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PHOSPHONATE ANALOGUES OF PYRUVATE

PROBES OF SUBSTRATE BINDING TO PYRUVATE OXIDASE AND OTHER THIAMIN PYROPHOSPHATE-DEPENDENT DECARBOXYLASES

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Summary

A number of enzymes catalyze the removal of carbon dioxide from pyruvate through covalent participation of the coenzyme thiamin pyrophosphate. The conversions of the decarboxylated adduct, hydroxyethyl thiamin pyrophosphate, to subsequent products distinguishes the function of these enzymes. Acetaldehyde is produced by pyruvate decarboxylase, acetic acid by pyruvate oxidase and acetyl coenzyme A by pyruvate dehydrogenase. Differences and details of steps prior to decomposition of hydroxyethyl thiamin pyrophosphate can be evaluated through the use of two substrate analogues, methyl acetylphosphonate and acetylphosphonate.

Methyl acetylphosphonate and acetylphosphonate are competitive inhibitors toward pyruvate with *Escherichia coli* pyruvate oxidase and *E. coli* pyruvate dehydrogenase but the value of the K_i for the oxidase is more than three orders of magnitude higher than for the dehydrogenase. Yeast pyruvate decarboxylase is not inhibited at all under the same conditions. The binding of methyl acetylphosphonate results in ligand-induced changes in the near ultraviolet circular dichorism spectrum of the oxidase. This spectral perturbation is only seen in the presence of the cofactor, thiamin pyrophosphate, strongly suggesting that the inhibitor is binding at the same site as the substrate, pyruvate, on the

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enzyme. Kinetic data suggest that lipid activators of pyruvate oxidase increase the affinity of the enzyme for pyruvate and its analogues.

Introduction

A variety of enzymes that utilize thiamin pyrophosphate as a cofactor have in common an initial reaction sequence involving addition of pyruvate to enzyme-bound thiamin pyrophosphate followed by rapid decarboxylation to form hydroxyethyl thiamin pyrophosphate (Fig. 1; Ref. 1). Distinctions between the reaction mechanisms of these enzymes have previously been based on the products of decomposition rather than on the formation of hydroxyethyl thiamin pyrophosphate. Thus, pyruvate decarboxylase produces acetaldehyde, pyruvate oxidase produces acetate, and pyruvate dehydrogenase produces acetyl coenzyme A. The nature of the common sequence leading to the formation of hydroxyethyl thiamin pyrophosphate is difficult to study by steady-state kinetics utilizing pyruvate since the proposed initial covalent intermediate, α-lactyl thiamin pyrophosphate (E-LTPP in Fig. 1) decomposes rapidly to E hydroxyethyl thiamin pyrophosphate [1], preventing an equilibrium from being established. It was anticipated that a substrate analogue that would not be subject to decarboxylation could be used to analyze these steps at equilibrium.

Phosphonate analogues of carboxylates as specifically designed inhibitors of

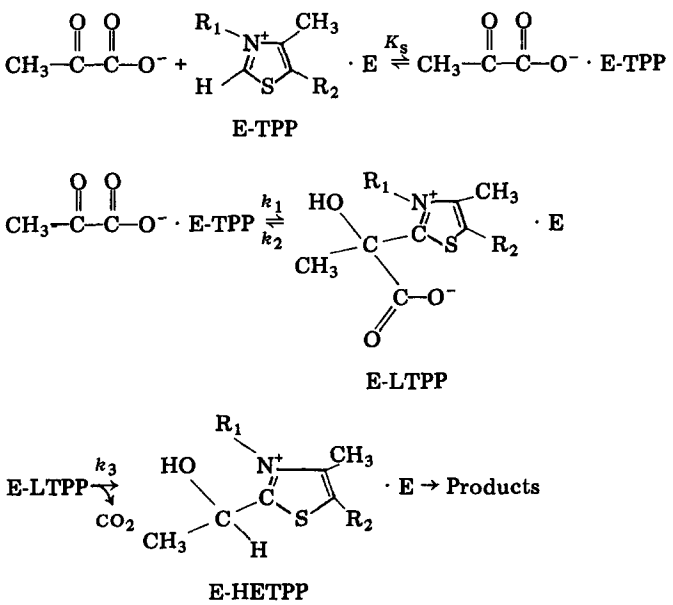
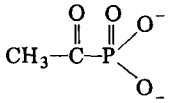
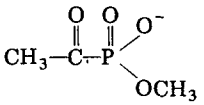


