The free radical in pyruvate formate-lyase is located on glycine-734

(EPR/protein-based radical/oxygen sensitivity/polypeptide fragmentation/Escherichia coli)

A. F. VOLKER WAGNER*, MANFRED FREY*, FRANZ A. NEUGEBAUER†, WOLFRAM SCHÄFER‡, AND JOACHIM KNAPPE§

*Institut für Biologische Chemie, Universität Heidelberg, D-6900 Heidelberg, Federal Republic of Germany; †Max-Planck-Institut für Medizinische Forschung, D-6900 Heidelberg, Federal Republic of Germany; and ‡Max-Planck-Institut für Biochemie, D-8033 Martinsried, Federal Republic of Germany

Communicated by Peter Reichard, October 23, 1991 (received for review August 22, 1991)

ABSTRACT Pyruvate formate-lyase (acetyl-CoA:formate C-acetyltransferase, EC 2.3.1.54) from anaerobic Escherichia coli cells converts pyruvate to acetyl-CoA and formate by a unique homolytic mechanism that involves a free radical harbored in the protein structure. By EPR spectroscopy of selectively 13C-labeled enzyme, the radical (g = 2.0037) has been assigned to carbon-2 of a glycine residue. Estimated hyperfine coupling constants to the central 13C nucleus (A_H = 4.9 mT and A_A = 0.1 mT) and to 13C nuclei in α and β positions agree with literature data for glycine radical models. N-coupling was verified through uniform 15N-labeling. The large 1H hyperfine splitting (1.5 mT) dominating the EPR spectrum was assigned to the α proton, which in the enzyme radical is readily solvent-exchangeable. Oxygen destruction of the radical produced two unique fragments (82 and 3 kDa) of the constituent polypeptide chain. The N-terminal block on the small fragment was identified by mass spectrometry as an oxazyl residue that derives from Gly-734, thus assigning the primary structural glycyl radical position. The carbon-centered radical is probably resonance-stabilized through the adjacent carboxamide groups in the polypeptide main chain and could be comparable energetically with other known protein radicals carrying the unpaired electron in tyrosine or tryptophan residues.

Pyruvate formate-lyase (acetyl-CoA:formate C-acetyltransferase, EC 2.3.1.54) is the key enzyme of anaerobic glucose metabolism in Escherichia coli cells (1). Composed of two identical subunits of 85 kDa, this enzyme is posttranslationally interconverted between inactive and active forms. The active form (E-SH) carries a stable radical that is produced by pyruvate-formate-lyase-activating enzyme with adenosylmethionine as the H-atom-abstracting cosubstrate (2). From studies of a mechanism-based inhibitor (hypophosphite), we recently obtained direct evidence for the participation of the protein radical in catalysis (3, 4). It guides the crucial carbon–carbon bond cleavage/synthesis in the first halfreaction of the catalytic cycle along a radical-based chemical mechanism:

\[ \text{E-SH + pyruvate} \rightleftharpoons \text{E-S-acetyl + formate} \]  \[ \text{[1]} \]

\[ \text{E-S-acetyl + CoA} \rightleftharpoons \text{E-SH + acetyl-CoA}. \]  \[ \text{[2]} \]

The catalytic principle of pyruvate formate-lyase is unique among enzyme reactions of cellular metabolism.

Previous EPR spectroscopic studies (4) indicated that the protein radical (g = 2.0037) should be carbon-centered and located on a standard amino acid residue. Here we report its definitive assignment to glycine-734, as deduced from 13C-labeling experiments and from mapping of the regiospecific polypeptide fission that accompanies radical destruction by dioxygen. Pyruvate formate-lyase is an unusual case of an aliphatic amino acid-based protein radical.

