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Inhibition of Acetoacetate Decarboxylase by Ketophosphonates. Structural and Dynamic Probes of the Active Site†

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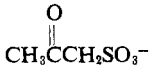
ABSTRACT: Acetonylphosphonate (**2**) (pK = 6.3) is a competitive, rapidly dissociating inhibitor of acetoacetate decarboxylase (K_i = 0.8 mM, pH 5.9). Inhibition decreases sharply with increasing pH due to the dissociation of a proton from the monoacid; compounds without a pK in this region do not show the sharp decrease. Similarly, the monoanion of acetyl phosphate is a much better inhibitor than the dianion. The monomethyl and monoethyl esters of **2** are much poorer inhibitors than the unesterified compound (about 60 times less effective), indicating the presence of steric interactions in the electrophilic region of the active site. Chemical tests for imine formation appear to be highly dependent upon electrostatic effects. Acetoacetate decarboxylase catalyzes the exchange of one of the two protons at the 2 position of the esters of **2** as well as the protons of the 4 position of ethyl acetoacetate but does not catalyze the exchange of the protons of **2**. These results suggest a charge-defined direction of binding of substrate and inhibitors and indicate that **2** binds in a manner unlike that of ketones which are poorer inhibitors. It is suggested that consideration of orbital overlap can account for these observations.

Acetoacetate decarboxylase from *Clostridium acetobutylicum* (Westheimer, 1969) has been the subject of extensive mechanistic studies. These have led to a proposed mechanism (for a discussion, see Fridovich (1972)) which specifies that the enzyme forms a covalent derivative of the substrate, presumably an imine, since complete loss of ^{18}O from that group accompanies decarboxylation (Hamilton and Westheimer, 1959) and sodium borohydride reductively traps a covalent derivative (Fridovich and Westheimer, 1962). The reduced product has been identified as the amine resulting from reduction of an imine derived from acetone and a lysine residue of the enzyme (Warren *et al.*, 1966). Studies of requirements for substrates and inhibitors have appeared and have been reviewed (Fridovich, 1972). The key result of the studies of inhibition which relate to the present work is that acetonylsulfonate (**1**) acts as a competitive



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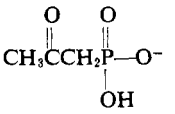
inhibitor with respect to substrate and appears to simulate the binding characteristics of the substrate (Fridovich, 1968; Autor and Fridovich, 1970). Although other inhibitors are known, none appear to be accurate mimics of the natural substrate in the manner of acetonylsulfonate since other compounds which are competitive inhibitors by kinetic criteria associate and dissociate with the enzyme very slowly (Tagaki *et al.*, 1968; Autor and Fridovich, 1970). In this work, we have examined the effects of a new substrate analog, sodium acetonylphosphonic acid (**2**) (Kluger and Wasserstein, 1973), and



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derivatives of this and related compounds as a probe of the steric and electronic forces determining the specificity of the enzyme for its substrate. We have also determined the effects of the enzyme upon the dynamic properties of the bound substrate analogs. The phosphonate is particularly useful for these studies since the availability of two anionic groups permits variations of structure and charge not accessible with carboxylate or sulfonate molecules. The carbon–phosphorus bond is not broken in the presence of acetoacetate decarboxylase, enabling us to study steps which are complicated by decarboxylation when acetoacetate is used.

Experimental Section

Materials. Heat activated acetoacetate decarboxylase from *Clostridium acetobutylicum*, prepared by the method described by Westheimer (1969), was obtained in the form of a crystalline suspension from Professor F. H. Westheimer of Harvard University and had been prepared by Mr. Jerome V. Connors. Quantities of enzyme used will be based on units of enzyme activity as defined by Westheimer (1969). Lithium acetoacetate was prepared using a method described by Hall (1963). Lithium acetyl phosphate was prepared by the method of Avison (1955). The sodium salt of acetonylphosphonic acid was syn-

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thesized by a published procedure (Kluger and Wasserstein, 1973) as was the monomethyl ester of sodium acetonylphosphonate (Kluger, 1973).