Fig. 1. The initial steps in the mechanism for pyruvate decarboxylation by pyruvate dehydrogenase, pyruvate decarboxylase, and pyruvate oxidase. The intermediates indicated are enzyme-bound α-lactyl thiamin pyrophosphate-(LTPP) and hydroxyethyl-thiamin pyrophosphate (HETPP).

enzymic reactions are potentially a general probe of enzyme mechanism [2—4]. For example, the two analogues utilized in this work were previously used to investigate pyruvate dehydrogenase, which catalyzes the formation of acetyl CoA. Acetylphosphonate and methyl acetylphosphonate were used to obtain information about the nature of the intermediates in the thiamin pyrophosphate decarboxylation process of this enzyme [3].



Acetylphosphonate



Methyl acetylphosphonate

In this paper the interaction between acetylphosphonate and methyl acetylphosphonate with *E. coli* pyruvate oxidase is investigated. A major goal is to further delineate similarities and differences between this and other thiamin pyrophosphate-dependent enzymes which decarboxylate pyruvate. Pyruvate oxidase is a peripheral membrane enzyme whose in vitro activity is dramatically enhanced by phospholipid and detergent activators [5—10]. Lipid activators increase V by 25-fold and decrease the K_m for pyruvate by approx. 6-fold [9]. It has previously been shown that lipids modulate the equilibrium binding isotherm of the cofactor, thiamin pyrophosphate, for pyruvate oxidase [10]. The pyruvate analogues are useful to investigate further the influence of lipid activators on the binding of ligands at the active site of the enzyme.

Materials and Methods

Materials. All reagents were purchased commercially and used without further purification. Pyruvate oxidase was prepared as described previously [8]. Pyruvate decarboxylase was prepared according to the procedure of Ullrich [11] using ale yeast from Labatt's Ontario Limited.

Sodium methyl acetylphosphonate was prepared by the reaction of NaI with methyl acetylphosphonate in acetone.

Disodium acetylphosphonate was prepared by a procedure analogous to that which was used by Kluger and Wasserstein [12] to convert methyl acetonylphosphonate to acetonylphosphonate.

Preparation of sodium methyl acetylphosphonate. 120.3 g (0.79 mol) dimethyl acetylphosphonate [13], were dissolved in 1.5 l acetone (dried over $MgSO_4$) with 135 g (0.90 mol) NaI, and the solution stirred at ambient temperature for 3 h. The resulting precipitate was filtered and dried in vacuo yielding 122 g (0.76 mol, 96%). The precipitate was recrystallized from ethanol/water (15 : 1) in 73% yield.

Preparation of disodium acetylphosphonate. Methyl acetylphosphonate (30 g, 0.19 mol) in 3-g portions was passed through a column of Dowex 50 X-2 ion-exchange resin (H^+ form). After water was evaporated in vacuo from the acidic fractions of eluent, residual water was removed by repeated vacuum evaporation of added dry acetone. The pale yellow residual syrup was stored overnight in a vacuum desiccator. The syrup was dissolved in 300 ml dry acetone with 70 g (0.47 mol) NaI and the solution refluxed for 6 h. The result-

ing pale yellow precipitate was filtered, dissolved in 100 ml H_2O, brought to pH 9 with NaOH and then added dropwise to 1.5 l absolute ethanol. The precipitate was collected by filtration and dried in vacuo.

Enzyme assays. Pyruvate decarboxylase was assayed using a coupled system in which the product, acetaldehyde, was converted to ethanol in the NADH-dependent reaction catalyzed by yeast alcohol dehydrogenase [11].

Pyruvate oxidase was assayed using a ferricyanide reductase assay [9].

