# Molecular mechanism of pyruvate-ferredoxin oxidoreductases based on data obtained with the *Clostridium pasteurianum* enzyme

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**Abstract Pyruvate-ferredoxin oxidoreductase oxidises pyruvate in many fermentative microorganisms. The enzyme from *Clostridium pasteurianum* is an air-sensitive homodimer of 2×120000 daltons, for which pyruvate is the best substrate found among several α-ketoacids. Each subunit contains eight iron atoms in two [4Fe–4S] clusters. Two distinct EPR signals, possibly associated with two ligand environments, arise from one of these clusters. Binding of pyruvate does not generate a radical. The results reported suggest a scheme for the electron flow in pyruvate ferredoxin oxidoreductases according to which the detailed reaction mechanism depends on the number (even or odd) of [4Fe–4S] clusters present in a given enzyme.**

*Key words:* Pyruvate; Electron paramagnetic resonance; Electron transfer; Iron-sulfur; Ligand exchange; *Clostridium pasteurianum*

## 1. Introduction

Pyruvate is a key intermediate in the energy metabolism of living cells. In most aerobes, pyruvate is oxidized by the pyruvate dehydrogenase multi-enzyme complex which generates carbon dioxide and acetyl-CoA and reduces $NAD^+$ ([1] and references therein). NADH is used as the major source of reducing power in oxidative phosphorylation. In anaerobes, pyruvate can be metabolized through a variety of pathways but it is often oxidized to carbon dioxide and acetyl-CoA with the concomitant reduction of a low-potential redox protein, like ferredoxin or flavodoxin [2]. The enzyme responsible for the oxidation of pyruvate in several bacteria, archaea or protists is pyruvate-ferredoxin oxidoreductase (PFO). PFO contains thiamine pyrophosphate (TPP) and iron-sulfur centers ([3,4] and references therein), but variations in quaternary structure, cofactor(s) content or sensitivity to inhibitors have been observed depending on the source of the enzyme. This report presents the main molecular properties of the enzyme purified from *Clostridium pasteurianum*, which reveal mechanistic differences among the enzymes from various sources. These data suggest the occurrence of mixed-ligand binding for one [4Fe–4S] cluster in the presence of substrates and disclose the sequence of intramolecular electron transfer events triggered by substrate turnover.

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*Abbreviations:* PFO, pyruvate-ferredoxin oxidoreductase; CoASH, reduced coenzyme A; DTT, dithiothreitol; TPP, thiamine pyrophosphate; PEG, polyethylene glycol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

## 2. Materials and methods

All experiments, including the purification of proteins and enzymes, have been carried out under strictly anaerobic conditions, either with argon lines or inside an anaerobic chamber (Jacomex, Livry-Gargan, France). Traces of oxygen in argon were removed by passage through a tower of BASF R3-11 catalyst.

### 2.1. Purification of PFO

*Clostridium pasteurianum* W5 (ATCC 6013) cells were grown, harvested, and processed in 300 g batches as described previously [5]. The supernatant was fractionated by precipitation with polyethyleneglycol 6000 (PEG) [6]. The supernatant at 30% (w/w) PEG contained the 2[4Fe–4S] ferredoxin. The pellet contained the [2Fe–2S] ferredoxin, hydrogenase, and PFO. It was dissolved in 20 mM Tris-Cl, pH 7.4, 0.1 M NaCl, 1 mM dithionite, and loaded on a 5×20 cm DE52 column equilibrated with the same buffer. All buffers used subsequently contained 20 mM Tris-Cl, pH 7.4 and 1 mM dithionite. The column was sequentially washed with 0.1 M, 0.15 M, 0.25 M and 0.4 M NaCl. The 0.15 M NaCl fraction contained hydrogenase and PFO. It was concentrated on an Amicon cell equipped with a PM30 membrane, and chromatographed on a 3×100 cm Sephadex G-100 (Pharmacia) column equilibrated with buffered 0.2 M NaCl. This step separated PFO from hydrogenase. The PFO-containing fraction was concentrated, while bringing the NaCl concentration down to 0.05 M, and purified on a 2×10 cm DE52 column by implementing a 0.05–0.15 M NaCl gradient. The fractions with highest specific activity were concentrated and chromatographed on a 2×100 cm Sephacryl S-300 (Pharmacia) column equilibrated with buffered 0.2 M NaCl. After this step PFO appeared to be essentially pure on SDS-PAGE gels, and its specific activity was in the 12–20 μmol pyruvate×(min×mg)$^{-1}$ range as measured with benzyl viologen as electron acceptor.

