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Cyclopropylglyoxylate as a Mechanistic Probe of Thiamine Pyrophosphate Dependent Pyruvate-Metabolizing Enzymes[1]

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Four pyruvate-decarboxylating enzymes with thiamine pyrophosphate (TPP) cofactors catalyze the decarboxylation of the cyclopropyl substrate analog cyclopropylglyoxylate. Pyruvate : ferredoxin oxidoreductase, an archaebacterial enzyme which catalyzes oxidation of the hydroxyethyl-TPP (HETPP) intermediate by two one-electron transfers to an iron–sulfur center, generates the coenzyme A thioester of cyclopropylcarboxylic acid. A long-lived free radical, HETPP is thought to be an intermediate in the pyruvate to acetyl-CoA conversion; however, cleavage of the cyclopropyl ring was not detected. Pyruvate decarboxylase, pyruvate oxidase, and pyruvate dehydrogenase also generate the corresponding cyclopropyl products. The applicability of cyclopropyl substrate analogs as indicators of free-radical enzyme mechanisms is discussed in light of these results.

INTRODUCTION

α-Keto acid decarboxylases require thiamine pyrophosphate (TPP) as cofactor. The TPP C_2 anion attacks the ketone group of bound substrate to yield an adduct with an iminium ion beta to the substrate carboxylate and thus facilitates low-energy generation of CO_2 via stabilization of the incipient carbanion as an eneamine. The subsequent fate of the C_2 eneamine-TPP differs with specific TPP enzymes, and the pyruvate-decarboxylating enzymes can be divided into nonoxidative and oxidative categories (*1*). Yeast pyruvate decarboxylase decomposes the adduct nonoxidatively, unraveling it to the initial TPP C_2 anion and acetaldehyde. Three other pyruvate-decarboxylating enzymes catalyze electron transfer before adduct decomposition and yield not an aldehyde product but an acid species. The FAD-containing pyruvate oxidase (PO) yields free acetate, and acetyl-CoA is generated by pyruvate dehydrogenase (PDH) and the pyruvate : ferredoxin oxidoreductases (PFO), of which the *Halobacterium halobium* enzyme is a well-characterized example (*2*).

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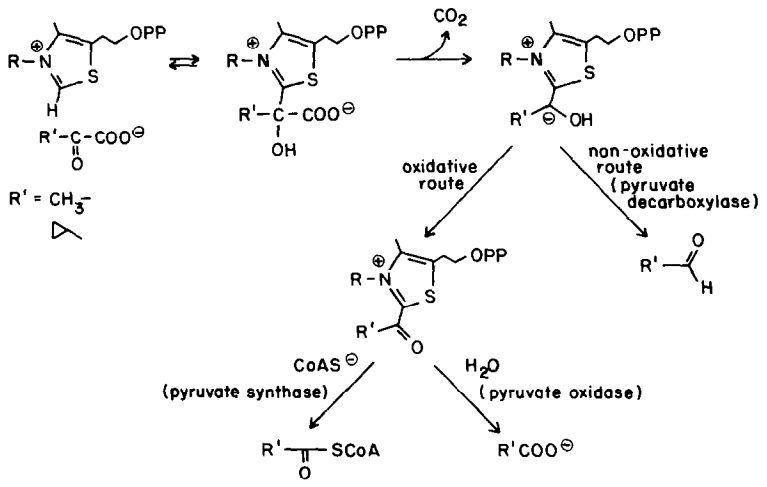
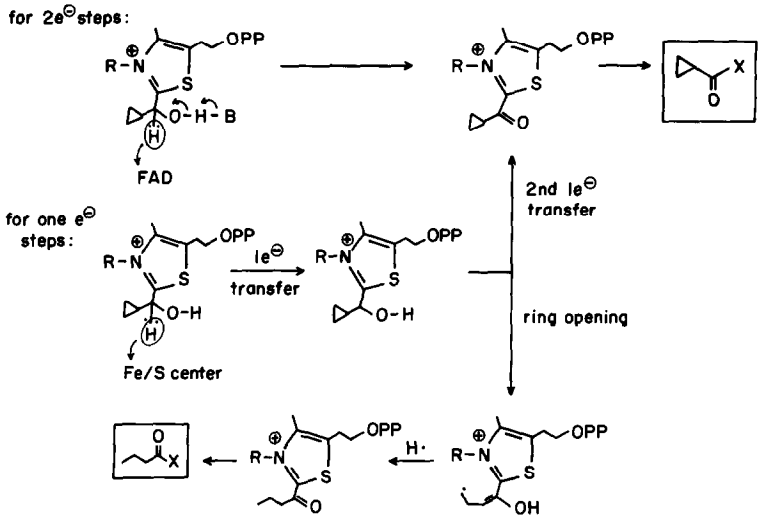