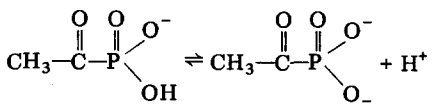
pK'_a determination. The pK'_a of acetylphosphonate monoanion was determined to be 5.6 by titration in 1 M KCl solution using NaOH as a titrant. Radiometer Autoburette 12 controlled by a Radiometer TT11 titrator and Radiometer pH meter 28 were used. Temperature was maintained at 25°C. pH volume curves were recorded on a Heath recorder connected to the Autoburette by a potentiometer. The pK'_a was determined by fitting the data to the Henderson-Hasselbach equation.

The pK'_a of methyl acetylphosphonate was determined by a spectrophotometric titration. The absorbance of the nonhydrated form (92% of total between pH 3 and 10, determined by NMR) at 333 nm vs. pH in solutions buffered to a total ionic strength of 1.0 M was plotted to give a best fit to the Henderson-Hasselbach equation. This yielded a pK'_a value of 0.5. Thus, in all solutions less acidic than pH 3, methyl acetylphosphonate is more than 99% dissociated. The decrease in absorbance with decrease in pH is due to the fact that the conjugate acid of methyl acetylphosphonate is almost completely hydrated and thus has no ultraviolet absorbance at 333 nm.

Circular dichroism studies were performed using a Jasco J-40 spectropolarimeter at room temperature. Methyl acetylphosphonate, thiamin pyrophosphate or their combination in aqueous solution exhibit no circular dichroism.

Results

The pyruvate analogues acetylphosphonate and methyl acetylphosphonate both inhibit the reaction of pyruvate oxidase with its normal substrate, pyruvate. The inhibitor patterns (not shown) indicate pure competitive inhibition. At pH 7 the K_i of methyl acetylphosphonate (K_i = 1.1 mM) is considerably smaller than that of acetylphosphonate (K_i = 10.5 mM), whereas at pH 6 the K_i values of the two analogues are essentially the same (1 mM). Pyruvate oxidase has a pH optimum for activity at pH 5.9 and manifests 70% of the maximal activity at pH 7. The pK'_a values of acetylphosphonate and methyl acetylphosphonate were measured to be 5.6 and 0.5, respectively.



monoanion dianion

The effects of both acetylphosphonate and methyl acetylphosphonate on yeast pyruvate decarboxylase at pH 6.5 were also investigated. No inhibition was observed. Methyl acetylphosphonate (8 mM) had no effect on the decar-

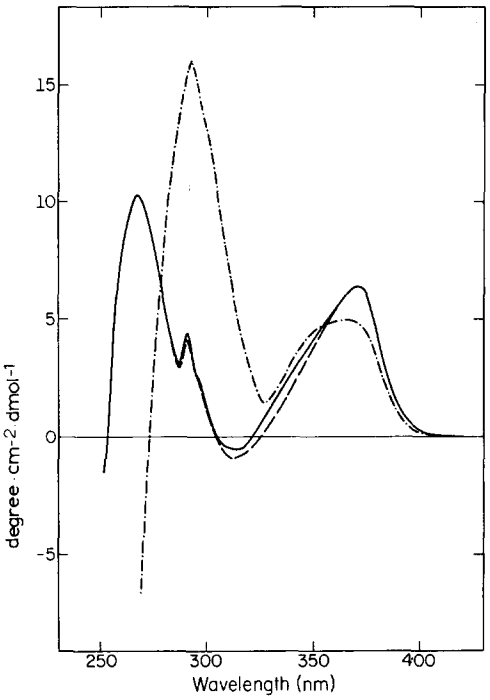


Fig. 2. The effect of methyl acetylphosphonate on the CD spectrum of pyruvate oxidase in the presence and absence of thiamin pyrophosphate. All samples contained the following: ———, 0.1 M Pipes buffer, 50 mM $MgCl_2$, and 0.53 mg/ml pyruvate oxidase at pH 5.7; - - - - -, plus 11 mM methyl acetylphosphonate; — · — · —, plus 11 mM methyl acetylphosphonate and 24 μM thiamin pyrophosphate. The conditions are designed so that the oxidase is saturated with the cofactor thiamin pyrophosphate (Mg^{2+}) at 24 μM thiamin pyrophosphate.

boxylase activity using pyruvate concentrations between 0.5 and 5 mM. If methyl acetylphosphonate is a competitive inhibitor of this enzyme, the K_i value would have to be greater than 31 mM [14]. Similarly, the minimum value for the K_i of acetylphosphonate with yeast pyruvate decarboxylase is 27 mM.

