 min illumination, respectively.

The glycyl radical spectra were recorded at 60 K, a temperature at which the [4Fe-4S]^{1+} signal of PFL-AE is not observable.

(17) Spin quantitations were done as described previously (Aasa, R.; Väningård, T. J. Magn. Reson. 1975, 19, 308). A solution of 0.1 mM Cu(II) and 1 mM EDTA was used as a standard for the cluster signals. K_{2}(SO_{3})_{2}NO (1.04 mM) was used as the standard for glycyl radical signals, and the concentration of this standard was determined using the optical extinction coefficient (Munih, J. H.; Ritter, D. M. J. Am. Chem. Soc. 1952, 74, 339). The spin quantitations reported for the [4Fe-4S]^{1+} signals are 0.5 x the actual spin quantitations calculated for these spectra. This was done to correct for the difference in protein concentration in the -PFL and +PFL samples.

(18) The [4Fe-4S]^{3+} spectra reported here have g = 2.01, 1.89, and 1.88.

(19) Based on 112 μM spin for 200 μM protein with 2.65 Fe/PFL-AE.18

(20) The g value (2.007) and splitting (17 g) of the gly* EPR signal are similar to values previously reported: Wagner, A. F. V.; Frey, M.; Neugebauer, F. A.; Schäfer, W.; Knappe, J. Proc. Natl. Acad. Sci. U.S.A. 1992, 89, 996.

However, EPR spectra recorded at 12 K for the same samples also showed no [4Fe-4S]^{1+} signal.21 This observation demonstrates that the [4Fe-4S]^{1+} cluster has been converted to an EPR-silent state upon addition of PFL to the [4Fe-4S]^{1+}/PFL-AE/AdoMet and subsequent generation of the glycyl radical. Cleavage of AdoMet is stoichiometric with PFL glycyl radical generation, and requires a source of electrons.22 Our results strongly suggest that the required electrons come from the [4Fe-4S]^{1+} cluster, thereby converting it to an EPR-silent [4Fe-4S]^{2+} cluster. This conclusion is supported by the observation of a UV−vis spectrum which is typical of a [4Fe-4S]^{2+} cluster.21 In addition, the increase in ε_{00} upon addition of PFL is consistent with oxidation of the [4Fe-4S]^{1+} to a [4Fe-4S]^{2+} cluster.22 Furthermore, re-illumination of samples containing the EPR-silent cluster can regenerate the [4Fe-4S]^{1+} EPR signal, indicating an ability to cycle readily between the [4Fe-4S]^{1+} and [4Fe-4S]^{2+} states.21

The involvement of adenosyl radical intermediates in both the Fe-S/AdoMet and adenosylcobalamin-dependent enzymes raises intriguing mechanistic questions regarding the role of iron−sulfur clusters in radical generation.23-26 The data presented here support the conclusion that the [4Fe-4S]^{1+} cluster of PFL-AE is the source of the electron necessary for reductive cleavage of AdoMet and subsequent generation of the glycyl radical on PFL. However, the role of the [4Fe-4S]^{1+} likely goes beyond a simple electron transfer, since the [4Fe-4S]^{1+} is stable in the presence of excess AdoMet, and is not oxidized until PFL is added and the PFL glycyl radical is generated. The full mechanistic details by which the iron−sulfur cluster promotes adenosyl radical formation in these fascinating enzymes therefore awaits further studies.

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(21) Data not shown. Further characterization of the cluster product of turnover by Mössbauer spectroscopy and other techniques is underway, as are more extensive studies of the redox cycling of the cluster.


