

Pyruvate Formate-Lyase Activating Enzyme Is an Iron–Sulfur Protein

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Only a few enzymes are known to utilize stable protein-based radicals as part of their catalytic cycles.¹ Among these are both the aerobic and anaerobic ribonucleotide reductases and pyruvate formate-lyase from *Escherichia coli*. Aerobic and anaerobic ribonucleotide reductases utilize tyrosyl and glycy radicals, respectively, to generate a transient active site thiyl radical that effects hydrogen atom abstraction in the initial step of ribonucleotide reduction.² Pyruvate formate-lyase (PFL) has been shown to require a stable glycy radical to effect the rearrangement of pyruvate to formate.³ The mechanism of generation of these catalytically essential radicals is of considerable interest, but has only been elucidated in any detail for aerobic ribonucleotide reductase. For PFL, an iron-dependent activating enzyme (PFL-AE) is required for activation under reducing conditions in the presence of DTT, via an (S)-adenosylmethionine-dependent hydrogen atom abstraction, to generate a glycy radical.^{4,5} However, the nature of the iron center in PFL-AE has until now remained elusive. We report here the first evidence for the presence of an iron–sulfur cluster in PFL-AE. When a combination of absorption, variable temperature magnetic circular dichroism (VTMCD), EPR, and resonance Raman (RR) spectroscopies is used, anaerobically prepared PFL-AE is shown to contain a mixture of diamagnetic [2Fe–2S]²⁺ and [4Fe–4S]²⁺ clusters. Since only [4Fe–4S]²⁺ clusters remain in dithionite-reduced samples and (S)-adenosylmethionine (SAM) is required to effect reduction to the [4Fe–4S]⁺ state, these results are interpreted in terms of a subunit-bridging [4Fe–4S]^{2+,+} cluster in active PFL-AE and suggest that this cluster is involved with generating the 5'-deoxyadenosyl radical from SAM.

Purified PFL-AE⁶ has a distinct red-brown color and a UV–vis absorption spectrum consistent with the presence of an Fe–S cluster (Figure 1a). Analysis of four distinct preparations indicated 1.5 ± 0.1 mol of iron and 1.7 ± 0.2 mol of acid-labile sulfide per mole of enzyme monomer.⁷ The specific activity of purified PFL-AE containing 1.5 mol of iron per mole

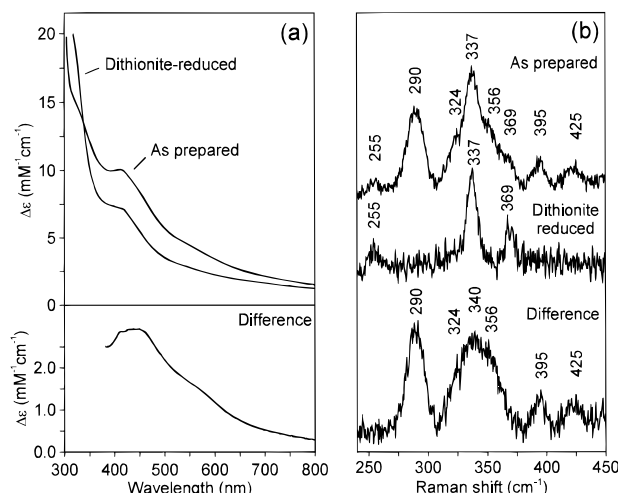


Figure 1. UV–vis absorption spectra (a) and resonance Raman spectra (b) of anaerobically purified PFL-AE. As prepared, dithionite-reduced and as-prepared minus dithionite-reduced difference spectra are shown for each. The PFL-AE samples, 0.23 mM for absorption and ~ 3 mM for RR, were in 50 mM Tris-HCl buffer, pH 7.2 with 200 mM NaCl and 1 mM DTT and were reduced anaerobically with a 5-fold excess of sodium dithionite. The RR spectra were recorded with 457 nm excitation using a 10 μ L frozen droplet at 25 K with 70 mW laser power at the sample. Each scan involved photon counting for 1 s at 0.5 cm^{-1} increments with 0.7 cm^{-1} spectral resolution and the spectra shown are the sum of 85 scans. The vibrational modes originating from the lattice modes of the frozen buffer solution have been subtracted after normalization of the intensities of the “ice-band” at 231 cm^{-1} , and a linear ramp fluorescence base line has also been subtracted.

