Reconstitution and Characterization of the Polynuclear Iron-Sulfur Cluster in Pyruvate Formate-lyase-activating Enzyme

MOLECULAR PROPERTIES OF THE HOLOENZYME FORM*

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The glycyl radical (Gly-734) contained in the active form of pyruvate formate-lyase (PFL) of Escherichia coli is generated by the S-adenosylmethionine-dependent pyruvate formate-lyase-activating enzyme (PFL activase). A 5′-deoxyadenosyl radical intermediate produced by the activase has been suggested as the species that abstracts the pro-S hydrogen of the glycine 734 residue in PFL (Frey, M., Rothe, M., Wagner, A. F. V., and Knappe, J. (1994) J. Biol. Chem. 269, 12432-12437). To enable mechanistic investigations of this system we have worked out a convenient large scale preparation of functionally competent PFL activase from its apoform. The previously inferred metallic cofactor was identified as redox-interconvertible polynuclear iron-sulfur cluster, most probably of the [4Fe-4S] type, according to UV-visible and EPR spectroscopic information. Cys → Ser replacements by site-directed mutagenesis determined Cys-29, Cys-33, and Cys-36 to be essential to yield active holoenzyme. Gel filtration chromatography showed a monomeric structure (28 kDa) for both the apoenzyme and holoenzyme form. The iron-sulfur cluster complement proved to be a prerequisite for effective binding of adenosylmethionine, which induces a characteristic shift of the EPR signal shape of the reduced enzyme form ([4Fe-4S]*) from axial to rhombic symmetry.

Pyruvate formate-lyase (PFL)1 is a key enzyme of the anaerobic glucose fermentation in Escherichia coli and other microorganisms, catalyzing the CoA-dependent cleavage of pyruvate to acetyl-CoA and formate (1). In its active form, this enzyme contains a stable glycyl radical (Gly-734) required for catalysis (2). The glycyl radical (Gly-734) contained in the active form of PFL activase, which employs adenosylmethionine (AdoMet) and dihydroflavodoxin (or artificial 1e− donors) as co-substrates, yielding 5′-deoxyadenosine and methionine as stoichiometric co-products (Equation 1).

$$[-\text{NH-CH}_2\text{-CO-}] + \text{AdoMet} + e^- \rightarrow [-\text{NH-CH-CO-}] + 5′\text{-deoxyadenosine} + \text{Met} \quad \text{(Eq. 1)}$$

Because the abstracted H atom is recovered in the 5′-CH3 of deoxyadenosine, a 5′-deoxyadenosyl radical intermediate has been proposed as the actual H abstracting species in this system. How this nucleoside radical is generated from AdoMet is an intriguing problem, requiring in the first place PFL activase to be fully characterized at the molecular level.

Previous work has identified PFL activase as a monomer of 28 kDa, and its primary structure, as deduced from the DNA-nucleotide sequence (246 amino acids), has been established by Frey, M., Rothe, M., Wagner, A. F. V., and Knappe, J. (1994) J. Biol. Chem. 269, 12432-12437. Studies of mutants and substrate analogs propose that on substrate binding, the spin is transferred from Gly-734 to the reaction center (Cys-418/Cys-419), where a thiyl radical initiates the homolytic cleavage of the pyruvate C-C bond (3, 4). The previously inferred metallic cofactor was identified as redox-interconvertible polynuclear iron-sulfur cluster, most probably of the [4Fe-4S] type, according to UV-visible and EPR spectroscopic information. Cys → Ser replacements by site-directed mutagenesis determined Cys-29, Cys-33, and Cys-36 to be essential to yield active holoenzyme. Gel filtration chromatography showed a monomeric structure (28 kDa) for both the apoenzyme and holoenzyme form. The iron-sulfur cluster complement proved to be a prerequisite for effective binding of adenosylmethionine, which induces a characteristic shift of the EPR signal shape of the reduced enzyme form ([4Fe-4S]*) from axial to rhombic symmetry.