The monoethyl ester of acetonylphosphonate was synthesized by refluxing the diethyl ester (Jacobson *et al.*, 1957) with 1 equiv of sodium hydroxide for 12 hr. The product was isolated by evaporation and washed with acetone. The white powder was recrystallized from ethanol–ether. Sodium ethyl acetonylphosphonate melted at 156°. *Anal.* (Galbraith Laboratories): Calcd for $C_5H_{10}O_4PNa$: C, 31.94; H, 5.36; P, 16.47. Found: C, 31.83; H, 5.36; P, 16.31.

The method of Terent'ev and Preobrazhenskaya (1956), involving the addition of sulfur trioxide–dioxane to acetone, was found to be a superior procedure for the preparation of sodium acetonylsulfonate. Sodium borohydride was obtained from K and K Laboratories and sodium cyanoborohydride from the Aldrich Chemical Co. Ethyl acetoacetate from the Aldrich Chemical Co. was redistilled. Inorganic chemicals were reagent grade and used as obtained. Deuterium oxide was purchased from Merck Sharp and Dome of Canada.

Methods. Enzyme assays were conducted with a Unicam SP 1800A ultraviolet-visible spectrophotometer with a Unicam AR 25 recorder. The cell holder of the spectrophotometer was maintained at 30.0 ± 0.1° with a Heto circulating water bath. The procedure for the assay was essentially that of Fridovich (1963) involving observation of the decrease in optical density at 272 nm which we attribute to the weaker $n \rightarrow \pi^*$ absorption of acetone compared to acetoacetate (see Matsen *et al.*, 1960, for a discussion of the absorption properties of carbonyl groups). It has also been considered that the absorption of acetoacetate results from the presence of enol (Guthrie and Jordan, 1972). Assays were performed at pH 5.92 in phosphate buffer unless otherwise noted. Nmr experiments involved the utilization of deuterated media whose pD was determined using a Radiometer PHM 26 meter and appropriate pH meter corrections (Glasoe and Long, 1960). The deuteration of a particular position was followed by observing the integrated intensity of the signals corresponding to the protons which were being replaced. A Varian A60 nmr spectrometer or Varian HA100 nmr spectrometer was used for all experiments. Samples were maintained at 30.0 or 35.0°. When constant amplitude and sweep settings were used for a particular experiment, absolute values of integrated signals were more reproducible and gave less scatter on a first-order plot than ratio comparison with an internal standard.

The ability of hydrogen cyanide to interact with various enzymic intermediates was studied according to the procedure described by Autor and Fridovich (1970). Plots of hydrogen cyanide concentration *vs.* per cent inhibition in the presence and absence of added ketonic compounds were used to monitor these effects. Experiments were conducted at 30.0° with pH 5.9 phosphate buffer at inhibitor levels of 3 mM acetonylphosphonate, 14.7 mM acetonylsulfonate, 15 mM monomethyl acetonylphosphonate, and 180 mM acetone with cyanide concentrations varying from 0 to 8.33×10^{-6} M.

Reductions using borohydride were performed by the method of Warren *et al.* (1966). Dialyses were conducted using buffer solutions kept at 4°. Dialysis tubing was boiled prior to use.

Results

Inhibition Studies. Acetonylphosphonate monoanion (pK = 6.3) was found to be a competitive inhibitor of acetoacetate decarboxylase (see Figure 1). Inhibition levels were independent of time or order of mixing and final OD values were stable, indicating that the inhibitor combines and dissociates with the enzyme rapidly. K_i for acetonylphosphonate at pH 5.92 was determined to be 0.8 mM (see Dixon and Webb (1964) for an exposition on the determination of K_i); at the same pH, K_i for acetonylsulfonate is 8.0 mM and K_m for acetoacetate is 8.0 mM (Fridovich, 1968).