EXPERIMENTAL PROCEDURES

Chemicals. [U-13C]glucose (98 mol % 13C), [15NH4]Cl (95 mol % 15N), [1-13C]glycine (99 mol % 13C), and [2-13C]glycine (99 mol % 13C) were obtained from MSD Isotopes; 2H2O (99.8%) was from Sigma; and [U-13C]glycine (104 mCi/mmol; 1 Ci = 37 GBq) and [1-13C]glycine (53 mCi/mmol) were from Amersham.

E. coli Strains and Growth Conditions. Prototrophic and amino acid-auxotrophic strains used are listed in Table 1. The chromosomal act gene (encoding pyruvate-formate-lyase-activating enzyme) (9) was inactivated by insertion of the pACYC184-derived chloramphenicol-resistance (Cmr) gene by homologous recombination via bacteriophage P1; construction details will be reported elsewhere. Cells were transformed with p153E1 to overproduce pyruvate formate-lyase (nonradical form).

Cell growth was on sterile-filtered minimal medium (10), in 2H2O (96% final concentration) where appropriate, with 50 mM glucose or [13C]glucose, 0.1 mM thiamin, 20 μg of chloramphenicol per ml, and 50 μg of kanamycin sulfate per ml. For 15N substitution, a medium with 10 mM 15NH4Cl as sole nitrogen source was used. With auxotrophic strains, the appropriate 13C-labeled amino acid (3 mM) and traces of the corresponding 14C-labeled compound (10 μCi/liter) were added. All cultures were grown with aeration and adjustment to pH 6.5–7.4, and centrifuged cells stored frozen at 200 K for 1 day to several weeks.

Purification of Pyruvate Formate-Lyase (Nonradical Form). Normal (protiated) and deuterated enzyme forms were purified to homogeneity by previous procedures (11). 13C-labeled amino acid-substituted enzymes were carried to about 70% purity by Sepharose Q-chromatography (pH 7.2; 0.1–0.25 M KCl) of the polyme-treated cell extract. Studies of fully 13C-labeled protein were with the centrifuged cell extract (enzyme content, about 20%).

To check glycine/serine labelings, the 14C/13C-containing proteins were acid-hydrolyzed, the amino acids were resolved by standard techniques, and their specific radioactivities were determined.

Preparation and Quantification of Radicál Enzyme. Following previous procedures (4, 11), we converted 1–25 mg of purified pyruvate formate-lyase to the radical form, E-SH, by incubation with 0.05–1 mg of activating enzyme in 0.5–2 ml of anaerobic buffer [0.1 M 3-(N-morpholino)propanesulfonic acid (Mops); adjusted with KOH to pH 7.8] containing 5 mM dithiothreitol and 0.1 mM Fe(NH4)2(SO4)2 under argon with 0.5 mM adenosymethionine, 50 μM continuously phot-

Abbreviations: E-SH, active radical form of pyruvate formate-lyase; Cmr', chloramphenicol resistance.

*To whom reprint requests should be addressed.
Table 1. E. coli strains and plasmid used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Ref. or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC4100</td>
<td>act&lt;sup&gt;+&lt;/sup&gt;</td>
<td>5</td>
</tr>
<tr>
<td>AT2457</td>
<td>glyA6 act&lt;sup&gt;+&lt;/sup&gt;</td>
<td>6</td>
</tr>
<tr>
<td>JC158</td>
<td>serA6 act&lt;sup&gt;+&lt;/sup&gt;</td>
<td>7</td>
</tr>
<tr>
<td>234M1</td>
<td>MC4100 act:&lt;sup&gt;Cm&lt;/sup&gt;&lt;sup&gt;t&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>WFG501</td>
<td>AT2457 act:&lt;sup&gt;Cm&lt;/sup&gt;&lt;sup&gt;t&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>WF8501</td>
<td>JC158 act:&lt;sup&gt;Cm&lt;/sup&gt;&lt;sup&gt;t&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmid</td>
<td>p153E1</td>
<td>8</td>
</tr>
</tbody>
</table>

Km<sup>t</sup>, kanamycin resistance.