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1 The abbreviations used are: PFL, pyruvate formate-lyase; PFL activase, pyruvate formate-lyase-activating enzyme; RNR, ribonucleotide reductase; AdoMet, S-adenosylmethionine; DTT, dithiotheitol; Mops, 3-(N-morpholino)propanesulfonic acid.

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Bacterial Strain, Plasmids, and Site-directed Mutagenesis—E. coli strain 234M1 (2), a MC1400 derivative carrying the Cm" gene inserted into the chromosomal act gene, was used as host for the overproduction of activase proteins (wild type or mutants). The parental expression plasmid pX-1 contained the act gene encompassing the 1-kilobase pair DNA fragment (26) that was purified from the parental strain and linked to the tac promoter of pKK223-3 (Pharmacia). This construct differs from the earlier pKE-1 (5) by deleting 0.1 kilobase pairs of the act downstream region.

Site-directed mutagenesis was performed with the phosphorothioate method (12) using the Sculptor<sup>®</sup> in vitro mutagenesis system (RPN 1500, Pharmacia) according to the supplier's manual. This comprised single Ser substitutions of the six Cys residues: TGT codons (for Cys in amino acid positions 12, 36, and 92) and TGC codons (for Cys in amino acid positions 29, 33, and 94) were mutated to TCT and TCC codons, respectively, by using 24–30-mer antisense mutagenic oligonucleotides that were synthesized by R. Frank (Zentrum fu¨r Molekulare Biologie, Heidelberg, Germany). All mutant genes were proof sequenced.

Activase Assays—The reaction mixtures (0.5 ml) for PFL conversion to the radical form contained in general: 0.15 ml Tris-HCl, pH 7.5, 0.1 ml KCl, 5 mM DTT, 10 mM potassium oxamate, 0.2 ml AdoMet, 50 mM 5-deazariboflavin, 1 mM riboflavin, 10 mM of catalase, 60 mM (340 pmol) of PFL, and 0.1–2 mM of the activase sample. A slide projector light was used for continuous photoreduction of deazaflavin, and reactions were run at 30 °C under argon, usually for 10 min. In the assay version termed "holoactivase assay" ("Results"), the reaction was started by the addition of 10 mM EDTA, and the reaction was started with the activase sample. In the "standard assay," which was used to determine intact activase together with iron core-defective enzyme forms, the reaction mixture contained additionally 0.2 mM Fe(NH₄)₂(SO₄)₂ and optionally 0.1 mM Na₂S, and was preincubated in the presence of activase, in the dark for 20 min; the reaction was started by illumination. The produced radical form of PFL was determined by activity measurement (coupled optical assay with a 5–10-µl aliquot); 35 IU correspond to 1 nmol of PFL dimer containing one Gly radical.

One unit of activase activity was defined to produce 1 nmol of the PFL radical/min. (Activity data reported here are lower by a factor of approximately 5 with respect to previous work (6), which used a different unit definition.)

Chemical Assays—Protein concentrations of purified apoenzyme solutions were determined by the 280-nm absorption using a 1 mg/ml protein solution and a 1.38 value of 1.38 (as calculated from the Trp and Tyr residues in the polypeptide and verified by amino acid analysis). On reconstituted enzyme and other enzyme fractions, the Bradford assay (13) or a biuret reaction was used for continuous photoreduction of deazaflavin, and reactions were run at 30 °C under argon, but column operations were not strictly anoxic. Enzyme purifications were made at 0–6 °C, except for the Q-Sepharose columns.

Bacterial Growth and Isolation of the Aponzyme—E. coli 234M1/pX-1 cells were grown in LB medium with 50 µg/ml ampicillin and 30 µg/ml chloramphenicol at 30 °C under aeration, maintaining pH 7 by addition of 5 mM KOH. Cells were harvested at the transition to the stationary growth phase (λ<sub>max</sub> = 4.8) and frozen in liquid nitrogen (yield = 5 gliter).