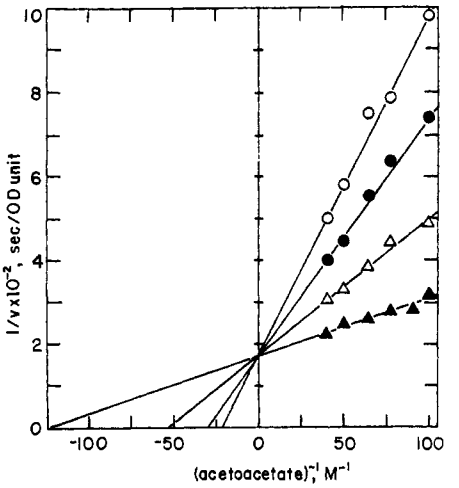

FIGURE 1: Inhibition of acetoacetate decarboxylase by sodium acetonylphosphonate (pH 5.92) in potassium phosphate buffer. Rates are initial rates of conversion of acetoacetate to acetone based on the change in absorbance at 272 nm. Concentrations of acetonylphosphonate used were 5 mM (○), 3.3 mM (●), 1.7 mM (△), and none (▲).

Table I contains a summary of data regarding the inhibitory power of various compounds. It is clear that the effect of conversion of a monoanionic acid to its conjugate dianion results in a marked reduction in inhibition, a change which is many times greater than the effects of the change of pH on the inhibitory power of the control compound which remains monoanionic throughout the pH range studied. Furthermore, it can be seen that the monoanionic monoesters are much poorer inhibitors than the corresponding monoanionic monoacid. Acetyl phosphate inhibits with a time-dependent component which is probably due to its acetylating a group at the active site. The monoanionic form is again the potent form of the inhibitor.

Reaction with Borohydride. The ability of the borohydride

TABLE I: Concentration of Inhibitors Necessary to Decrease Rate of Acetoacetate Decarboxylase Catalyzed Decarboxylation of 25 mM Acetoacetate by 10%.

Compound	pK'	pH	Concn (mM)
Acetonylsulfonate	~0	4.2[a]	3.0
		5.9	4.6
		7.2	7.1
Acetonylphosphonate	6.3	5.9	0.2
		7.2	9.0
Methyl acetonylphosphonate	0.6	5.9	12.0
Ethyl acetonylphosphonate	0.6	5.9	12.0
Acetyl phosphate	4.8	4.2[a]	0.3[b]
		5.9	25.0[b]

[a] Sodium acetate buffer (0.1 M). [b] Time-dependent value. After 15-min preincubation with enzyme.

TABLE II: Comparison of Acetoacetate Decarboxylase Activity after Treatment with Borohydride or Cyanoborohydride in the Presence of Acetonylphosphonate, Acetonylsulfonate, or Acetoacetate in pH 5.9, 0.1 M Potassium Phosphate Buffer.

Enzyme (10^{-8} M)	Acetoacetate (10^{-4} M)	Acetonylphosphonate (10^{-4} M)	Acetonylsulfonate (10^{-4} M)	$NaBH_4$ (10^{-4} M)	$NaBH_3CN$ (10^{-4} M)	% Activity
12		4.2		2.1		93[a]
12		4.2		2.1		98[b]
12		4.2		2.1		100[a]
7.3		60		30		94[c]
7.3	60			30		0
7.3		1000		500		85
7.3				500		80
21		16.7		83.3		92
21	16.7			83.3		0
21				83.3		97
29		7		3.5		91
29	7			3.5		52
29				3.5		98
29			7	3.5		73
29	7			3.5		25
29				3.5		92
36			16.7	83.3		3
36	16.7			83.3		2
36				83.3		94
7.3		4.2			2.1	82[a]
7.3	4.2				2.1	95[a]
9.5	5.3				2.65	120[c]
29		7			3.5	89
29	7				3.5	90
29					3.5	87
220		2200[e]		360		18[d]
350		960		160		97[a]
114		2000		360		98[a]

[a] Compared to control with acetonylphosphonate and without $NaBH_4$. [b] Repeated treatment on above sample. [c] Compared to control with $NaBH_4$ and without acetonylphosphonate. [d] Compared to control with ethyl acetonylphosphonate and without BH_4^-. [e] Monoethyl ester of acetonylphosphonate.

