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Radical reactions of thiamin pyrophosphate in 2-oxoacid oxidoreductases[^]

George H. Reed^{a,*}, Stephen W. Ragsdale^b, and Steven O. Mansoorabadi^c

^aDepartment of Biochemistry, University of Wisconsin-Madison, 1710 University Avenue, Madison, WI 53726, USA

^bDepartment of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109, USA

^cDivision of Medicinal Chemistry, College of Pharmacy, University of Texas at Austin, Austin, Texas 78712, USA

Abstract

Thiamin pyrophosphate (TPP) is essential in carbohydrate metabolism in all forms of life. TPP-dependent decarboxylation reactions of 2-oxo-acid substrates result in enamine adducts between the thiazolium moiety of the coenzyme and decarboxylated substrate. These central enamine intermediates experience different fates from protonation in pyruvate decarboxylase to oxidation by the 2-oxoacid dehydrogenase complexes, the pyruvate oxidases, and 2-oxoacid oxidoreductases. Virtually all of the TPP-dependent enzymes, including pyruvate decarboxylase, can be assayed by 1-electron redox reactions linked to ferricyanide. Oxidation of the enamines is thought to occur via a 2-electron process in the 2-oxoacid dehydrogenase complexes, wherein acyl group transfer is associated with reduction of the disulfide of the lipoamide moiety. However, discrete 1-electron steps occur in the oxidoreductases, where one or more [4Fe-4S] clusters mediate the electron transfer reactions to external electron acceptors. These radical intermediates can be detected in the absence of the acyl-group acceptor, coenzyme A (CoASH). The π -electron system of the thiazolium ring stabilizes the radical. The extensively delocalized character of the radical is evidenced by quantitative analysis of nuclear hyperfine splitting tensors as detected by electron paramagnetic resonance (EPR) spectroscopy and by electronic structure calculations. The second electron transfer step is markedly accelerated by the presence of CoASH. While details of the second electron transfer step and its facilitation by CoASH remain elusive, expected redox properties of potential intermediates limit possible scenarios.

1. Introduction

Thiamin pyrophosphate (TPP), the coenzyme form of vitamin B1, promotes reversible cleavage of carbon-carbon bonds between carbonyl and alcoholic groups or between vicinal carbonyl groups (Scheme 1) [1].

TPP provides the means for stabilization of the acyl anions that are implicit in these cleavages [1]. TPP is essential for the metabolism of carbohydrates and is therefore

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*Corresponding author: ghreed@wisc.edu.

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ubiquitous to living organisms. Reactions of TPP-dependent enzymes are invariably multistep processes. The first step is ionization of the thiazolium moiety of TPP to form a zwitterion or ylide-like species that subsequently adds to the carbonyl carbon of the substrate (Scheme 2) [2].

The resulting adducts serve as the precursors for the cleavages shown in Scheme 1. The decarboxylated products (such as hydroxyethylidene-TPP (HE-TPP), Scheme 3) are central intermediates in all TPP-dependent reactions [3, 4].

These intermediates are capable of giving up electrons one-at-a-time, as evidenced by the fact that even TPP-dependent enzymes, including pyruvate decarboxylase, which physiologically catalyzes decarboxylation without oxidation, can be assayed by the reduction of ferricyanide, which is an obligate one-electron acceptor [5, 6].

In the 2-oxoacid oxidoreductases and in the pyruvate oxidases [7], a one electron oxidation of the enamine intermediate results in a radical that achieves some degree of stabilization by delocalization onto the thiazole/thiazolium ring of the coenzyme [6, 8]. This HE-TPP radical can exist as a cation or neutral species (Scheme 4) and both species have multiple resonance structures that distribute the unpaired spin density over the hydroxyethylidene and thiazolium moieties. Persistent π -radicals have been generated electrochemically from thiamin analogs and active aldehydes [9].