SCHEME I

While the stabilized carbanion of HETPP is accepted to be an intermediate common to the oxidative and nonoxidative decarboxylation sequences, it is less clear how the carbanion oxidation actually proceeds. The case with the *H. halobium* PFO has been perhaps most clear. The ferredoxin is an obligate one-electron acceptor and EPR resonances have been seen in whole cells and with purified enzyme and added pyruvate (*3, 4*). The $g = 2.006$ resonance has been assigned to the HETPP radical, which accumulates in the absence of coenzyme A (CoASH). This species is oxidized in a second one-electron step to a putative acetyl-TPP with subsequent acyl transfer to CoASH. The *Escherichia coli* PO, having FAD as built-in redox cofactor, might oxidize the HETPP carbanion to acetyl-TPP either by two one-electron steps or by a single two-electron step. Flavins can act as electron-transfer mediators between one- and two-electron donor/acceptor pairs. Finally, the pyruvate-decarboxylating component, enzyme I, of the PDH complex, may use the HETPP carbanion directly as nucleophile to attack the disulfide of oxidized lipoamide on enzyme II (the transacetylase). Alternatively, enzyme I could transfer one electron from HETPP to the lipoamide disulfide of enzyme II, followed by radical recombination between cofactors.

To probe for radical intermediates in the oxidative decarboxylation of pyruvate catalyzed by these enzymes, we synthesized cyclopropylglyoxylate (CPG), the cyclopropane analog of pyruvate, as a probe. Our rationale was, first, that CPG represents only a small steric departure from pyruvate and so might function as an alternative substrate. Second, it is known that carbon-centered radicals adjacent to cyclopropanes fragmentatively isomerize to the butenyl radical at a rate of 1.3×10^8 sec^{-1} (*5*). Transfer of a hydrogen atom to the lower energy acyclic radical will yield an isomerized product as a diagnostic for one-electron steps in the reaction. Cyclopropyl-containing substrate analogs have been used recently for monoamine oxidase (*6*) and for liver microsomal cytochrome *P*-450 monooxy-



SCHEME II

genase (*7, 8*) to inactivate the target enzymes irreversibly, processes interpreted as reflecting the suicidal rerouting of radical cation intermediates. CPG, specifically, has been used to probe the mechanism of NAD-dependent L-lactate dehydrogenase (*9*). The keto acid was smoothly reduced to cyclopropylglycolic acid without detectable ring-opened products. Since *n*-butyltin hydride reduction of CPG in the presence of azoisobutyronitrile yielded, via precedented radical rearrangement, the anticipated cleaved 2-oxopentanoate isolated as the methyl ester, it was concluded that NADH reduction most probably did not involve radical intermediates.

In this study we have used CPG as an alternate substrate and probe for radical intermediates in the reactions catalyzed by the TPP enzymes yeast PDC, *E. coli* PO and PDH, and *H. halobium* PFO.

EXPERIMENTAL PROCEDURES

Substrates

CPG was synthesized by permanganate oxidation of cyclopropyl methyl ketone (*10*). Cyclopropyl carboxaldehyde was synthesized by pyridinium chlorochromate oxidation of cyclopropyl carbinol (*11*). Compounds were identified by TLC, ^{1}H-NMR, and IR spectroscopy.

Enzymes

Yeast pyruvate decarboxylase, yeast alcohol dehydrogenase, and horse heart cytochrome *c* were purchased from Sigma and used without further purification.

E. coli pyruvate oxidase was purified as described elsewhere (*13*). Pyruvate dehydrogenase was a kind gift from Dr. Perry Frey, University of Wisconsin. *H. halobium* pyruvate : ferredoxin oxidoreductase and ferredoxin were prepared by the method of Kerscher and Osterhelt (*2*). The PFO was found to be ca. 60% pure by both specific activity and SDS–PAGE.