The purification of the enzyme was made at 6 °C, except for the Q-Sepharose step. Buffer solutions were previously deagitated and flushed with argon, but column operations were not strictly anaerobic. Enzyme fractions collected were stored under argon.

A 50-µl portion of cell paste was suspended in 100 ml of 50 mM Mops/KOH, pH 7.5, 5 mM DTT, 0.1 mM EDTA, and 0.2 mM phenylmethanesulfonyl fluoride. After sonication, DNAse I (1 mg/ml) and RNAse A (1 mg/ml) were added, and the sample was centrifuged at 100,000 × g for 60 min. The supernatant (138 ml, 45 mg of protein/ml) was adjusted to pH 7.5, and 5 mM DTT, 10 mM potassium oxamate, 0.2 mM AdoMet, 0.2 mM Fe<sup>2+</sup>, and 0.05 mM sulfide (flow rate = 0.4 ml/min). The eluate was monitored for protein (280 nm) and holoactivase activity (see Fig. 3). For the apoenzyme sample monitored at 280 nm, the column was run with anoxic buffer that lacked Fe<sup>2+</sup> and sulfide. Proteins for column calibration were bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and cytochrome c.

AdoMet Binding Assays with Penefsky Columns—The centrifuged column procedure (16) for separating enzyme-bound and free small molecules was used to monitor binding of [³¹⁵C]AdoMet to various activase forms. Bio-Spin columns (Bio-Rad) in which the plastic support was replaced by a glass fiber disc (GF-C, Whatman) were employed with a 1-ml filling of Sephadex G-25 fine (Pharmacia), which was equilibrated with anaerobic buffer composed of 50 mM Mops/KOH, pH 7.5, 0.15 mM KCl, 10 mM mercaptoethanol, 0.2 mM Fe<sup>2+</sup>, and 0.05 mM sulfide (flow rate = 0.4 ml/min). The eluate was monitored for protein (280 nm) and holoactivase activity (see Fig. 3). For the apoenzyme sample monitored at 280 nm, the column was run with anoxic buffer that lacked Fe<sup>2+</sup> and sulfide. Proteins for column calibration were bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and cytochrome c.

RESULTS

Overproduction and Functional Assay of PFL Activase—To study the molecular properties of wild-type and mutant forms of the starting buffer, 90 mM Tris-phosphate pH 8.0 (with DTT and EDTA as before) was applied, which eluted the apoenzyme as a symmetric protein peak (275 ml), whereas the iron-based color remained column-bound. The colorless solution was concentrated (in the cold) over a PM-10 membrane, then gel filtered into 50 mM Mops/KOH, pH 7.5, 0.1 mM KCl, 5 mM DTT, and 0.1 mM EDTA (18 ml, 8.7 mg/ml). Long term storage was at −70 °C. Analysis by SDS-polyacrylamide gel electrophoresis (12%) gel showed the 28-kDa band of PFL activase (6, 7) in addition to a few minor bands comprising ≤5% of the total protein.

The same protocols (but down-scaled) of bacterial growth and isolation as apoenzyme were applied for C125S, C295S, C313S, C685, and C1202S mutant enzymes. The C945S mutant was carried only to the Ultrogel AcA44 stage (protein purity = 85%).

Reconstitution of Holoenzyme—Samples of holoenzymes were prepared in batches up to 20 ml composed of 50 mM Mops/KOH, pH 7.5, 0.1 mM KCl, 0.1 mM mercaptoethanol, and 1 mg/ml chloramphenicol at 30 °C under aeration, maintaining pH 7 by addition of 5 N KOH. Cells were harvested at the transition to the stationary phase (λ<sub>max</sub> = 4.8) and frozen in liquid nitrogen.

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of PFL activase, we overproduced the protein in the E. coli act null strain 234M1 carrying the expression vector pXA-1, wherein the act gene is under control of the Tac promoter. Optimal yields of activase protein, amounting to 15% of the soluble protein fraction, were obtained by aerobic cell growth in LB medium at 30 °C. Glucose/minimal medium, at aerobic or anaerobic conditions, proved unsuitable because this produced the enzyme chiefly deposited in inclusion bodies.