The electron paramagnetic resonance (EPR) spectra of the corresponding radicals obtained from pyruvate: ferredoxin oxidoreductase (PFOR) and 2-oxoglutarate: ferredoxin oxidoreductase (OFOR) for the enzymes from the archaeon, *Halobacterium salinarum* (formerly *Halobacterium halobium*) were reported more than 30 years ago [10]. These enzymes purify from the organism with a significant amount of the radical signal present even under aerobic conditions, which indicates that the radicals are unusually robust. The HE-TPP radicals derived from PFORs of most species have shorter lifetimes. The HE-TPP radical of the PFOR from *Desulfovibrio africanus* appears upon addition of pyruvate to the reductively activated enzyme, and the radical persists for at least several minutes [11]. In contrast, the HE-TPP radical from *Moorella thermoacetica* has a half-life of ~ 1.5 min at room temperature under anaerobic conditions [12]. Despite differences in apparent stability, EPR spectra of radicals derived from pyruvate with PFORs from different organisms are virtually identical (see Fig. 1). The partially resolved features in the EPR spectra reflect hyperfine interactions with nuclear spins in the radical and are therefore indicators of the location of unpaired spin density and associated electronic structure [13]. The EPR spectra, therefore, indicate that the HE-TPP radicals from PFORs of diverse organisms have the same electronic structure.

As noted above, the capacity to undergo one-electron oxidation is not restricted to the enamine intermediates in the 2-oxoacid oxidoreductases. For example, a radical corresponding to the one-electron-oxidized enamine-thiazolium intermediate has been characterized in the E1o component of the 2-oxoglutarate dehydrogenase complex from *Escherichia coli* under aerobic conditions [14]. In this case, O₂ intercepts the enamine-thiazolium intermediate in an “off-pathway” reaction that would produce the radical and superoxide eventually leading to production of other “reactive oxygen species” (ROS). This review will focus on the 2-oxoacid oxidoreductase, PFOR, its radical intermediate, and electron transfer properties. Inferences for other members of the 2-oxoacid oxidoreductases and pyruvate oxidases will be considered. Recent comprehensive overviews of TPP-dependent enzymes and of PFOR are available [1, 4, 15–19].

2. Pyruvate:ferredoxin oxidoreductase (PFOR)

2.1 Reaction and physiological context

PFOR (EC 1.2.7.1) catalyzes the TPP-dependent oxidative decarboxylation of pyruvate with production of acetyl-CoA and reducing equivalents typically harvested by electron transfer to external ferredoxins (Fd) (see eq. 1).

The stoichiometry of 2 Fd in eq. 1 is used to emphasize the fact that two electrons are produced in the reaction; however, the external Fd entities that receive these electrons sometimes contain a pair of [4Fe-4S] clusters that capture both reducing equivalents produced in the reaction.

Phylogenetic analyses suggest that PFOR is an ancient enzyme that appeared before the divergence of archaea and eukaryotes [16]. PFOR is ubiquitous among archaea, has a significant representation among bacteria, and is found in amitochondriate protists such as *Giardia lamblia* and *Trichomonas vaginalis* [20]. In the forward direction, the reaction allows for the anaerobic oxidative decarboxylation of pyruvate and harvesting of the “low potential” reducing equivalents to drive other reactions such as reduction of sulfate, N₂, or protons [16]. The reaction is reversible [21, 22] –the pyruvate synthase activity assimilating CO₂ in organisms, including methanogens [23], that use the Wood-Ljungdahl or the reductive tricarboxylic acid pathways [24, 25]. The presence of PFOR in numerous pathogenic microbes and its absence in higher animals make it a drug target. Many current therapies exploit the low potential electrons generated in the reaction to activate reductively pro-drugs such as metronidazole [26, 27].