Product Analysis

The identities of the enzymatic products were determined by co-injection with authentic standards on a HPLC. A resin-based Aminex HPX-87H, 300 × 7.8-mm column was used in a Waters HPLC system with a R40a differential refractometer detector. Running conditions were 0.6 ml/min in 0.01 N H_2SO_4. This analytical system enabled separation of the keto acids, carboxylic acids, and alcohols described in this report, with a practical limit of detection of 1–5 μg per injection.

Enzyme Incubations

1. Pyruvate decarboxylase was assayed with pyruvate or CPG at 30°C under conditions described by Jordan (*12*). The production of acetaldehyde was coupled to NADH-dependent reduction catalyzed by alcohol dehydrogenase. Oxidation of NADH was monitored at 340 nm. Enzymatic incubations contained 30 mM citrate buffer (pH 6.2), 0.4 mg alcohol dehydrogenase, 50 mM NADH, 0.12 mg PDC, and 50 mM keto acid in a final volume of 3.0 ml. The mixture was incubated at 37°C for 30 min.

Products were partially purified by adding Chelex resin (Bio-Rad) to remove metals and applying the mixture to a Centricon ultrafiltration unit (Amicon) with a 10,000 MW cutoff. The unit was centrifuged for 60 min at 5000*g*. The filtrate was acidified to pH 2–3 with 1 N H_2SO_4. Two hundred microliters of the mixture was injected into the HPLC column.

2. Pyruvate oxidase was assayed with pyruvate or CPG at 37°C under conditions developed by Gennis *et al.* (*13*). Ferricyanide was used as electron acceptor and the reaction was monitored by decrease in absorbance at 450 nm. Incubations were performed at 37°C in 3.0 ml total volume containing 60 mM sodium phosphate, pH 6.0, 60 mM keto acid, 6 mM $MgCl_2$, 6 μM TPP, 4 mM SDS, 2.3 mg PO, and 8 mM $Na_3Fe(CN)_6$. The solution was incubated for 10 min prior to addition of ferricyanide to activate the enzyme. Incubations were held at 37°C overnight.

The product was partially purified by addition of Chelex and ultrafiltration as described above for PDC. SDS and salts were removed by chromatography on Dowex AG-1 X8 anion-exchange resin. A 2-ml column of resin was washed with H_2O and the filtrate was applied. After washing with 8 ml H_2O, the column was eluted first with 5 ml 0.1 N HCOOH, which elutes acetate, followed by 6 ml 0.3 N HCOOH, which elutes butyrate and cyclopropyl carboxylate. Five hundred microliters of the eluate was applied to the HPLC column.

3. Pyruvate dehydrogenase was assayed at 30°C with 2 mM pyruvate or CPG, 0.12 mM CoA, 2 mM NAD, 0.5 mM $CaCl_2$, 2.6 mM cysteine, and 50 mM MOPS, pH 7.6, to make 1.5 ml. The reaction was monitored for NAD reduction at 340 nm. Incubation reactions to isolate product from CPG contained 6 mM keto acid, 10

mM NADH, 6 mM CoA, 10 mM cysteine, 0.2 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 200 mM MOPS, pH 7.6, to make 10 ml. Enzyme was added in three 0.8-mg aliquots over a 12-h period at 30°C.

The products were purified by ultrafiltration, followed by hydrolysis of the CoA thioester by incubation at 50°C for 30 min after adjusting the solution to pH 12 with 1 N NaOH. The hydrolysate was applied to a Dowex column and eluted as described above. Aliquots (500 μl) of the eluant were injected into the HPLC column.

4. Pyruvate : ferredoxin oxidoreductase was assayed according to Kerscher and Osterhelt (*2*). Assays were performed at room temperature and contained 1 M KCl, 18 μM *H. halobium* ferredoxin, 50 μM cytochrome *c*, 1 mM keto acid, 50 μM CoA, 20 μg enzyme, and Tricine-K^+ to make 1 ml. Incubations of 20 ml to isolate product were the same with substrate concentrations increased to 200 μM CoA and 200 μM cytochrome *c*. Two 1-ml aliquots of enzyme solution (250 nkat total) were added over a 12-h period. Incubation was at 35°C and progress of reactions was monitored by visible spectroscopy of cytochrome *c*.