Two protocols were used to assay PFL activase activity via the production of the radical form of PFL. In the first protocol, which has been used in previous work (6, 8) and will be termed the production of the radical form of PFL. In the first protocol, the enzyme chiefly deposited in inclusion bodies.

| Purification step | Protein | mg | nmol/mg | Holoactivase assay units/mg | Standard assay units/mg |
|------------------|---------|----|---------|-----------------------------|------------------------|
| Centrifuged extract<sup>a</sup> | 3700 | 16 | 0.2 | 4.3 | 8.6 |
| Ultrogel AcA44 | 490 | ≤0.4 | | 4 | 33 |
| Q-Sepharose (apoenzyme) | 160 | | | 0 | 60 |
| Reconstituted holoenzyme<sup>b</sup> as prepared gelfiltered | | 92 | 60 | |

<sup>a</sup> From 30 g of packed cells.

<sup>b</sup> Reconstitution was performed on 20-mg batches.

The specific activity values of reconstituted enzyme were in the range of 60 ± 5 units/mg. This calculates the turnover number to be 1.7 min<sup>-1</sup> at assay conditions with 0.7 μM PFL ($K_m = 1.4 \mu M$) or 5 min<sup>-1</sup> at a saturating PFL concentration. It is interesting to note that the presence of up to 5 mM EDTA in place of dithiothreitol as protective thiol reagent in the reconstitution mixture, the sulfide requirement was total (Fig. 2), thus immediately demonstrating a polynuclear Fe-S cluster in the catalytically competent enzyme form.

Molecular Size, Iron-Sulfur Content, and Stability Properties of Reconstituted Holoenzyme—To structurally characterize the functional competent enzyme form, we determined in the first place its molecular size using a calibrated Superdex-75 column. Holoactivase activity migrated as a single band, together with the principal protein band, according to 25 ± 1 kDa (Fig. 3). For the apoenzyme (data not shown) the molecular size was found to be 23 ± 1 kDa. These values compare with the sequence-derived molecular mass of the polypeptide of 28.1 kDa (7), indicating that activase is monomeric with or without the Fe-S complement.

On Superdex-75 chromatography, which was operated with anaerobic reconstitution medium containing iron and sulfide, the recovery of holoactivase activity was routinely ≥95%. However, yields dropped to ≤10% when free Fe<sup>2+</sup> and sulfide was omitted from the running buffer, even at 4 °C column operation. The residual activity was again found exclusively in the fractions corresponding to a monomeric size.

Removal of excessive ligand components contained in the reconstitution mixture without damaging the Fe-S center proved technically difficult and has as yet been accomplished only partially. With Sephadex G25 columns run at 4 °C with anaerobic Mops buffer, pH 7.5 (see “Experimental Procedures”), activase preparations were obtained that showed activity values from 40 to 50 units/mg in the holoactivase assay and about 60 units/mg in the standard assay. Iron and sulfide contents were determined in such samples to be 2.6 ± 0.2 iron/polypeptide chain and 2.5 ± 0.2 sulfur/polypeptide chain. Taken that the deficiency of holoactivase activity is due to partial loss of the iron-sulfur center, a content of 3.3 ± 0.3 iron.
and 3.2 \pm 0.3 sulfide/polypeptide would result for the fully complemented enzyme.

In SG25 gel filtered enzyme samples stored under argon at 0 °C, the functionally competent Fe-S cluster, as monitored by the holoactivase activity, proved to be totally stable for at least several days. However, exposure to air under slight agitation destroyed the activity with a half-time of about 2 min. Likewise, incubation with metal chelators under anaerobic conditions caused rapid inactivation, the half-life with 0.3 mM EDTA being 5 min.