2.2 Structures and cofactor content

Quaternary structures of PFORs vary among species [28–30]. The quaternary structures separate into four groups: homodimers (α_2); dimers of heterotetramers ($\alpha\beta\gamma\delta$)₂; dimers of heterotrimers ($\alpha\beta\gamma$)₂; and heterodimers ($\alpha\beta$)₂. The dimers are proposed to have arisen from various gene fusions from an ancestral heterotetrameric protein such as those found in archaea [18]. In some cases, e. g., in the *H. salinarum* enzyme, a fusion of the α and γ subunits can be isolated as a homodimer containing only the binding site of TPP and Cys residues corresponding to a single [4Fe-4S] cluster. A separate Fd containing two [4Fe-4S] clusters binds to the heterodimer [29–32]. Thus, in terms of cofactor content, there are two classes of PFOR—those having three [4Fe-4S] clusters and those having just a single [4Fe-4S] cluster.

Currently, ten of the eleven entries for crystallographically determined structures of PFOR in the PDB are for the homodimeric enzyme from *D. africanus*. One view of the homodimer is provided in Figure 2. The 3-D structures show that the three [4Fe-4S] clusters in the homodimeric protein are optimally spaced [33] to provide an effective internal electron transport pathway that can shuttle electrons between the active site and the external surface of the protein [19]. The redox potentials of the three [4Fe-4S] clusters in *D. africanus* PFOR are reported to be –390 mV, –515 mV, and –540 mV [11]. Addition of pyruvate to the “resting state” (i. e., three [4Fe-4S]²⁺ clusters) of the homodimeric PFOR from *M. thermoacetica* generates the HE-TPP radical and a single reducing equivalent in the chain of Fe-S clusters. The magnitude of the electron-electron dipole-dipole interaction between the HE-TPP radical and the [4Fe-4S]¹⁺ cluster, as determined in electron-electron double resonance experiments, corresponds to an interspin distance of ~32 Å. This distance correlates with a model in which the medial cluster harbors the unpaired electron following

the one-electron oxidation of HE-TPP [34]. Thus electron transfer from HE-TPP through the proximal cluster to the medial cluster occurs in the first stage of the oxidation.

2.3 Reaction sequence and intermediates

The sequence of reactions outlined in Schemes 2–4 leads to the HE-TPP radical, CO₂, and one reducing equivalent. The second electron transfer step from the radical to the [4Fe-4S]²⁺ acceptor is chemically gated [35] by the acetyl group acceptor, CoA. CoA stimulates the rate of electron transfer from the HE-TPP radical to the iron sulfur clusters in the PFOR from *M. thermoacetica* by 10⁵ fold [36]. In the related enzyme, pyruvate oxidase (POX) from *Lactobacillus plantarum*, the acetyl group acceptor, P_i, produces a 10²–10³ -fold enhancement in the rate of electron transfer from the HE-TPP radical to the semi-flavin FAD moiety in the active site [7]. The reaction scheme of PFOR is outlined in Scheme 5.

Intermediates in this scheme include the lactyl-TPP, HE-TPP, and the HE-TPP radical. Crystallographic investigations of the related enzyme, POX, have provided electron density maps consistent with lactyl-TPP and with HE-TPP bound in the active site [37]. Crystals of PFOR from *D. africanus* grown and maintained at pH 6.2 show electron density of pyruvate near to C2 of TPP [38]. Interpretations of difference electron density maps in pyruvate-soaked crystals of PFOR crystals at higher pH values [39, 40] have not been as straightforward as in the case of POX. Assignments based on the difference density have led to a hypothesis for the structure of the HE-TPP radical that has a C2–C2 α bond of unprecedented length as well as unusual tautomerizations around the thiazol ring [39]. Moreover, these maps have led to the suggestion that, in PFOR, the HE-TPP (enamine) intermediate is somehow by-passed in the overall reaction [40, 41].

Interpretations of difference density maps from crystals expected to contain the HE-TPP radical intermediate have led to the proposal that the radical has an unconventional electronic structure described as a σ/n cation radical (Scheme 6) [39, 40].