The incubation mixture was ultrafiltered and the CoA ester was hydrolyzed as above. The mixture was then evaporated under reduced pressure at 50°C until salt began to precipitate (ca. 5 ml volume). This suspension was acidified to pH < 2 with 3 N HCl and extracted 5× with 5 ml diethyl ether. The combined ether extracts were then extracted with 3 × 1 ml 12 mM NAOH. The basic solution was rotary evaporated to dryness and the residue dissolved in 400 μl 0.01 N H_2SO_4. A few drops of concentrated H_2SO_4 were added to adjust the pH to 2. Aliquots (80 μl) of this solution were applied to the HPLC column. By integration of the peak and comparison with internal standard, we estimated that 80 μg of product was present per injection. Theoretical yield was 72 μg.

RESULTS AND DISCUSSION

The turnover rates and products of CPG incubations with the four enzymes examined are summarized in Table 1. The cyclopropyl keto acid is turned over by each of the enzymes, but with varying degrees of selectivity. PFO is the least

TABLE 1

Products of CPG Reaction for TPP-Containing Enzymes

Enzyme	Rate of conversion[a]	Product
PDC	0.17	Cyclopropylcarboxaldehyde[b]
PO	0.12	Cyclopropyl carboxylate
PDH	0.013	Cyclopropylcarboxyl-CoA
PFO	0.55	Cyclopropylcarboxyl-CoA

[a] Rates are expressed as V_{max}(CPG)/V_{max} (pyruvate).

[b] Cyclopropylcarbinol is the product in the coupled assay with alcohol dehydrogenase.

selective and PDH is the most selective enzyme for the increase in carbon skeleton with V_{max} values of 1.3 and 55% of the V_{max} rate with pyruvate, respectively. In no case was irreversible inactivation induced by the cyclopropyl substrate. Thus, if radicals are generated in catalysis, they are not inactivating any of the enzymes. To determine if any rearrangement of the cyclopropyl ring occurred during catalysis, we isolated the product from each enzyme incubation and it was identified by HPLC retention times and by comparison with co-injected authentic standards. Each case is noted below.

Pyruvate Decarboxylase

This is the prototypic nonoxidative decarboxylation of pyruvate to acetaldehyde in yeast and should be the positive control enzyme, for no radicals are expected. Indeed CPG was converted to cyclopropylcarboxaldehyde and reduced in situ with alcohol dehydrogenase and NADH to cyclopropylcarbinol, the only detected product. Suckling and co-workers have previously shown ADH processes cyclopropyl aldehydes without ring opening (*14*).

Pyruvate Oxidase

This FAD-linked enzyme from *E. coli* converts pyruvate to acetate and CO_2 in an oxidative process. Electrons are transferred to generate $FADH_2$, which is reoxidized by O_2 (to H_2O_2) or by membrane respiratory chain components. CPG was converted to cyclopropylcarboxylate as sole detectable product by HPLC. A radical-mediated ring opening at the stage of hydroxycyclopropyl-TPP should have given butyrate as product, well resolved by HPLC, and none was detected.

Pyruvate Dehydrogenase

The three-enzyme pyruvate dehydrogenase complex converts pyruvate to acetyl CoA, bridging glycolysis and the citric acid cycle. Again, the acyclic butyryl-SCoA should be produced if the oxidation of cyclopropylglyoxylate passes through a species that generated the equilibrating radical shown in Scheme II. If no ring-opening sequences intervene, the thiolysis product from CoASH attack on the proposed cyclopropyl acyl thiolester of dihydrolipoamide on enzyme II should be cyclopropyl carboxyl-CoA. The enzymatic thioester products were converted to the corresponding carboxylic acid by basic hydrolysis with KOH, followed by acidification. Only cyclopropyl carboxylic acid was detected by HPLC.