**Spectral Characteristics**—Gel filtered holoenzyme samples, which are greenish brown in color, showed a UV-visible spectrum (Fig. 4) comprising a prominent 280 nm peak followed by a shoulder at 320 nm and a broad peak at 370–420 nm, with extensions of the absorbance to wavelengths \( \geq 600 \text{ nm} \), which is typical for Fe-S proteins containing \([4\text{Fe}-4\text{S}]^{2+}\) clusters. The difference spectrum of holo versus apoenzyme calculates absorption coefficients for iron at maximum wavelengths of 310 and 420 nm to be 5.3 and 3.8 mM\(^{-1}\) cm\(^{-1}\), respectively. These spectral data closely match those reported for \([\text{Fe}_6\text{S}_4(\text{SEt})_4]^{2-}\) (18) but are different from those for \([\text{Fe}_2\text{S}_2(\text{SEt})_4]^{2-}\) (19). Addition of \( \geq 0.1 \text{ mM dithionite} \) (\( \geq 5\)-fold excess) reduced the 420-nm absorption by 45% (Fig. 4, inset), and a subsequent short exposure (1–2 min) to air restored the original spectrum, which, however, became bleached upon prolonged admission of air.

For comparison, Fig. 5 shows the optical spectrum of the Ultrogel AcA44 fraction of the enzyme purification scheme, which is largely defective as to holoenzyme activity (Table I). The prominent feature is a pronounced peak at 416 nm (estimated absorption coefficient for iron = 6 M\(^{-1}\) cm\(^{-1}\)), followed by shoulders at 470 and 580 nm, giving such samples a characteristic red-brown color appearance. These spectral data suggest the presence of 3Fe-4S clusters, possibly a mixture of linear and cubic forms, when the spectrum is compared with the optical spectra of the 3Fe-4S forms ofaconitase (20). This is supported by the EPR spectrum of the Ultrogel fraction (not shown).
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Fig. 5. Electronic spectrum of the Ultrogel AcA44 fraction. The Ultrogel fraction of the enzyme purification scheme (see "Experimental Procedures" and Table I) containing 25 mg/ml and 16 nmol Fe/mg, was recorded in a 0.1-cm cuvette.

Fig. 6. X-band first derivative EPR spectrum of PFL activase (reduced enzyme form). The spectra are from a sample of holoenzyme (15 mg/ml or 0.5 mM; 2.6 iron/polypeptide and 2.5 sulfur/polypeptide; holocatalytic activity, 46 units/mg) contained in gel filtration buffer as indicated in Fig. 4 and incubated with 10 mM dithionite (5 mM). The EPR conditions were: temperature as indicated; microwave power, 2 mW; modulation of 0.5 mT at 100 kHz. The g values are shown in the 20 K spectrum.

Table II

Catalytic properties of Cys → Ser mutants

| Mutation | Catalytic activitya | % of wild type |
|----------|---------------------|---------------|
| C12S     | 34 ± 3              | 57 ± 5        |
| C29S     | <0.05               | <0.1          |
| C33S     | <0.05               | <0.1          |
| C36S     | <0.05               | <0.1          |
| C94S     | 49 ± 5b             | 82 ± 8        |
| C102S    | 40 ± 5              | 67 ± 8        |

a Measurements were with both the holocatalytic assay and the standard assay protocols, which yielded identical values.

b Specific activity value by accounting for the protein purity of 0.85.

EPR spectroscopy of reconstituted holoenzyme samples as obtained after gel filtration showed a very weak, nearly isotropic signal at \( g = 2.01 \) which we ascribe to a small content of \([3Fe-4S]^+\) species. On reduction with dithionite, an intense EPR signal of axial symmetry, \( g_1 = 2.029 \) and \( g_2 = 1.925 \), was obtained suggestive of an abundant \( [4Fe-4S]^+ \) cluster (Fig. 6). (A small shoulder at \( g = 1.95 \), registered on various samples, could arise from a minor component, the origin of which is presently unknown). Signal intensity was maximal in the temperature range 15–20 K and virtually undetectable above 45 K. Spectral saturation effects were observed only for microwave powers above 20 mW (at 20 K). Using a HIPPI (high potential iron-sulfur protein) reference sample for spin quantification, we estimated that the axial resonance accounts for \( 0.4 \) spins/four-iron atoms in the enzyme preparations.

