Mechanism-Based Inactivation of Pyruvate Formate-Lyase by Fluoropyruvate: Direct Observation of an α-Keto Carbon Radical

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Received July 19, 1995

Pyruvate formate-lyase (EC 2.3.1.54; PFL) is a glycyl radical-containing enzyme that catalyzes the CoA-dependent conversion of pyruvate into acetyl-CoA and formate.[1] In addition to this unusual α-carbon radical, the enzyme contains two cysteines, C418 and C419, which are essential for catalysis.[2] A radical mechanism for the reaction has been proposed in which the transient formation of a thiyl radical at C419 occurs, followed by its addition to the keto group of pyruvate with subsequent homolytic carbon−carbon bond cleavage.[3] Our search for radical intermediates formed during this conversion has led to investigations of mechanism-based inactivators. In this report, we provide EPR evidence for the formation of an α-keto carbon radical intermediate in the mechanism-based inactivation of PFL by fluoropyruvate.

In the presence of formate or CoA, fluoropyruvate inactivates PFL in a time- and concentration-dependent manner, obeying saturation kinetics (Figure 1).[4,5] The addition of pyruvate protected PFL against inactivation (data not shown). The kinetics of inactivation are similar to those of two other substrate analogs, mercaptopyruvate[5a] and acetylphosphinate.[5b] Thus, a similar kinetic mechanism can be proposed.[6]

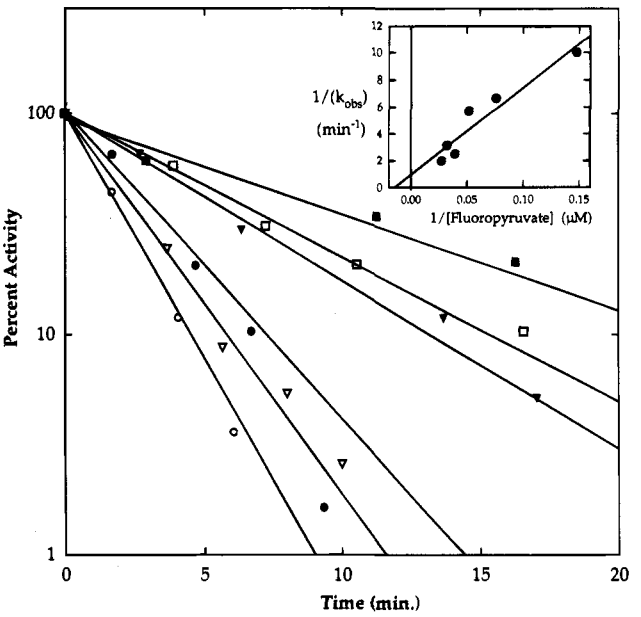

Figure 1. Kinetics of inactivation of PFL by fluoropyruvate. Fluoropyruvate at the following concentrations, (○) 36.1, (▽) 30.8, (●) 25.2, (▼) 19.4, (□) 13.2, and (■) 6.8 μM, was incubated with the enzyme in the presence of 25 mM formate. At indicated time intervals, residual activities were assayed according to the published procedure.[5a] Plots of percent remaining activity as a function of time were fitted to a single exponential decay using Sigma Plot (Jandel Scientific). The kinetic parameters for inactivation were calculated from a plot of $1/k_{obs}$ vs reciprocal of the concentration of fluoropyruvate (inset). The calculated kinetic parameters for inactivation of PFL by fluoropyruvate are 68 μM and 1.05 min^{-1} for K_I and k_{inact}, respectively. The K_m values for pyruvate and formate are 2 and 25 mM, respectively.[1c]

EPR studies of the inactivation provide direct evidence for a homolytic mechanism. Upon addition of fluoropyruvate to active PFL in the presence of formate, a long-lived radical intermediate is generated which exhibits a complex line shape in its frozen solution EPR spectrum (Figure 2a).[7] The EPR spectra of this intermediate were identical in H_2O and in D_2O (data not shown), indicating that the observed splittings are not due to solvent exchangeable hydrogens. Deuterium isotope substitution for the methylene hydrogens of fluoropyruvate,[8] on the other hand, resulted in a change in the splitting pattern, indicating that the radical is on or near the methylene group originating from fluoropyruvate (Figure 2b). This conclusion was corroborated by perdeuteration of the enzyme.[9]

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(4) (a) Fluoropyruvate has been previously reported to inhibit PFL activity, although no detailed kinetics were reported (Knappe, J.; Blaschkowski, H.-P.; Edenharder, R. *Int. Symp. Metab. Interconvers. Enzymes* **1972**, 319−329). The inhibition was previously reported to be reversible upon removal of excess inhibitor via gel filtration; in contrast, we find the inhibition to be irreversible under our experimental conditions, although nearly complete activity can be restored by the reactivation procedure.[3] Thus, the earlier observation of reversibility can be explained by reactivation in the crude extracts that were used.