Reduced 5-deazariboflavin, and 20 mM pyruvate or oxamate. When required the samples were stored at 200 K. Transfer into anaerobic 2H<sub>2</sub>O buffer was performed by gel filtration through a Sephadex G-25 column. Final concentrations of radical enzyme were determined by activity assays using the relationship that an amount of 35 units (µmol of pyruvate conversion per min at 303 K) contains 1 nmol of unpaired electron spin and 1 nmol of active site (as quantified by [14C]acetyl charging) (4). With homogeneous preparations, this quantity corresponded to 0.17 mg (1 nmol) of homodimeric enzyme (α<sub>2</sub>).

Isolation and Tryptic Digestion of the γ Fragment of E-SH Produced by O<sub>2</sub>. Twenty-five milligrams (154 nmol) of the radical enzyme in 2 ml of anaerobic buffer was supplemented with 10 mM EDTA and gassed with air for 12 min at 273 K. After gel filtration into 20 mM ammonium formate, guanidinium chloride (GdmCl) was added to a final concentration of 3 M, and the sample was filtered through a SepPak C<sub>18</sub> cartridge (Waters/Millipore). Washings with GdmCl/formate and 10 mM formate removed all large polypeptides [unfragmented α (85 kDa), β fragment (82 kDa), pyruvate-formate-lyase-activating enzyme (28 kDa)]. The γ fragment (3 kDa) was then eluted with 3 ml of 80% (vol/vol) CH<sub>3</sub>CN in 4 mM ammonium formate and concentrated under reduced pressure. The yield was about 300 µg (100 nmol).

Digestion with 20 µg of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Serva) was performed for 10 hr in 0.1 M NH<sub>4</sub>HCO<sub>3</sub>. The peptides were purified by reverse-phase HPLC on Nucleosil C<sub>18</sub> (Macherey & Nagel) with a 0–20% CH<sub>3</sub>CN gradient containing 0.1% CF<sub>3</sub>C<sub>6</sub>COOH; the elution sequence of the tryptic peptides was T<sub>1</sub>, T<sub>3</sub>, T<sub>2</sub>, and T<sub>4</sub>.

Peptide Sequencing. Edman degradation, performed by R. Frank, used a gas-phase sequenator and on-line identification of phenylthiohydantoin by HPLC. C-terminal sequencing was carried out with carboxypeptidase Y (Boehringer Mannheim); amino acids released were identified as the dabsyl derivatives by HPLC analysis.

Mass Spectrometry of Tryptic Peptide T<sub>1</sub>. Liquid secondary-ion mass spectra were obtained with a mass spectrometer HSQ 30 (Finnigan-MAT, Bremen, F.R.G.) equipped with a cesium gun (AMD Intectra, Harpstedt, F.R.G.). Acceleration voltage was 3 kV, resolution was 1000, and the gun operated at 10.5 kV and 1.5 mA. The sample was dissolved in 0.1 M HCl and was mixed with glycerol on the probe tip. For esterification, the peptide was incubated with 1 M HCl in isopropanol for 12 hr at room temperature.

EPR Spectroscopy. EPR first-derivative spectra were recorded with a Bruker ESP 300 spectrometer operating at X-band with 100-kHz modulation and using the Bruker variable-temperature device ER 4111 VT. Measurements were performed usually at 255 K (20-mT scan, range, about 0.1-mT field modulation, 20–100 accumulations) on 0.2-ml samples of 10–40 µM radical enzyme, which were filled into 0.2-cm quartz tubes under argon and sealed with a plastic film. At 295 K the aqueous solutions were contained in a flat quartz cell (0.2 mm). Simulations of anisotropic EPR spectra were performed by using a computer program set up by P. M. R. Trousson (University of Leicester, U.K.).

We report hyperfine coupling constants as follows: A (mT), for isotropic and dipolar interactions; a (mT), for isotropic interaction in solutions of low viscosity.