ion to reduce (protonated) imines more rapidly than it does other functional groups led to the use of this reagent for the "trapping" of Schiff bases formed in enzymic reactions (Fischer *et al.*, 1958; for a review, see Fischer (1964)). The reagent has been used to trap the acetone-derived imine of acetoacetate decarboxylase (Fridovich and Westheimer 1962; Warren *et al.*, 1966) but the imine of acetoacetate has not been trapped. Acetonylsulfonate has supported the inactivation of acetoacetate decarboxylase with borohydride, but V_{max} for the reduction is five times less than for the reduction of the acetone imine of the enzyme (Autor and Fridovich, 1970). We find that acetonylphosphonate cannot be trapped as an imine; that is, borohydride does not produce irreversible inhibition in the presence of acetonylphosphonate, even with large amounts of borohydride, although the monoethyl ester, which does not bind nearly as well to the enzyme, is trapped (see Table II). Sodium cyanoborohydride, a reagent particularly suited to the reduction of imines in water (Borch *et al.*, 1971), had little or no effect as a trapping agent, even with compounds which are readily trapped with borohydride (in model systems, the reagent has been employed successfully (Guthrie and Jordan, 1972)).

Synergistic Inhibition with HCN. Autor and Fridovich (1970) made use of the fact that hydrogen cyanide adds rapidly to imines to probe imine formation with acetoacetate decarboxylase. Inhibition in the presence of ketones that form imines with acetoacetate decarboxylase was markedly enhanced in the presence of hydrogen cyanide and was shown to be greater than would be expected if separated sites were involved. Following the procedure of Author and Fridovich (1970), we were able to observe synergistic enhancement by hydrogen cyanide of inhibition of acetoacetate decarboxylase with acetone. However, with acetonylphosphonate and acetonylsulfonate absolutely no synergism upon addition of hydrogen cyanide was observed.

Enzymic Catalysis of Deuteration. Since acetoacetate decarboxylase catalyzes the deuteration of acetone in deuterium oxide, presumably *via* tautomerization of the enzymic imine (Tagaki and Westheimer, 1968), catalysis of deuteration by acetoacetate decarboxylase of the position adjacent to a carbonyl function can be used as a probe of mechanism. For rapidly dissociating inhibitors, the catalysis of exchange by acetoacetate decarboxylase indicates that a dynamic equilibrium between imine and enamine forms probably exists and is catalyzed by a group at the active site. With acetonylphosphonate in pD 5.9, 0.1 M phosphate buffer, however, we observed no enhancement of exchange over the relatively slow nonenzymic rate at either α position (see Table III). Acetonylphosphonate strongly inhibited the deuteration of acetone, indicating that the two molecules probably do bind to a com-

mon site. Acetoacetate decarboxylase catalyzes the exchange of one of the two protons of the 2 position of both methyl and ethyl acetonylphosphonate but not of the protons in the 4 position of either compound. Our conclusion that only one proton at the 2 position is exchanged enzymically is based on an extension of our studies on the deuteration of butanone and will be discussed in detail elsewhere. We saw no enhanced exchange of the protons in the 4 position of acetonylsulfonate nor of the protons in the 2 position of that species. The results with respect to the 2 position are not conclusive since the nonenzymic rate at the 2 position is relatively fast ($t_{1/2} \approx 20$ min). Acetoacetate decarboxylase also catalyzes deuteration of the 4 position of ethyl acetoacetate; the rate at the 2 position is very rapid in the absence of enzyme ($t_{1/2} > 5$ min). A summary of conditions and results is presented in Table III.

TABLE III: Effect of Acetoacetate Decarboxylase on Proton Exchange Reactions of Inhibitors.[a]