From these electronic and EPR spectroscopic characteristics we conclude that the functionally defective Fe-S core is usually retained on carrying the Ultrogel fraction through conventional protein purification protocols. This occurred most probably also with the first isolation of chromosomally encoded PFL activase from wild-type *E. coli*, where an associated color had been ascribed to an organic factor (6). However, this possibility was dismissed later on by studies of overproduced or heterologically expressed enzyme (3, 8).

Essential Cysteine Residues of PFL Activase—Because cysteine residues are the prominent ligands of Fe-S cores in iron-sulfur proteins, we investigated the effect of Cys mutations as indicated in Fig. 4 and incubated with 10 mM dithionite (5 min). The EPR conditions were: temperature as indicated; microwave power, 2 mW; modulation of 0.5 mT at 100 kHz. The \( g \) values are shown in the 20 K spectrum.

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| C94S     | 49 ± 5b             | 82 ± 8        |
| C102S    | 40 ± 5              | 67 ± 8        |

a Measurements were with both the holocatalytic assay and the standard assay protocols, which yielded identical values.

b Specific activity value by accounting for the protein purity of 0.85.

on purification to the Ultrogel AcA44 stage, with spectral features resembling that of the wild-type enzyme at this stage (Fig. 5). Although C12S and C102S were readily convertible to the apoenzyme form, the C94S mutant protein became insoluble upon removing the iron center by the Q-Sepharose step and therefore was analyzed with the Ultrogel sample. All three mutants displayed full holocatalytic activity (as compared with standard assay activities), albeit absolute values were slightly lower, by a factor of \( \approx 2 \), than the wild-type value. In contrast, C29S, C33S, and C36S mutants proved catalytically incompetent. These proteins, which were colorless at the Ultrogel purification stage, behaved normally throughout the isolation procedure, suggesting that they are unimpaired with respect to global protein-folding. These observations suggest that C29, C33, and C36 constitute cysteine sulfur ligands of the Fe-S core in PFL activase.
Iron-Sulfur Cluster of PFL Activase

Studies of Adenosylmethionine Interaction with PFL Activase—The Fe-S cluster in PFL activase is a prerequisite for effective binding of AdoMet, the crucial co-substrate of this enzyme. This was demonstrated by assays using $^{14}$C-labeled AdoMet and the convenient column centrifugation technique (16) as shown in Fig. 7. No interaction was detected with the apoenzyme sample or with the critical Cys mutants (C29S, C33S, and C36S) previously subjected to Fe-S cluster reconstitution conditions. The data for the holoenzyme sample yielded a tentative $K_{m}$ value of $3 \pm 1 \mu M$, which compares to reported $K_{m}$ values of 2.8–7 $\mu M$ (8, 22). (The maximal quantity of 0.55 AdoMet bound per polypeptide, as calculated for this experiment, is lower than expected, but our analytical technique is insufficient to give accurate data.) The holoenzyme-AdoMet complex was also detectable by conventional gel filtration at 4 °C on Sephadex G-25 run with anoxic Mops buffer, pH 7.5. It should be noted that the $^{14}$C label associated with the protein fraction in these binding experiments was verified by high pressure liquid chromatography analysis to be due to intact AdoMet.