RESULTS

EPR Spectroscopic Assignment by Isotopic Substitutions. The multiplet structure in the EPR spectrum of pyruvate formate-lyase is produced by hyperfine couplings of the unpaired electron spin to different protons. A readily solvent-exchangeable proton is responsible for the dominating doublet splitting (A<sub>iso</sub>(H) = 1.5 mT), whereas the partially resolved substructure comes from two nonexchangeable protons detected by electron-nuclear double resonance (ENDOR) spectroscopy. These assignments were achieved previously by 2H substitution experiments (4), which we have reproduced with the present enzyme preparations (Fig. 1). Computer simulation of spectrum I estimated the isotropic A values for the less strongly coupled protons to be about 0.6 and 0.45 mT.

Assuming that the nonexchangeable protons would be associated with the skeleton of the radical-carrying amino acid residue, initially we addressed its identity by supplementation of 2H<sub>2</sub>O-based bacterial growth media with individual 1H amino acids. When this hydrogen-isotope approach proved to be unsuccessful (for reasons explained below), we turned to 12C/13C replacements. Selected sets of 12C amino acids were applied to E. coli cultures growing on [13C<sub>6</sub>]glucose, and the enzyme preparations were examined for any narrowing of the broad (8 mT-encompassing) EPR signal that characterizes the fully 13C-labeled enzyme (4, 12). Glycine and serine, indistinguishable because of their meta-

![Fig. 1. EPR spectra of the normal (protiated) enzyme (spectra I and II) and the deuterated enzyme (spectra III and IV). The enzyme preparations were from 234M1/p153E1 cells that had been grown in H<sub>2</sub>O and D<sub>2</sub>O medium, respectively.](image-url)
bolic interconversion in protrophic strains, were both effective. This result was consistent with concurrent investigations of the O₂-mediated polypeptide cleavage (see below), indicating Gly734 as the radical site.

To delineate the unpaired spin locus, we prepared selectively ¹³C-substituted enzyme samples using auxotrophic E. coli strains. Traces of ¹³C-labeled amino acids were applied together with the ¹³C-labeled compounds to verify the specificity and extent of the labeling procedures. In Figs. 2 and 3, we present the EPR spectra obtained for [2-¹³C]glycine- and [1-¹³C]glycine-substituted pyruvate formate-lyase, which proved that the radical is centered at C-2 of a glycine residue (Fig. 4).

The two distinct EPR features, separated by 4.9 mT, of the [2-¹³C]glycine-containing enzyme in ²H₂O (spectrum VI in Fig. 2) clearly display strong coupling of the unpaired electron to the ¹³C (J = 1/2) nucleus. We assign these lines to A₂ (¹³C); the A₄ (¹³C) value would be too small to be resolvable, a common property of π-type organic radicals. This is confirmed by computer simulations of the EPR spectra of the enzyme samples in H₂O and ²H₂O buffer; the data are given in Fig. 2. By taking the perpendicular ¹³C-coupling to be smaller than the linewidth of 0.7 mT, the isotropic coupling constant Aiso(¹³C) should lie in the range from 1.6 to 2.1 mT. It should be noted that the anisotropic EPR spectra in Fig. 2 were observed invariably in the frozen state (253 K) and in solution at 293 K (at which temperature the correlation time of enzyme tumbling is about 2 × 10⁻⁷ s).

Couplings to ¹³C nuclei in the α position (glycine carboxyl) and β position (serine carboxyl), which support the proposed radical structure (Fig. 4), are indicated by the significant line broadening effects shown in Fig. 3. The peak-to-trough linewidth of 1.17 mT of the EPR signal of normal enzyme in ²H₂O (spectrum II in Fig. 1) is increased by 0.17 and 0.57 mT upon substitutions by [1-¹³C]glycine and by [1-¹³C]glycine and [1,¹³C]serine, respectively. Moreover, coupling to N was confirmed via global substitution of ¹⁴N (J = 1) by ¹⁵N (J = 1/2) in perdeuterated enzyme. This narrows the linewidth from 0.73 mT (spectrum IV in Fig. 1) to 0.68 mT (spectrum not shown).