### 2.2. Other methods

Iron was determined as reported [7]. UV-visible and low temperature EPR spectroscopies were implemented according to previously published procedures [8,9]. The molecular mass of PFO was determined on a 600 ml-column of Sephacryl S-300 (Pharmacia) equilibrated in 20 mM Tris-Cl, 0.2 M NaCl, pH 7.4. The column was calibrated by filtration of bovine serum albumin, lactate dehydrogenase and component I (MoFe protein) of *C. pasteurianum* nitrogenase.

### 2.3. Enzymatic assays

The activity of PFO was measured by monitoring the reduction of 5 μM ferredoxin at 425 nm or 1 mM benzyl viologen at 540 nm or 1 mM methyl viologen at 604 nm, in an anaerobic chamber ensuring an oxygen concentration in the Ar gas phase of less than 2 ppm. The 500 μl-assay contained 50 mM potassium phosphate, pH 7.5, 2 mM DTT, 140 μM CoASH, 50 μM TPP, 1 mM $MgCl_2$, the electron acceptor and 4 μg of PFO. The reaction was started by adding 2 mM sodium pyruvate.

## 3. Results

### 3.1. Catalytic properties.

Among α-ketoacids, α-ketobutyrate was the only substitute found for pyruvate in the methyl viologen reduction assays. The apparent $K_m$ and $V_{max}$ of the reaction with α-ketobuty-


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rate (5 mM and 0.9 μmol(min·mg)$^{-1}$) are 6-fold higher and 3-fold lower, respectively, than in the reaction with pyruvate (0.8 mM and 2.6 μmol(min·mg)$^{-1}$), indicating that the latter compound is most probably the physiological substrate of the enzyme. Several other α-ketoacids with 5 (α-ketovalerate, α-ketoisovalerate, α-ketoglutarate) or 6 carbon atoms (α-ketocaproate, α-ketoisocaproate) were not substrates of the enzyme. Indeed, oxidative decarboxylation of other 2-oxoacids exists but it is carried out by other enzymes [10]. Clostridial ferredoxins are the type of electron acceptor displaying the highest specificity for the enzyme [11].

The activity of *C. pasteurianum* PFO always displayed a lag time of variable length, from a few seconds to two minutes. Preincubation of the enzyme with components of the assay, like CoASH, TPP (50 μM) and $MgCl_2$ (1 mM), or with 1 mg/ml bovine serum albumin or with up to 5 mM DTT did not suppress the period of latency. *C. pasteurianum* PFO exhibited a sharp optimum at pH 7.2, close to the value reported for the *C. acetobutylicum* enzyme [12]. The enzyme was rapidly inactivated by air, but oxygen was not the only factor involved. The activity of a 8 mg/ml enzyme solution remained constant over 6 days in the absence of oxygen, but a 0.8 mg/ml solution lost 85% of its activity in 3 h in the same conditions. 0.3 mM CoASH decreased the loss to 45%, while 50% (w/w) PEG completely stabilized the same solution for over 24 h. Therefore, dilution and absence of substrates are likely to contribute to the inactivation of the enzyme.

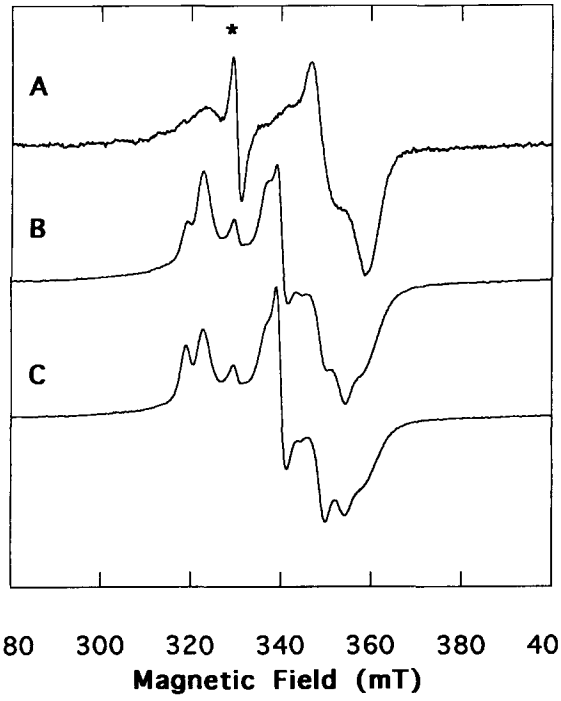


Fig. 1. EPR spectra of *C. pasteurianum* PFO. Purified PFO (100 μM) was reacted with: (A) 20 mM pyruvate; however, no EPR signal was observed under these conditions with most enzyme preparations; (B) 20 mM pyruvate and 13 mM CoASH; (C) as (B) with 10 mM $Na_2S_2O_4$. The starred signal indicates a minor radical. The spectra are not drawn to scale: the total intensity of the spectrum in (A) is about one sixth of that of the spectrum in (B). Instrument settings: microwave frequency 9.229 GHz, modulation frequency 100 kHz, modulation amplitude 1 mT, microwave power 1 mW and temperature 10 K.