(5) Kinetics of inactivation of PFL by fluoropyruvate were obtained by the method described in the following: (a) Parast, C. V.; Wong, K. K.; Kozarich, J. W.; Peisach, J.; Magliozzo, R. S. *Biochemistry* **1995**, *34*, 5712−5717. (b) Ulissi-Demario, L.; Brush, E. J.; Kozarich, J. W. *J. Am. Chem. Soc.* **1991**, *113*, 4341−4342. Sodium fluoropyruvate (Aldrich) was dissolved in 100 mM Tris/HCl, 100 mM KCl, pH 7.6, and the solution was rendered anaerobic by passing a constant stream of argon through it for 5−10 min. DTT was excluded from this solution to minimize decomposition of the inhibitor prior to addition to the enzyme.

(6) Fluoropyruvate is proposed to be a substrate analog which partitions between turnover and inactivation (Scheme 1). Turnover leads to formation of fluoroacetyl-PFL, which is protected from further inactivation. Formate or CoA is required for deacylation of the fluoroacetyl-PFL, thereby permitting multiple cyclings of active PFL until complete inactivation is achieved. In the absence of formate or CoA, slower and more complex kinetics of inactivation are observed. In support of the prediction that fluoropyruvate is a substrate for PFL, we have observed that the enzyme catalyzes the exchange of [^{14}C]formate into fluoropyruvate. Quantitation suggests that complete inactivation of PFL requires ~30 turnovers of fluoropyruvate.

(7) The conversion of the glycyl radical to the new radical species is ~1:1 in the presence of 25 mM formate. In the absence of formate, a complex spectrum is observed that is a composite of the starting glycyl radical and the new radical species. This behavior is similar to that observed for mercaptopyruvate inactivation of PFL and is consistent with the partitioning between turnover and inactivation.[5a]

(8) Dideuteriofluoropyruvate was prepared as described by Cheung and Walsh: Cheung, Y. F.; Walsh, C. *Biochemistry* **1976**, *15*, 3749. The EPR spectrum obtained for PFL inactivated with deuteriofluoropyruvate, while lacking the multiplicity found for protiofluoropyruvate, was not analyzed quantitatively due to the presence of some signal intensity arising from the fraction of monoprotio-/monodeuteriofluoropyruvate (~33%) contained in the sample.

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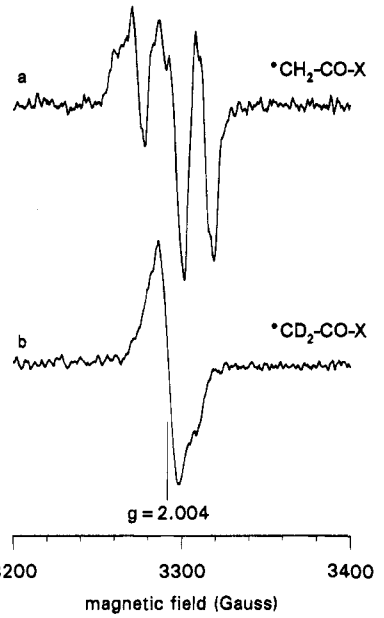
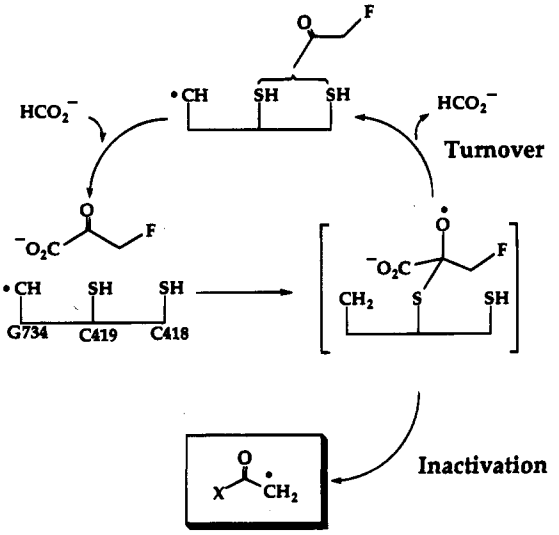