The large ¹H hyperfine splitting of 1.5 mT (Fig. 1) is undoubtedly produced by the H₄ atom of the radical center. Its solvent exchangeability (T₁/₂ at 293 K of ≤1 min (4)) is a remarkable property. (In glycine crystals a rapid intraradical exchange reaction has been reported (16): ND₅CHCO₂⁻ → NH₄⁺CO₂⁻.) The additional ¹H hyperfine splittings of

![Fig. 2. EPR spectrum of the [2-¹³C]glycine-substituted enzyme.](image)

The enzyme preparation was from WFG501/p153E1 cells grown in the presence of [2-¹³C]glycine and was measured in H₂O (spectrum V) or ²H₂O (spectrum VI). The simulated spectra depicted below the experimental spectra were obtained with the following data: gₓ = gᵧ = 2.0047, gₗ = 2.0017; linewidth, J = 0.7 mT; Aₓ = Aᵧ = 0.1 mT, Aₛ = 4.9 mT (¹³C); Aₓₓ = Aᵧᵧ = 1.5 mT (¹H) for spectrum V and Aₓₓ = Aᵧᵧ = 0.2 mT (¹H) for spectrum VI.

![Fig. 3. EPR spectra of [1-¹³C]glycine-substituted enzyme (spectrum VII) and of [1-¹³C]glycine/[1-¹³C]serine-substituted enzyme (spectrum VIII). The samples, measured in ²H₂O, were prepared from p153E1-transformed WFG501 (glyA6) cells (spectrum VII) or WFS501 (serA6) cells (spectrum VIII), each grown in the presence of [¹³C]glycine.](image)

0.45 and 0.6 mT are yet unassigned but could be from H₄ (NH) and/or from δ protons located on the adjacent amino acid residues. In attempting to obtain experimental evidence for this, the serine amino acid, as applied to cell cultures, proved to undergo metabolic solvent-exchange of its α-carbon proton.

In conclusion, our EPR spectroscopic data for the pyruvate formate-lyase radical agree well with data published for glycine radical models, which are given in Fig. 4. The optical absorption at 365 nm (4) matches that reported for the 2,5-dioxopiperazin-3-yl radical at pH 5 (17).

**Oxygen Fragmentation of the Polypeptide Chain at the Radical Site.** Contact with O₂ has long been known to destroy rapidly the EPR signal and the catalytic activity of pyruvate formate-lyase; in air-saturated buffer at 293 K, the half-life is about 10 s (2). By gel electrophoretic analysis, this process has now been found to be accompanied by a fission of the radical-carrying polypeptide chain (85 kDa) into unique pieces of 82 kDa and 3 kDa (Fig. 5, lane 2). This opened the possibility to map the primary structural position of the protein radical. That only about half of the applied enzyme protein becomes cleaved is consistent with the quantity of 1 electron spin per dimer measured previously (4).

Fig. 6 summarizes our chemical characterization of the polypeptide cleavage site. N- and C-terminal sequencing showed the large fragment, 82 kDa to comprise the N-terminal portion of the constituent polypeptide chain (α, 85 kDa). The small fragment (γ, 3 kDa), which was isolated in 0.1-μmol quantities, proved unamenable to Edman degradation. To facilitate structure analyses, the peptide was digested with trypsin, and the crucial N-terminally blocked subfragment (T₁) was then analyzed by liquid secondary-ion mass spectrometry. We found a (M + H)⁺ ion of 580 mass units, which matched with the value of 580.27 calculated for the oxalylated tetrapeptide, encompassing the residues 735-738: HOOC-CONH-Tyr-Ala-Val-Arg-COOH. This structure was confi-
FIG. 5. Polypeptide fragmentation by O$_3$. E-S-acetyl (25 μM) in anaerobic buffer containing 20 mM pyruvate was either directly (lane 2) or after transformation to the 1-hydroxy-1-phosphoryl-ethyl radical derivative (4) (lane 3) gassed with air for 1 min at 0°C in the presence of 10 mM EDTA. The samples were resolved by NaDodSO$_4$/polyacrylamide gel electrophoresis using an 8% gel (18) for the high molecular weight range (a) or a 16% gel (19) for the low molecular weight range (b). Four micrograms (a) or 240 μg (b) was applied per lane; visualization was by Comassie blue staining. Lane 1 contains a reference with the nonradical enzyme form. Marker protein migration is shown in kDa.