Spectroscopic examinations found that binding of AdoMet slightly decreased the holoenzyme absorbance between 320 and 440 nm (maximally by 5% at 375 nm). Drastic effects, however, were found with the EPR spectrum as shown in Fig. 8A. On incubation with AdoMet, the axial signal of dithionite-reduced enzyme (Fig. 6) changed to a distinctive rhombic symmetry ($g_\perp = 2.009, g_\parallel = 1.921, g_{5/2} = 1.886$). A similar but subtly different signal change to rhombicity, with $g$-factors of 2.038, 1.930, and 1.899, was obtained with S-adenosylhomocysteine (Fig. 8B). In the activity assays, this compound is a competitive inhibitor of PFL activase with respect to AdoMet. (In both samples, weak resonances with $g$ factors of 1.91 and 1.87 (Fig. 8A) or 2.014 and 1.988 (Fig. 8B) again indicated the presence of a minor, second component.)

It is apparent that binding of either nucleoside significantly affects the environment of the iron-sulfur core, inducing modifications of its electronic and magnetic properties. In the sample of Fig. 8A, containing dithionite (10 mM), AdoMet (1 mM) and holoenzyme (0.5 mM), we determined after the EPR measurement a 5'-deoxyadenosine content of about 30 $\mu$M, i.e. a small fraction of AdoMet underwent reductive cleavage.

DISCUSSION

Through isolation as apoenzyme and chemical reconstitution of the iron-sulfur cluster, substantial quantities of PFL activase in its catalytically competent form have now been made available enabling closer structure-function analyses of this radical-generating enzyme. The reconstituted enzyme has been determined to be monomeric (28 kDa), and data reported here are strongly indicative of a 4Fe-4S$^{2+}$ cluster content (rather than a 2Fe-2S type). The electronic spectrum of the greenish brown colored solution shows features (shoulder at 320 nm and broad absorption in the 420 nm region) typical of a 4Fe-4S$^{2+}$ cluster type, which is reducible by dithionite. Accordingly, enzyme samples as obtained are largely EPR silent but on reduction yield an axial 1/2 signal ($g = 2.03, 1.92$) indicative of a 4Fe-4S$^{2+}$ cluster. Integration of the fast relaxing EPR signal, observed optimally at 20 K, estimated ~0.4 spin/four-iron cluster as calculated from the protein-bound iron. Iron and sulfide analyses regularly found 2.6 ± 0.2 iron/polypeptide and 2.5 ± 0.2 sulfur/polypeptide, values that are too low for a 4Fe-4S stoichiometry/monomer. However, allowance has to be made for the fact that the samples of reconstituted enzyme, on which all cluster characterizations were made, were previously gel filtered, an operation that losses between 15 and 30% of the catalytic holoenzyme activity.

For PFL activase samples prepared by anaerobic enzyme isolation from E. coli extracts, Broderick et al. (9) have recently described the iron center content as a mixture of [4Fe-4S]$^{2+}$ and [2Fe-2S]$^{2+}$ clusters, the latter form representing an oxidative degradation product. Neither enzyme samples as obtained nor dithionite-treated enzyme contained a paramagnetic Fe-S center. Cluster reduction to the [4Fe-4S]$^{+}$ state required the

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2 A. Becker and J. Knappe, unpublished results.
The unique utilization of AdoMet as a radical generating cofactor had been recognized at first in 1984 by studies of the pyruvate formate-lyase system, when we identified 5'-deoxyadenosine and methionine as co-products of the post-translational radical introduction into PFL (27). The chemical mechanism of this process, proposed to involve intermediary formation of a H abstracting 5'-deoxyadenosine radical (3, 5), should be approachable now more directly because the metal center of PFL activase has been characterized, and functionality correct enzyme samples are available in abundance. The ligands of the Fe-S core must still be identified. It is tempting to speculate that the metal cluster is not completely cysteinyl coordinated, thus enabling close interaction with the AdoMet co-substrate. Its effective binding to PFL activase has been demonstrated to require the Fe-S complement. Reductive cleavage of AdoMet occurs presumably through the Fe-S cluster; however, this process is largely suppressed when the PFL substrate or Gly-734 site analogous peptide substrates are not present simultaneously (5). The essential role of the metal center for AdoMet reduction has recently been shown also for the parallel case of anaerobic RNR (28).

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