The atomic models proposed to fit into the difference density had C2–C2 α distances of 1.95 Å and 1.75 Å for molecules A and B in asymmetric unit. Exceptionally long carbon-carbon bonds are a continuing curiosity [42, 43]. An empirical correlation of carbon-carbon bond lengths and dissociation energies predicts that bonds longer than 1.748 Å will have zero bond dissociation energy [44]. Moreover, precedent cited for the exceptionally long bonds in σ/n cation radicals was based largely on computations [45]. Higher-level treatments of the “title member” of this group of radicals, the ethane cation, have subsequently concluded that the carbon-carbon bond distance is “normal” [42, 46] as opposed to the 1.908 Å distance suggested from earlier calculations. Neither of the C2–C2 α distances in the above model of the σ/n cation radical is consistent with the established persistence of the HE-TPP radical in *D. africanus* [11] or in *H. salinarium* [10] at room temperatures. Catalytic groups that would be expected to effect the required tautomerizations and resulting proton transfers at C2, C4, C4 α and C5 are not in evidence in the active site.

The characteristic EPR spectrum of the HE-TPP radical (see Figure 1) is an envelope of partially resolved hyperfine structure arising from interactions of the unpaired electron with ¹H and ¹⁴N nuclear spins. This underlying structure can be elaborated by subjecting spectra collected at high signal-to-noise ratios to resolution enhancement [47]. Changes in the basic splitting pattern upon regio-specific introduction of ¹³C or ¹⁵N into the substrate or thiazolium moieties (isotope editing) can be elaborated and hyperfine-splitting tensors determined through spectral simulation as illustrated in Fig. 3. The magnitudes of the hyperfine splitting constants are measures of unpaired spin density and hence the electronic structure of the radical. Electronic structure calculations can then be used to compare experimentally determined tensors with those expected for different models of the radical.

Analysis of the nuclear hyperfine splitting in the EPR spectra of the radical generated with ^2H , ^{13}C , and ^{15}N substitutions in the substrate, pyruvate, and in the thiazolium moiety of TPP were fully consistent with a π -radical having the unpaired spin delocalized over the hydroxyethylidene and thiazolium moieties [48]. The unpaired spin distribution in the HE-TPP radical suggested that the ionization state of the radical was intermediate between that expected for the cation and neutral radical species, which indicated that the radical was likely participating in a hydrogen bond. Given its expected proximity to C2 α O of the radical [39], the exocyclic amino group of the substituted pyrimidine moiety of TPP was proposed as a logical intramolecular H-bonding partner. A planar π -radical provided a closer fit to the X-ray coordinates than did a properly formulated model of the σ/π cation radical [48].

Although the aminopyrimidine moiety of TPP is not conjugated to the π -electron system of the hydroxyethylidene/thiazolium moieties, its potential H-bonding interaction with C2 α O(H) can influence unpaired spin distribution in the radical-bearing part of the system. Truncated models of TPP (lacking the aminopyrimidine portion) were used in the earlier electronic structure calculations. This truncation simplification of TPP and the absence of the protein matrix have been suggested as potential sources of discrepancies between competing models of the radical [41]. DFT calculations (*Gaussian 09*) [49] were performed that included the aminopyrimidine moiety and active site residues Arg114 and Glu64 in order to assess their influences on the structure of the radical. An isosurface plot of spin densities of the resulting energy minimized structure is shown in Fig. 4. In the earlier calculations, it was noted that a primary effect of protonation at C2 α was a shift in spin density from C2 to C2 α [48]. As shown in Figure 4, a similar shift of spin density occurs upon inclusion of H-bonding interactions at C2 α O such that the calculated ^{13}C hyperfine splitting tensors at C2 and at C2 α are closer to the experimentally determined values and about midway between the those calculated for models of C2 αO^- or C2 αOH . In E1o component of the 2-oxoglutarate dehydrogenase complex, a similar shift in unpaired spin upon formation of the C2 αOH tautomer of the TPP radical intermediate was calculated [14].

The possibility of differences in the electronic structures of the HE-TPP radical among PFORs from different species (e. g., *M. thermoacetica* and *D. africanus*) has also been suggested as a potential source of discrepancies in proposed structures of the HE-TPP radical [41]. However, as shown in Fig. 1, the EPR spectra of the HE-TPP intermediate are identical not only for PFORs from *M. thermoacetica* and *D. africanus*, but also for PFORs from other organisms. The EPR spectra leave no doubt that the same radical is responsible for the spectra and therefore that the electronic structures are identical.