firmed through (di)esterification of T1 with isopropanol; the product yielded (M + H)$^+$ of 664 (calculated, 664.29).

That Gly-734 is converted to an oxalyl residue upon oxidative destruction of the protein radical unambiguously places the EPR-spectroscopically identified glycine radical to that particular position. The O$_3$-mediated fission of the polypeptide backbone should be initiated by formation of a peroxy radical on carbon-2 of the amino acid residue. Such intermediates were suggested previously for γ-radiolyses of proteins and peptides in the presence of oxygen (OH/O$_3$), which yield mainly a peptide amide and a carbonyl peptide (for a review, see ref. 20):

R$^1$CONHCR$^2$(O$_2$)CONHR$^3$ \[\rightarrow R^1\text{CON} = CR^2\text{CONHR}^3 \]

\[\rightarrow R^1\text{CONH}_2 + R^2\text{COCONHR}^3.\]

Sensitive carbonyl reagents, when immediately applied to our O$_3$-reaction system, indeed gave a weak response, but attempts to directly characterize the putative glyoxylate residue [via reductive amination or transamination to glycl (21) or as dinitrophenylhydrazone] were not successful. While the oxalyl residue is the principal product of the glycine carbons in this system (isolation yield, ~70%), further (oxidative) modifications are likely, as the serine-733 residue was not recovered with our analyses.

The oxygen-mediated polypeptide cleavage invariably takes place with either of the two functional states of the radical enzyme, E-SH or E-S-acetyl. It no longer occurs, however, when E-S-acetyl is previously converted with hypophosphite to the protein-linked 1-hydroxy-1-phospho-

β-fragment

\[\text{SEINEK} --- \text{ACC} \quad \text{T1 by oxalyl-TAVR} \quad \text{T3} \quad \text{EQQD}^{***} \quad \text{T4} \quad \text{TPH2}^{**} \quad \text{CP-Y} \]

\[\text{SEINEK} --- \text{LVR} \quad \text{T1 by oxalyl-TAVR} \quad \text{T3} \quad \text{EQQD}^{***} \quad \text{T4} \quad \text{TPH2}^{**} \quad \text{CP-Y} \]

\[\text{SEINEK} --- \text{LVR} \quad \text{T1 by oxalyl-TAVR} \quad \text{T3} \quad \text{EQQD}^{***} \quad \text{T4} \quad \text{TPH2}^{**} \quad \text{CP-Y} \]

FIG. 6. Mapping of the radical site by the O$_3$-mediated polypeptide fission. The bold-line type designates relevant stretches of the amino acid sequence (759 residues) of pyruvate formate-lyase (9). Results of N-terminal sequencing, carboxypeptidase (cp-y) digestion, or mass spectrometric analysis are given in single-letter amino acid code under the underlined peptide designations (β-fragment, 82 kDa; tryptic peptides T1–T4 from the γ-fragment, 3 kDa). Asterisks indicate incomplete sequencing.

FIG. 7. Amino acid residues of pyruvate formate-lyase involved in substrate or suicide substrate conversions.

FIG. 8. Polypeptide fragmentations of E-S-acetyl (4) (see structure in Fig. 7), as shown in Fig. 5, lane 3. This demonstrates that the polypeptide fragmentation requires the protein radical to be backbone-centered.