The effect of the lipid activator on the inhibition of pyruvate oxidase was also examined. When the steady state assay is performed in the presence of 20 μM sodium dodecyl sulfate, V increases about 25-fold. The detergent activator also affects both the K_m of the substrate, pyruvate, as well as the K_i of methyl acetylphosphonate. The K_m for pyruvate is reduced by about a factor of six (from 133 to 22 mM), and the K_i value for methyl acetylphosphonate drops by about a factor of four from the value obtained with unactivated oxidase (from 4.5 to 1.2 mM). Similar results have been obtained with another competitive inhibitor, 3-fluoropyruvate [15]. In this case the K_i was reduced from 8.7 to 1.4 mM in the presence of sodium dodecyl sulfate. Other lipid or detergent activators have been shown to have a similar effect on the K_m of pyruvate, and this is characteristic of the activation process [9].

Spectroscopic evidence suggests that methyl acetylphosphonate is binding at the active site of the enzyme. When methyl acetylphosphonate is added to a solution of pyruvate oxidase containing saturating amounts of thiamin pyro-

phosphate (K_d = 6 μM), the CD spectrum changes dramatically in the near ultraviolet region (Fig. 2). The substantial changes in the CD spectrum in the region near 290 nm is strongly suggestive of a tryptophan residue. In the absence of the cofactor, thiamin pyrophosphate, no induced CD due to methyl acetylphosphonate is observed.

Discussion

The three enzymes which have been examined which catalyze the thiamin pyrophosphate-dependent decarboxylation of pyruvate respond very differently to the phosphonate analogues of pyruvate. Methyl acetylphosphonate is a very potent inhibitor of pyruvate dehydrogenase, is effective only at much higher concentrations with pyruvate oxidase, and appears not to inhibit yeast pyruvate decarboxylase at even higher concentrations. Preliminary results indicate that pyruvate decarboxylase from wheat germ is inhibited by methyl acetylphosphonate and acetylphosphonate (Smyth, T., unpublished data).

The K_i for methyl acetylphosphonate with pyruvate dehydrogenase is $5 \cdot 10^{-8}$ M [3]. The analogue appears to bind to this enzyme better than does the substrate, pyruvate, by a factor of 10^4. This extremely tight binding is consistent with the formation of a phosphonate analogue of α-lactyl thiamin pyrophosphate being generated enzymatically [3]. If the rate determining step in the pyruvate dehydrogenase-catalyzed decarboxylation of pyruvate is the formation of α-lactyl thiamin pyrophosphate, then a major function of that enzyme involves stabilization of the transition state leading to α-lactyl thiamin pyrophosphate. In effect, pyruvate dehydrogenase appears to generate its own transition state analogue with methyl acetylphosphonate.

The ability of methyl acetylphosphonate and acetylphosphonate to inhibit pyruvate oxidase competitively at pH 6 with K_i values near 1 mM is consistent with those species binding to form a Michaelis complex but not proceeding to form the covalent adduct (Fig. 1). Apparently, methyl acetylphosphonate, acetylphosphonate (at pH 6), 3-fluoropyruvate, and perhaps pyruvate all bind to pyruvate oxidase with comparable affinities. The presumed inability of thiamin pyrophosphate to add to the carbonyl group of methyl acetylphosphonate and acetylphosphonate in this case may be the result of the differences in stereoelectronic alignment of the phosphonate monoester compared to the carboxylate [4]. The CD studies strongly suggest that methyl acetylphosphonate is binding to the thiamin pyrophosphate-containing site on the oxidase, since the CD spectral changes observed upon methyl acetylphosphonate addition require the presence of thiamin pyrophosphate. It is likely that the observed CD changes are due to a tryptophan at the active site. Other data suggest the presence of a tryptophan is at or near the cofactor binding site. Thiamin pyrophosphate-binding to the enzyme results in fluorescence quenching of one or more of the tryptophan residues [10]. Also, the binding of thiamin pyrophosphate protects the oxidase against inactivation by *N*-bromosuccinimide, apparently by protecting a single tryptophan residue which is essential for enzyme activity (O'Brien, T.A. and Gennis, R.B., unpublished data).