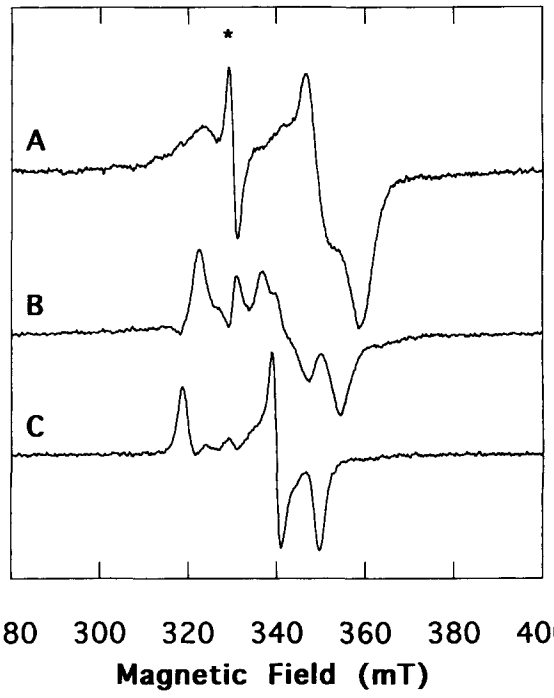


Fig. 2. Deconvoluted signals contributing to *C. pasteurianum* PFO EPR spectra. (A) signal I (2.04; 1.89; 1.84) as observed in Fig. 1A; (B) signal II (2.04, 1.94, 1.86) obtained by subtracting signal I and signal III from Fig. 1B; (C) signal III (2.07, 1.94, 1.89) obtained by subtracting the spectrum of Fig. 1B from the spectrum of Fig. 1C.

### 3.2. Molecular properties

The molecular mass of purified PFO was estimated at 240000 daltons by passage through a calibrated gel filtration column. The enzyme gave a single band of ca. 120000 daltons on PAGE-SDS indicating a homodimeric structure.

The sequencing of several proteolytic peptides provided satisfactory alignments (in the 50–100% range of identity) with segments of bacterial *nifJ* (nitrogen fixation) gene products or of PFO from amitochondriate protists available in the protein databanks. In contrast, no similarities were detected between these peptides and the sequenced enzymes from archaea.

Determination of the iron content of several enzyme preparations gave an average value of 14.5 mol iron/mol of enzyme (dimer). *C. pasteurianum* PFO as purified above was EPR-silent and remained so when 2 mM CoASH but no pyruvate were added to the enzyme. Addition of 20 mM of pyruvate together with 2 mM CoASH generated a complex EPR spectrum (Fig. 1B), which could be described as composed of three different $S = 1/2$ signals (see below, Fig. 2), at $g = 2.04$; 1.89; 1.84 (signal I), at $g = 2.04$, 1.94; 1.86 (signal II), and at $g = 2.07$; 1.94; 1.89 (signal III). Signal III was found to increase when 10 mM dithionite was added to the mixture of the enzyme and substrates (Fig. 1C). However, this signal was hardly detected when *C. pasteurianum* PFO as isolated was reduced by dithionite alone or by a mixture of dithionite and CoASH. Of these three signals, signal I was occasionally found to be the only one present in samples reduced with a large excess of pyruvate alone (Fig. 1A). The spectrum of Fig. 1A also shows a very weak isotropic signal, most likely arising from a radical, but without the hyperfine structure contributed by α-ketoacids [4]. All spectra of Fig. 1 were significantly broadened above ca. 25 K, as expected from the EPR signals

of $[4Fe–4S]^{+}$ clusters, with signal II relaxing faster than the other two.