Pyruvate : Ferredoxin Oxidoreductase

This archaebacterial enzyme seemed the prime candidate for generation of radical intermediates during catalysis. The electron acceptor in the oxidative decarboxylation of pyruvate to acetyl-CoA is the obligate one-electron acceptor ferredoxin, and the oxidoreductase is itself an iron protein. Further, a stable HETPP radical intermediate forms stoichiometrically in the absence of CoASH, detectable by EPR, with hyperfine structure contributed by the pyruvate moiety, which indicates that significant spin density is localized at the HETPP C2 carbon

(*3, 4*). However, once again no butyric acid, the tell-tale product from rearrangement of a TPP- substrate cyclopropyl radical, was observed. The only product detected after alkaline hydrolysis of the CoA ester is the intact cyclopropylcarboxylic acid. Our practical limit of detection, dependent upon peak resolution, was 10 μg of a ring-opened butyric acid and 1–2 μg of 2-butenoic acid. Therefore, we would not have detected the former ring-opened product if it were present at less than 15% of the total CoA ester product.

The results with cyclopropylglyoxylate appear unambiguous. The molecule is a substrate of all four pyruvate-decarboxylating enzymes we have tested and in no case did we detect acyclic products that are indicative of radical rearrangement of the cyclopropyl ring. On the surface, this would argue against kinetically competent radical species in the oxidations of the HETPP–carbanion adducts in the three oxidative decarboxylation enzymes. This assumes, however, that cyclopropyl radicals are faithful reporters of radicals at carbons alpha to the ring, and the radicals, once cleaved, pick up an H atom rather than reclose.

The rate for ring opening of cyclopropylcarbinyl radical is $\sim 1.3 \times 10^8\ s^{-1}$ (*5*) which is quite rapid compared to the turnover number of $k \sim 10^2\ s^{-1}$ for these TPP enzymes. How accessible the proposed ring-opened butenyl radical terminus may be to a quenching hydrogen atom or other one-electron equivalent is unclear. It may be that if the active sites are relatively sequestered and an acyclic radical forms, ring closure, with $k \sim 4.5 \times 10^3\ s^{-1}$, may be its only kinetically significant fate. To date, then, there are no enzymes known that catalyze oxidative ring opening of CPG.

Only for halobacterial PFO is there independent evidence in favor of a radical process. We have reproduced the reported EPR spectrum in the presence of pyruvate or CPG (data not shown). The kinetic competence of the EPR-detectable species in catalysis is yet to be addressed, although the species is quenched upon addition of CoA. The formal, but unlikely, possibility still exists that one-electron species are not on the main reaction pathway. Christen and colleagues have used oxidants to trap the HETPP carbanion (*15*), but it is unclear whether those reactions proceed by one- or two-electron pathways.

Sherry and Abeles (*16*) have recently studied the inactivation of the flavin-containing enzyme methanol oxidase by cyclopropanol. Inactivation leads to an N_5C_{4a}–flavin adduct, with cyclopropyl ring cleavage suggested to accompany inactivation. Reconstitution of apoenzyme with 5-deaza-FAD (a coenzyme restricted to hydride transfer) produces a holoenzyme refractory to inactivation by cyclopropanol. This protection suggests that one-electron pathways are required to produce the flavin adducts seen in inactivation of native enzyme. However, when native methanol oxidase was incubated with cyclopropyl carbinol, the cyclopropylaldehyde product ensued without ring opening, in analogy to the TPP enzymes discussed in this report. Sherry and Abeles argue that failure to see ring cleavage *does not exclude* a radical process since an enzyme-based radical may be conformationally restricted such that it only undergoes ring closure and no other radical captures.

We conclude that our data for the four TPP enzymes processing CPG do not exclude radical mechanisms, *but we adduce no data in favor of them.* While

radical processes remain permissible for PO and PDH, they will require other probes for detection. A more sensitive analytical technique than the HPLC analysis of the free acids reported herein might be useful for detection of minute amounts of ring-opened products derived from CPG (e.g., GC–MS of the esterified acids). It is clear from our data, however, that if any ring opening does occur without subsequent closure, it represents a minor kinetic pathway (<15% of the flux), even for PFO which generates stoichiometric amounts of HETPP radical species during turnover conditions. Although there is significant spin density at the carbon alpha to the cyclopropane ring, no relevant data for model compounds are available to estimate the free energy for opening of a delocalized radical cyclopropylcarboxylate–thiazole adduct.

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