pH effects

The effects of changing solution pH on the inhibition of pyruvate oxidase by methyl acetylphosphonate and acetylphosphonate give information about the binding site in a manner analogous to what has been found in analogue studies of pyruvate decarboxylase [2]. The K_i for methyl acetylphosphonate at pH 6 and 7 is the same within experimental error. Since this monoanionic species binds equally well under both conditions, it is unlikely that a group responsible for binding on the enzyme undergoes a change in ionization state.

The unesterified material, acetylphosphonate, is an equilibrium mixture of monoanion and dianion. The pK'_a of the monoanion is 5.6. The K_i of acetylphosphonate is about a factor of ten greater at pH 7 than it is at pH 6, the lower value being close to that for methyl acetylphosphonate. This suggests preferential binding of the monoanion. Assuming exclusive binding of the monoanion, the K_i of monoanion would be 0.3 mM. This analysis suggests the monoanion is a slightly better inhibitor than methyl acetylphosphonate (K_i = 1.1 mM).

A similar analysis can be applied to the effects of pH on the K_m value for pyruvate. The 50% difference between the value at pH 6 (22 mM) and pH 7 (31 mM) is large compared to the change in K_i for the analogue methyl acetylphosphonate. This implies that the K_m is not purely a binding constant, since the binding of the inhibitor, which is not involved in further reaction, is not affected by the pH change. The difference in activity of the enzyme at the two pH values, concomitant with the change in K_m, suggests that kinetic phenomena subsequent to formation of an enzyme-coenzyme-substrate complex control the observed pH dependencies.

Pyruvate dehydrogenase is also inhibited more potently at pH 7 by the monoanionic methyl acetylphosphonate ($K_i = 5 \cdot 10^{-8}$ M) than it is by the acetylphosphonate mixture of anion and dianion ($K_i = 4 \cdot 10^{-6}$ M) (Harrison, R.A., Perham, R.N. and Slater, D.M., unpublished data, quoted in Ref. 16). This can be explained by assuming that a selectivity for monoanion is involved as it is for pyruvate oxidase.

Lipid effects

Lipid and detergent activators affect the binding of thiamin pyrophosphate to the active site of pyruvate oxidase. Both the dissociation constant and the degree of cooperativity (Hill coefficient) are modulated by the amphiphilic activators [10]. In addition, a reciprocal effect has been observed, in that the affinity of the oxidase for phospholipids (Schrock, H.L. and Gennis, R.B., unpublished data) and detergents [17] is enhanced in the presence of both pyruvate and thiamin pyrophosphate. It is in this context that it is important to measure the effect of lipid binding on the affinity of the oxidase for its substrate, pyruvate. The direct measurement of substrate binding has not been successful. However, lipid activation has a similar effect on both the K_m value for pyruvate, and the K_i values of pyruvate analogues. It was initially believed that 3-fluoropyruvate was a competitive dead-end inhibitor of the oxidase. However, recent work by Shaw-Goldstein [18] has demonstrated that this compound actually undergoes an eliminative decarboxylation with fluoride and acetate as products. The flavin is not directly involved in this reaction, and the

turnover number is approx. 10% of the value found for pyruvate under similar conditions. The same reaction has been shown to be catalyzed by pyruvate decarboxylase [19]. The kinetic data suggest that the lipid activators increase the affinity of pyruvate oxidase both for pyruvate and the substrate analogues. Hopefully, in the near future direct measurements of pyruvate binding to the oxidase will be made to determine the effect of lipid activators on this parameter.

The studies reported in this work further indicate the usefulness of phosphonate substrate analogues in probing the active sites of carboxylate-requiring enzymes. The three enzymes involved in pyruvate decarboxylation which we have analyzed show very distinct responses to the analogues of pyruvate. Therefore, the common feature catalytically is not due to structural invariance of the active sites. This information can be used as a basis for understanding the energetics of binding and catalysis by these enzymes and, in addition, can be used to probe the detailed nature of the substrate binding sites.

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