of enzyme was 3800 U/mg, compared to 540 U/mg for enzyme with a low cluster content (<0.2 mol of iron per mole of enzyme), indicating a direct correlation between cluster content and enzyme activity.^{8,9} In common with most biological Fe–S centers, reduction with dithionite results in partial bleaching of the visible absorption. However, in contrast to all known types of Fe–S centers which are paramagnetic in at least one oxidation state, neither the as-purified or dithionite-reduced samples contain a paramagnetic Fe–S cluster, as evidenced by parallel and perpendicular X-band EPR and VTMCD studies over the temperature range of 4–50 K.¹⁰

The identity of the diamagnetic Fe–S clusters in these samples was revealed by RR studies in the Fe–S stretching region (Figure 1b). Although the signal-to-noise ratio is poor due to high background fluorescence, the RR spectrum of

(6) Overexpression of PFL-AE was carried out using *E. coli* N4830-1 transformed with pMG-AE.^{4c} The cells were grown at 30 °C to mid-log phase in Terrific Broth (Tartof, K. D.; Hobbs, C. A. *Bethesda Res. Lab. Focus* **1987**, 86, 184) and then induced by increasing the temperature to 42 °C. Cells were harvested and lysed by sonication, and the soluble extract was treated with Polymin (0.45% final concentration) to precipitate nucleic acids. The clarified soluble extract was loaded onto a Sephacryl S-200 column (5 \times 60 cm) and eluted with 50 mM Tris/200 mM NaCl/1 mM DTT, pH 7.2. Fractions were analyzed by SDS-PAGE, and those containing pure AE were pooled and concentrated. All purification steps were performed under an Ar atmosphere.

(7) Iron and sulfide analyses were carried out using the published procedures (Beinert, H. *Methods Enzymol.* **1978**, 54, 435–445. Beinert, H. *Anal. Biochem.* **1983**, 131, 373–378). Routine protein analyses were carried out using the published procedure (Bradford, M. *Anal. Biochem.* **1976**, 72, 248) and were converted to actual protein concentrations using a conversion factor of 0.65 derived from direct amino acid analysis carried out at the MCB Core Facility, University of Massachusetts.

(8) PFL-AE with a low cluster content is easily prepared by purification in the presence of EDTA. Specific activities were measured according to the published procedures.⁵

(9) The specific activities reported here for recombinant PFL-AE can be compared to 1300 U/mg for PFL-AE purified from inclusion bodies^{5a} and 17 800 U/mg for wild-type PFL-AE.^{4b} The apparent discrepancy in the specific activities of recombinant and wild-type enzymes is currently under investigation.

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dithionite-reduced PFL-AE is uniquely characteristic of a $[4\text{Fe}-4\text{S}]^{2+}$ cluster and the frequency of the totally symmetric breathing mode of the cubane Fe_4S_4 core, 337 cm^{-1} , is indicative of complete cysteinyl ligation.^{11,12} In contrast, the RR spectrum of as-prepared PFL-AE is best interpreted as a mixture of $[4\text{Fe}-4\text{S}]^{2+}$ and $[2\text{Fe}-2\text{S}]^{2+}$ clusters, and the as-prepared minus dithionite-reduced difference spectrum identifies bands at 290, 324, 340, 356, 395, and 425 cm^{-1} that are characteristic of a $[2\text{Fe}-2\text{S}]^{2+}$ center.^{11,13} Previous studies have shown that the frequency of the lowest energy Fe-S stretching mode (out-of-phase, symmetric stretching at the two Fe sites) is particularly sensitive to cluster ligation.¹⁴ In as-prepared PFL-AE, this band occurs at 290 cm^{-1} which is encompassed by the ranges of frequencies established for $[2\text{Fe}-2\text{S}]^{2+}$ clusters with four cysteinyl S-ligands ($281\text{--}291\text{ cm}^{-1}$) and with three cysteinyl S-ligands and one oxygenic ligand ($289\text{--}302\text{ cm}^{-1}$).

This interpretation of the RR results is supported by the UV-vis absorption data (Figure 1a), since the dithionite-reduced spectrum has a pronounced shoulder at 420 nm characteristic of a $[4\text{Fe}-4\text{S}]^{2+}$ cluster and the as-prepared minus dithionite-reduced difference spectrum has features at 420, 460, and 550 nm characteristic of $[2\text{Fe}-2\text{S}]^{2+}$ cluster. In accord with the iron and acid-labile sulfide analyses, the molar extinction coefficient of the dithionite reduced sample at 420 nm indicates that the Fe-S center is present in substoichiometric amounts, corresponding to approximately ~ 0.4 $[4\text{Fe}-4\text{S}]^{2+}$ clusters per monomer. Since EPR and VTCD studies give no evidence of a paramagnetic $[2\text{Fe}-2\text{S}]^{2+}$ cluster in dithionite reduced preparations, the absorption and RR results indicate that the $[2\text{Fe}-2\text{S}]^{2+}$ clusters are either degraded or converted to $[4\text{Fe}-4\text{S}]^{2+}$ clusters under reducing conditions.