2.4 Characteristics of the second electron transfer step

The HE-TPP radical is observed in the absence of the acetyl group acceptor substrate, CoASH. As noted above, the lifetime of the HE-TPP radical varies substantially from species to species. Although it has evidently not been established, one expects that an off-pathway, one-electron-oxidation of the radical would produce acetyl-TPP—the hydrolysis of which would lead to acetate and TPP. Lifetime differences of the radical among different PFORs might reflect accessibility of oxidants or accessibility of solvent to the active sites [39]. The origin of the 10^5 -fold enhancement in the rate of the second electron transfer step by CoASH is of obvious interest [36]. For PFOR from *M. thermoacetica*, decay of the radical in the absence of CoA behaved as a true electron-transfer reaction according to an analysis using Marcus theory. A similar analysis showed that electron transfer was chemically gated in the presence of CoA. Several possible explanations for gating by CoA were considered. The 10^5 -fold change in rate would require a change in distance between the donor (HE-TPP radical) and acceptor ($[\text{4Fe-4S}]^{2+}$) of $> 8 \text{ \AA}$ [50], and this was considered to be unlikely especially given the fact the desulfo analog of CoA decreases the rate of the electron transfer by 10-fold. The experiments with desulfo-CoA also make it

unlikely that some unusual electron transfer pathway is elaborated whenever CoA is bound. Two other possibilities for the CoA chemical gating were considered: 1) nucleophilic attack of the CoAS^- on C2 α of the radical to form an anion radical adduct; 2) one electron oxidation of the CoAS(H) to a thiyl radical by a $[\text{4Fe-4S}]^{2+}$ cluster and combination of the thiyl radical with the HE-TPP radical [36].

Formulations of the hypothetical anion radical adduct are shown in Scheme 7.

(The formulation in B Scheme 7 is more energetically feasible because the radical could be delocalized onto the thiazole ring.) The 10^5 -fold rate enhancement through a change in the thermodynamic driving force alone, would require an ~ 630 mV change in the redox potential of the anion radical adduct relative to the parent HE-TPP radical species [36]. Formation of an anion radical adduct between P_i and the HE-TPP radical was also proposed to account for the increase in electron transfer rate from the radical to the flavin in POX [7]. Preliminary electronic structure calculations using methane thiolate as a truncated form of CoAS^- suggest that the anion radical adduct would indeed enhance the reducing capacity of the radical by an amount greater than that required to promote the increase in electron transfer rate. Questions, however, arise regarding the susceptibility of C2 α in the radical towards nucleophilic substitution. Potential acetyl group transfers from synthetic acetyl-TPP to hydroxylamine, 2-mercaptoethanol, dihydrolipoic acid, and P_i have been investigated in aqueous solution. Hydroxylamine and to a much smaller extent, 2-mercaptoethanol are able to trap the acetyl group [51]. One expects that the reactivity of the one electron reduced version of acetyl-TPP to show somewhat lower reactivity than is observed with acetyl-TPP. Keeping in mind that the reaction is reversible, the addition product of the ylide of TPP and acetyl-CoA would need to undergo a one-electron reduction by reduced ferredoxins to reach the same anion radical intermediate state. Energetic issues associated with this reduction are yet to be determined although the attack of the ylide on the carbonyl of acetyl-CoA is a logical early step in the reverse reaction.

The intermediacy of a thiyl radical form of CoA leading to a radical combination event with the HE-TPP radical has been proposed [36, 39]. The scenario for this radical combination step is outlined in Scheme 8 wherein the protonation state at C2 α O is arbitrary.