Fig. 2 shows the deconvolution of the different spectra of Fig. 1 and displays the signature from each $S = 1/2$ species. The contribution of these signals to the spectra of Fig. 1 could thus be calculated. In the presence of pyruvate and CoASH (Fig. 1B), the intensity of signal II represented about half of the total spectrum, with signals I and III accounting for 36 and 13%, respectively. When dithionite was added (Fig. 1C), the total intensity underwent an increase of ca. 10%, which was almost equally distributed between signal II and signal III. Consequently, the relative contribution of signal II remained almost equal (47%) compared to the spectrum of Fig. 1B, but the proportions of signals I and III shifted to 29 and 24%, respectively. The effect of dithionite was thus the addition of ca. 10% more electrons to the enzyme reduced by substrates and the increase of the relative contribution of signal III, partly at the expense of signal I.

The UV-visible absorbance changes occurring upon reduction by substrates and dithionite were also monitored. The oxidized enzyme displayed a broad maximum at 390 nm and a shoulder at 320 nm, like other [4Fe–4S] proteins. Addition of both pyruvate and CoASH resulted in a ca. 30% decrease in absorbance at 390 nm. A further 10% loss of this absorbance occurred upon addition of dithionite.

## 4. Discussion

The quaternary structure and partial sequence of *C. pasteurianum* PFO resemble those of similar enzymes from bacteria or unicellular eucarya [4,13–16], but differ from the heterodimers or heterotetramers found in archaea [3,10,17,18].

Taken together, UV-visible absorbance and EPR spectra show evidence for only [4Fe–4S] centers, with no features reminiscent of [2Fe–2S], [3Fe–4S] or other clusters of known spectroscopic signature. The total integration of the EPR spectrum of Fig. 1C agrees with 4 spins per dimer. Assuming an average extinction coefficient of 15000 $M^{-1} \cdot cm^{-1}$ at 390 nm for each oxidized [4Fe–4S] center, as determined with ferredoxins [19], four such clusters are present per molecule of enzyme, in keeping with the results of EPR integrations. 16 Fe/mol from four [4Fe–4S] clusters is also a value in reasonable agreement with the chemical determination (14.5 Fe/mol). Among the enzymes of similar quaternary structures, these figures are similar to those found for *K. pneumoniae* and *C. thermoaceticum* PFO [20], but they are about twice those reported for the enzymes of *C. acidurici* [21] or *Trichomonas vaginalis* [15]. In contrast, *Desulfovibrio africanus* PFO has been shown to contain three [4Fe–4S] centers per subunit [4].

From the now available data, it appears that PFO from different sources have a variable number of [4Fe–4S] clusters whose spectroscopic properties provide clues to the relevant electron flow occurring upon the turnover of the substrates. In *C. pasteurianum* PFO, as in other such enzymes thought to contain two [4Fe–4S] clusters per subunit [17,20], pyruvate does not generate the EPR signal of a radical. In contrast, an EPR signal assigned to the hydroxyethyl-TPP radical [4,18,22] appears upon addition of pyruvate to PFO containing an odd (one or three) number of [4Fe–4S] clusters.

Upon addition of both substrates, CoASH and pyruvate, three distinct EPR signals are elicited in *C. pasteurianum* PFO (Figs. 1 and 2). Quantitation of the contribution of each signal agrees with two of these signals (labeled I and III) corresponding to two slightly different electronic structures of the same cluster, which appears to be the easiest to reduce. Two signals associated with a single cluster may be expected if one (or more) of the cluster's ligand is exchanged in the presence of substrates. According to the present EPR data, this exchange should not be limited to thiolate exchange but could involve other functional groups [23,24]. This situation is somewhat reminiscent of that observed with *Thermotoga maritima* PFO [17] for which three EPR signals were detected, although other data pointed to only two [4Fe–4S] clusters per mole. However, one of the EPR signals involved in *Thermotoga maritima* PFO appeared upon CoASH addition alone [18], whereas no signal was detected in *Clostridium pasteurianum* PFO under such conditions.

In conclusion, it is proposed that cluster I, which is responsible for EPR signals I and III in *C. pasteurianum* PFO, is close to the hydroxyethyl-TPP intermediate and is the primary electron acceptor from pyruvate when CoASH triggers the electron flow through the enzyme. This proposal is borne out by the unusual $g$-values of signal I (with an average value, $g_{av} = 1.92$, below the conventional 1.94) which might indicate a strong perturbation of the cluster, maybe via ligand exchange between a cysteinate and another anion [23,24], like a TPP or hydroxyethyl-TPP anion. Cluster II, contributing EPR signal II, is the most distant from the site of pyruvate binding and is the putative electron donor to the external electron acceptor, e.g. ferredoxin [11]. Cluster I is likely to be in equilibrium between two different forms, the one described above (signal I), and another one with more conventional coordination and $g$-values (signal III) when the acetyl group is formed.

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