Evidence that the $[4\text{Fe}-4\text{S}]^{2+}$ cluster is involved with generating the 5'-deoxyadenosyl radical via the one-electron reduction of SAM comes from the observation that the cluster can only be reduced to the $[4\text{Fe}-4\text{S}]^+$ state in the presence of SAM (Figure 2).¹⁵ Samples reduced with dithionite in the presence of SAM exhibited an almost axial $S = 1/2$ EPR signal, $g = 2.01, 1.89, 1.88$, in addition to a weak isotropic radical signal centered near 2.01. The axial resonance accounts for 0.38 spins/monomer and is attributed to a $S = 1/2$ $[4\text{Fe}-4\text{S}]^+$ cluster, since it is only observable at temperatures below 40 K.

(10) All samples of PFL-AE as purified exhibited a weak signal at $g = 4.3$ characteristic of adventitiously bound high-spin Fe^{3+} . Some, but not all, exhibited a fast-relaxing resonance, $g = 2.03, 2.01, 2.00$ (observable only below 20 K) characteristic of a $S = 1/2$ $[3\text{Fe}-4\text{S}]^+$ cluster and a slow-relaxing axial resonance, $g = 2.01, 2.01, 1.97$, that was still observable without broadening at 100 K. However, spin quantitations indicate that both are minor species accounting for <0.01 spins per monomer.

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(15) SAM-dependent reduction of a $[4\text{Fe}-4\text{S}]^{2+}$ cluster was first observed in lysine 2,3-aminomutase (P. A. Frey, personal communication) and may be a general property of SAM-dependent Fe-S enzymes that function by radical mechanisms.

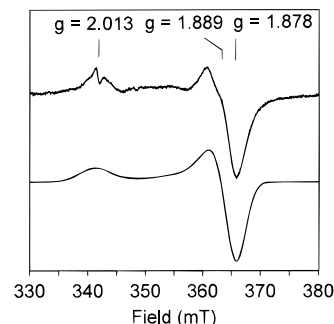


Figure 2. Perpendicular-mode X-band EPR spectrum of PFL-AE reduced with dithionite in the presence of SAM. PFL-AE, $\sim 0.5\text{ mM}$, in 50 mM Tris-HCl buffer, pH 7.2 with 200 mM NaCl and 1 mM DTT, was reduced with a 10-fold excess of sodium dithionite in the presence of a 10-fold excess of SAM. The spectrum was recorded at 14 K , using a microwave power of 5 mW , a modulation amplitude of 0.64 mT , and a microwave frequency of 9.61 GHz . The lower trace is a simulation of the dominant resonance; simulation parameters: $g_{1,2,3} = 2.013, 1.889, 1.878$; $I_{1,2,3} = 3.15, 2.45, 1.90\text{ mT}$.

The spectroscopic data reported here are interpreted in terms of a $[4\text{Fe}-4\text{S}]^{2+}$ cluster in active PFL-AE and suggest that this cluster undergoes facile oxidative degradation to $[2\text{Fe}-2\text{S}]^{2+}$ clusters as a result of incomplete anaerobicity during isolation. This type of oxidative cluster degradation has only been observed for the subunit-bridging $[4\text{Fe}-4\text{S}]$ clusters in nitrogenase Fe-protein¹⁶ and biotin synthase.¹⁷ In addition, the cluster stoichiometry indicated by analytical, EPR and absorption data (~ 0.4 $[4\text{Fe}-4\text{S}]$ clusters per monomer) are more consistent with one cluster per homodimer. Spectroscopic and analytical studies have also implicated subunit-bridging $[4\text{Fe}-4\text{S}]$ clusters in biotin synthase,¹⁷ lysine 2,3-aminomutase,¹⁸ and the activase subunits of anaerobic ribonucleotide reductase:¹⁹ three other members of the small but growing class of enzymes that utilize iron-sulfur clusters and SAM to catalyze radical-dependent reactions. While the primary sequence has yet to be determined for lysine 2,3-aminomutase, it is interesting to note that the other three enzymes all contain a $\text{C}-\text{X}_3-\text{C}-\text{X}_2-\text{C}$ motif near the N-terminus that is likely to be involved with cluster ligation.^{5a,17,19,20} The similarity of cofactor requirements for these four enzymes suggests a commonality of mechanism that may represent a new paradigm for radical generation in biological systems.

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