The thiyl radical of CoA in Scheme 8 is proposed to arise from one electron oxidation of CoA by one of the $[\text{4Fe-4S}]^{2+}$ clusters [36]. Although EPR analysis showed the presence of a single $[\text{4Fe-4S}]^{1+}$ cluster in PFOR from *M. thermoacetica* incubated with CoA, a thiyl radical was not detected [12]. Thiyl radicals are strong oxidants [52], and oxidation of CoA to a thiyl radical by a low potential bacterial ferredoxin raises energetic issues. Conditions for reconstitution of Fe-S clusters or repair of damaged ones invariably include various thiol containing compounds [53]. The reverse direction is problematic because homolytic cleavage of the carbon-sulfur bond is unlikely to occur spontaneously. Moreover, the radical recombination scenario is not available for the related chemical gating by P_i of the second electron transfer reaction from the HE-TPP radical in POX [7].

A variation of the thiyl radical recombination was proposed to exploit the hypothetical σ/n cation radical (Scheme 6) intermediate. In this proposal, the σ/n cation radical would fragment to an acetyl σ radical that would then combine with the thiyl radical to produce acetyl-CoA directly [39]. In addition to the issues related to the existence of a σ/n cation radical in PFOR, this mechanism runs into additional difficulties when viewed in the reverse direction. In the reverse reaction the carbon-sulfur bond of acetyl-CoA (expected bond dissociation energy 66–75 kcal M^{-1} [54]) would be required to undergo homolytic cleavage with a subsequent attack of the acetyl σ radical at C2 of the TPP moiety. The resulting

radical would need to tautomerize as required to achieve that non-planar structure shown in Scheme 6.

What can be concluded regarding the second electron transfer step of PFOR in the forward direction is that binding of CoA and its thiol/thiolate group greatly facilitates oxidation of the HE-TPP radical intermediate by the resident $[4\text{Fe-4S}]^{2+}$ cluster [36]. It is not yet clear whether the electron arises from the HE-TPP radical or from an adduct of this radical with CoA. The much slower oxidation of the radical that occurs in the absence CoA would lead to acetyl-TPP or its hydrate or carbinolamine with the aminopyrimidine moiety of the cofactor [51]. Of course, all of the players in the reaction scheme must reverse their roles in the synthesis of pyruvate, from acetyl-CoA, two reducing equivalents, and CO_2 .

2.5 Hydrogenase activity of PFOR

In some microbes, the ultimate fate of the reducing equivalents derived from the PFOR catalyzed oxidative decarboxylation of pyruvate is to serve as fuel for the hydrogenosomal production of H_2 [55]. Coupling of pyruvate oxidation to sulfate or proton reduction is also accomplished by species of PFOR containing sulfate-reducing bacteria [56]. In the absence of external electron acceptors, PFOR, itself functions as a hydrogenase [57]. The k_{cat} of H_2 evolution measured for PFOR from *M. thermoacetica* is 0.3 s^{-1} , and the products, H_2 and acetyl-CoA, are present at a 1:1 stoichiometry (eq. 2).

The same preparations of PFOR yielded a k_{cat} of $\sim 33 \text{ s}^{-1}$ using methyl viologen as the electron acceptor. Since an analogous side reaction hasn't been reported for POX, one can reasonably conclude that the reduced $[4\text{Fe-4S}]$ clusters of PFOR are sites of proton reduction. The hydrogenase activity of PFOR in the absence of external electron carriers serves as a "relief valve." This side activity also complicates detection of intermediates in the CoA dependent part of the reaction, although transient kinetic methods might still work.

3. Other 2-oxoacid Oxidoreductases

3.1 CoA-dependent 2-oxoacid oxidoreductases

The CoA-dependent 2-oxoacid oxidoreductases, PFOR, 2-oxoglutarate oxidoreductase (KGFOR), 2-oxoisovalerate oxidoreductase (VFOR), and indolepyruvate oxidoreductase (IPFOR) reinforce the concept that cognate enzymes facilitate the catalytic function of their coenzyme—in this case, TPP. Indeed in many cases for this group of enzymes, there is significant cross reactivity among the various substrates [23]. To the extent that they have been investigated, features of the proposed catalytic cycles of these other enzyme that are shared with PFOR include formation and decarboxylation of the TPP adduct to generate the respective enamines, one electron oxidation of the enamine to form the radical intermediate, and subsequent CoA promoted oxidation of the radical and formation of the respective acyl-CoA product. The EPR spectra of the enamine-derived radicals differ slightly from those of the HE-TPP radical in PFOR because of replacement of the methyl group at $\text{C2}\beta$ with the respective chain of the substrate. Hyperfine splitting of one of the β -protons of the methyl group is missing and the orientation of the R-CH_2 - may alter the extent of hyperconjugation of the remaining methylene protons at $\text{C2}\beta$ [10, 58]