Figure 2. X-band EPR spectra of (a) PFL inactivated with protiofluoropyruvate and (b) PFL inactivated with deuteriofluoropyruvate (5 mM). All enzyme samples (200 μL, 10 mg/mL) were activated according to the published procedure, and the resting glycyl radical signal was measured at 77 K prior to inactivation.[5a] The sample solutions were thawed at room temperature under a stream of argon. Fluoropyruvate (5 mM) and formate (25 mM) were then added, and the samples were quickly frozen in liquid nitrogen (~30 s). The assigned chemical structures corresponding to each EPR spectrum are shown. The identity of X remains to be established but could be the sulfur of C419. All spectra were obtained at 77 K with a microwave power of 2 mW and a frequency of 9.23 GHz.[5a]

The radical formed in our experiments has an EPR spectrum closely related to that assigned to the radical species, $^{\bullet}CH_2$-COOH, formed by elimination of SH from a thioglycolic acid radical precursor.[10] On the basis of this comparison, we suggest the structure $^{\bullet}CH_2-CO-X$ for the radical intermediate formed in the experiment presented here. A reasonable simulation of the spectrum (not shown) could be achieved using an anisotropic g-tensor ($g_{1,2,3} \approx 2.006, 2.0047, 2.0021$) and two anisotropic $S = {}^1/_2$ nuclear hyperfine coupling interactions for two hydrogens with isotropic components of 70 and 50 MHz. Consistent with the assignment is the spectrum of the radical formed in the deuterated inhibitor samples, which lacked a large splitting that would be present in a radical containing ^{19}F in an α-position.[11] These results strongly argue for the elimination of fluoride prior to the formation of the α-keto carbon radical intermediate.

The mechanism-based inactivation suggests that the initial steps leading to the α-keto radical may be similar to those occurring with pyruvate. The initial formation of a tetrahedral oxy-radical intermediate via the addition of an enzymic thiyl radical to C-2 of pyruvate has been proposed.[3] Formation of a similar intermediate with fluoropyruvate could provide the basis for partitioning between turnover and inactivation (Scheme 1). Turnover would proceed with C–C bond homolysis to afford fluoroacetyl-PFL and formate radical anion (which would be quenched with regeneration of the glycyl radical). Inactivation would proceed with fluoride elimination and the formation of the α-keto carbon radical. Fluoride elimination could be effected through a radical-mediated decarboxylation of C-1. The fate of the C-1 unit of fluoropyruvate during inactivation (i.e., CO_2 vs HCO_2^-) and the linkage site (possibly C419) of the radical to the enzyme (X, Figure 2) are currently under investigation.

Scheme 1. Proposal for the Mechanism-Based Inactivation of PFL by Fluoropyruvate[a]



[a] The identity of X as well as the fate of C-1 of the inhibitor remains to be established.

Radical-mediated halide elimination has been well documented for the model compounds ethylene glycol and chloroethanol, based on EPR spectroscopy as well as product isolation.[12] These model studies have long been the crux of the evidence for the proposed formation of α-keto carbon radicals in reactions catalyzed by enzymes such as ribonucleotide reductase, ethanol deaminase, and dioldehydrase; however, no such intermediates have been observed.[13] Thus, our findings constitute the first direct demonstration of the formation of this elusive radical in an enzymatic system and provide additional support for the postulation of radical intermediates in the normal catalytic process of PFL.

Acknowledgment. This research was supported by grants from the National Institutes of Health (GM35066 to J.W.K.; GM40168 and RR02583 to J.P.).

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(9) No decrease in the multiplicity of the splittings in the EPR spectrum of the radical intermediate using perdeuterated enzyme[5a] was found. However, the resolution of the splittings in the EPR spectrum was improved in these samples, indicating that the radical intermediate is intimately associated with nonexchangeable hydrogens in the active site.

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