DISCUSSION

Protein radicals have attained increasing interest during recent years, ever since the discovery of a tyrosyl radical in ribonucleotide reductase (22, 23). In all other protein-based radicals reported to date, the spin is located also on tyrosine [prostaglandin H synthase (24), photosystem II (25), galactose oxidase (26)] or on a tryptophan residue [cytochrome c peroxidase compound ES (27)]. Our results with pyruvate formate-lyase now give an example of a polypeptide main-chain-centered radical. The glycine C-2 radical is expected to be resonance-stabilized through the adjacent carboxamide groups and thus could be comparable energetically with the aromatic π-type radicals. Its remarkable stability of several days at 273 K or hours at 303 K is a fascinating property, since there is no metal center contained in pyruvate formate-lyase—in contrast to the systems cited above.

Although the glycine radical should be buried in the protein fold and thereby protected from exogenous quenching through the buffer medium (or by a spin-trap reagent like 5,5-dimethyl-1-pyrroline-N-oxide), a distinctive proton communication with the solvent phase must exist to explain the ready exchange of the C-2 hydrogen. Gly-734 is the ultimate of 57 glycine residues in the polypeptide chain, and secondary-structure predictions suggest its location in a turn stretch (Val-Ser-Gly-Tyr) between two β-strands.

The distance to the acetyl transfer site Cys-419 (3) is another open question that concerns the catalytic function of the glycine radical. The spin is located on the substrate skeleton after reaction of E-S-acetyl with the formate-analog hypophosphite (3, 4) (Fig. 7). The same radical intermediate, which is finally quenched without regeneration of the glyycl radical, is produced from E-SH and the pyruvate-analog acetyl phosphinate [M.F. and J.K., unpublished data; a detailed kinetics of enzyme inactivation by acetyl phosphinate has been reported (28)]. The remarkable feature of the analog reactions is a covalent-labeling of the SH group of Cys-418, which is adjacent to the acetyl-transferring Cys-419. Cys-418 is also of importance for the physiological substrate conversion, since Cys-418 → Ser or Cys-418 → Ala mutant enzymes, although carrying the glyycl radical, are inactive (S. Elbert and J.K., unpublished data). With these findings, we tentatively propose a reversible hydrogen transfer, induced by substrate binding, between the Gly-734 resting-state spin localization and Cys-418 (Gly$^-$ + RSH ⇌ Gly(H) + RS$^-$), whose thyl radical will function as the "working radical" for substrate processing. For ribonucleotide reductase, x-ray structure data (29) ruled out a direct H abstraction from the substrate by the Tyr-122 resting-state radical. Instead, a long-range electron transfer into the substrate site to possibly a cysteine residue has been proposed (see ref. 30).
The oxygen-sensitivity of the protein radical clearly places pyruvate formate-lyase as an obligate anaerobic enzyme. [Metabolic signals not yet fully recognized guarantee that the posttranslational radical introduction will only operate in strictly anoxic *E. coli* cells (1).] The unique polypeptide fission by O₂ represents an irreversible modification that is characteristic for pyruvate formate-lyase and is a useful measure of the enzyme radical for *in vivo* or *in vitro* studies. Is the glycol radical unique to pyruvate formate-lyase? The recently discovered anaerobic (class IV) ribonucleotide reductase of *E. coli* (31) and the clostridial lysine 2,3-aminomutase (32) show requirements for enzyme activation that resemble those for pyruvate formate-lyase activation (adenosylmethionine, metal, reducing agents). Therefore, they could represent other candidates for the glycol radical motif.

We thank Dr. Matthias Rothe for providing pyruvate-formate-lyase-activating enzyme, Dr. Rainer Frank (Zentrum für Molekulare Biologie, Heidelberg) for peptide analyses, Dr. Barbara J. Bachmann (New Haven, CT) for providing mutant strains, and Dr. Chris Sander (European Molecular Biology Laboratory, Heidelberg) for secondary-structure predictions. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Bonn) and the Fonds der Chemischen Industrie (Frankfurt/Main).