3.2 Oxalate oxidoreductase

The recently discovered oxalate oxidoreductase (OOR) from *M. thermoacetica* catalyzes the reaction given in eq. 3 [59].

The enzyme contains TPP and three [4Fe-4S] clusters; however, unlike the other 2-oxoacid oxidoreductases, it does not require CoA. Addition of oxalate causes reduction of the [4Fe-4S] clusters to the +1 paramagnetic state as observed by EPR. Any organic radical intermediates generated decay within the time required for manual mixing and freezing of the sample (~30 s). A plausible mechanism for OOR is sketched in Scheme 9.

M. thermoacetica is a strict anaerobe. OOR allows the bacterium to grow on oxalate as a sole source of carbon and energy. The CO₂ that is generated in the reaction and the reducing equivalents are linked to the Wood-Ljungdahl pathway of autotrophic acetyl-CoA formation [60]. OOR catalyzes oxidation of glyoxalate at rate comparable to that observed with oxalate. 2-Oxobutyrate, pyruvate, oxaloacetate, 2-oxovalerate, and 2-oxoglutarate are also oxidized at about a ten-fold slower rate [59].

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Fig. 1. EPR spectra (X-band) of the radical formed upon addition of pyruvate to PFORs from diverse organisms. **A** *Moorella thermoacetica* [48], **B** *Desulfovibrio africanus* [39], **C** *Halobacterium salinarum* [10], **D** *Methanosarcina barkeri* [61].

Fig. 2.
Ribbon representation of the homodimer of PFOR from *D. africanus* (PDB ID: 1KEK). The Fe-S clusters, divalent cations (green), and TPP-pyruvate adduct are highlighted as space filling models.

Fig. 3. EPR spectrum (77 K) of the HE-TPP radical obtained from samples made up from [2- ^{13}C]-pyruvate with PFOR from *M. thermoacetica*. The experimental spectrum was collected at high signal-to-noise ratio by scan averaging and subjected to resolution enhancement using Fourier methods [47]. The calculated spectrum was generated using the g values and hyperfine tensors, and line shape parameters described previously [48].

Fig. 4. Isosurface plots of spin density in an energy-minimized model of the HE-TPP radical obtained from DFT calculations (B3LYP/6-31G*//B3LYP/TZVP using *Gaussian 09*) [49] that include the aminopyrimidine moiety of TPP and the active site residues Arg 114 and Glu 64. The initial positions of atoms in the model were taken from PDB ID: 1KEK. The plot was created using the program *Molekel 4.3* [62]. ^{13}C hyperfine splitting tensors arising from the model were [8,7,21] (G) for $^{13}\text{C}2\alpha$ (experimental [6,6,18] (G)) and [5,4,3] (G) for $^{13}\text{C}2$ (experimental [4,4,9] (G)). These values are approximately midway between extremes calculated for the neutral and cationic HE-TPP radicals [48].

Scheme 1.
Examples of bonds cleaved in TPP-dependent reactions

Scheme 2.
Formation of lactyl-TPP

Scheme 3.
Decarboxylation of lactyl-TPP to give HE-TPP

Scheme 4.

One-electron oxidation of HE-TPP to produce the HE-TPP radical

Scheme 5.
Overall reaction of PFOR

Scheme 6.

Proposed model of a σ/n cation radical for the HE-TPPP radical

Scheme 7.
Hypothetical CoA anion radical adducts

Scheme 8.
Hypothetical radical combination mechanism

Scheme 9.
Proposed mechanism of OOR