Species	Concn (M)	Acetoacetate Decarboxylase (μM)	k_{obsd} (min^{-1})
$CH_3CCD_2PO_3D^-$ [b]	0.2	2.3	0.0
O	0.2	0.0	9.7×10^{-4}
$CH_3CCH_2PO_3H^-$	0.2	1.4	0.0
O	0.2	11.3	0.0
	0.2	0.0	2.1×10^{-3}
$CH_3CCH_2SO_3^-$ (35°)	0.3	2.0	0.0
O	0.2	2.0	0.0
	0.3	0.0	2.0×10^{-2}
	0.2	0.0	2.3×10^{-2}
CH_3CCH_3 [c]	0.5	6.0	3.0×10^{-3}
O	0.5	0.0	Not observed
$CH_3CCH_2PO_2(OCH_3)^-$	0.2	~4.0	3.0×10^{-3}
O	0.2	0.0	1.8×10^{-3}
$CH_3CCH_2PO_2(OC_2H_5)^-$ [d]	0.4	~4.0	2.0×10^{-3}
O	0.4	0.0	6.9×10^{-4}
$CH_3CCH_2COC_2H_5$	0.2	~4.0	6.0×10^{-5}
O O	0.2	0.0	4.6×10^{-6}

[a] Data obtained at pD 5.9 in D_2O in the presence of 0.1 M potassium phosphate buffer at 30.0°, unless otherwise noted. The values of k_{obsd} for the enzymic processes are net rate constants, corrected for nonenzymic background rate. Enzyme concentration is given in terms of subunits (Westheimer, 1969). Where exact enzyme concentration was not determined, an approximate value (±20%) is given and noted as such. [b] In H_2O, pH 5.9, 0.1 M potassium phosphate buffer. [c] 2-Picoline–2-picolinium sulfate buffer (0.15 M). [d] Phosphate buffer concentration was reduced to 0.03 M to minimize general base catalyzed background rate.

Discussion

It is clear that acetonylphosphonate monoanion is a much better inhibitor of acetoacetate decarboxylase than the corresponding monoester or dianion. It had previously been postulated (Fridovich, 1972) that there is an electrophilic binding area in the active site of acetoacetate decarboxylase. Our results indicate that the site is extremely selective for monoanions over dianions, even for those of structurally similar carbonyl-containing compounds. Furthermore, the site is sterically restricted, limiting the binding of monoesters of acetonylphosphonate.

The failure of borohydride to trap an enzymic imine has been reported in another system. Shaltiel and Cortijo (1970) explain the inability of borohydride to reduce the Schiff base of pyridoxal phosphate and glycogen phosphorylase as being due to the location of the Schiff base in an inaccessible hydrophobic pocket. However, that explanation is unlikely to apply to acetoacetate decarboxylase since the facility of trapping is a function of the choice of substrate and not of the enzyme itself. Although acetonylsulfonate can be trapped with borohydride onto acetoacetate decarboxylase, we have not been able to observe the synergism with hydrogen cyanide that would also be expected to be a consequence of imine formation. We can consider the failure of borohydride to trap the imine of acetonylphosphonate as being due to electrostatic effects, in analogy to Fridovich's explanation for the slow reduction of acetonylsulfonate (1968). Perhaps since the hydrolysis of borohydride generates hydroxide, this leads to local pH increases and the loss of the acidic proton of the bound phosphonate, producing a dianion which is especially difficult to reduce. The monoesters and the sulfonate cannot undergo such an ionization. Furthermore, the failure of hydrogen cyanide to synergize its inhibition with that of acetonylphosphonate or acetonylsulfonate indicates that there is probably an electrostatic sensitivity in the addition of cyanide to the enzymic imine. All previously successful applications of this method with acetoacetate decarboxylase involved uncharged inhibitors (Autor and Fridovich, 1970). Preliminary experiments in our laboratory indicate that the effect of cyanide in the absence of inhibitors is due to its addition to the imine which results after decarboxylation and tautomerization have occurred. Cash and Wilson (1966) showed that relatively high concentrations (10 mM) of hydrogen cyanide and dihydroxyacetone phosphate are necessary to produce inhibition in an aldolase system. The electrostatic similarity of dihydroxyacetone phosphate and the compounds in our tests suggests a common explanation for the phenomena. We were not able to test inhibition by cyanide and acetonylphosphonate at high concentrations with acetoacetate decarboxylase because inhibition is so great at low concentration levels.

The absence of enzyme-catalyzed proton exchange at the 4 position of the charged ketonic inhibitors of acetoacetate decarboxylase indicates that an electrophilic group on the enzyme specifically directs the mode of binding of keto acid anions, including the substrate. In contrast, acetoacetate decarboxylase does catalyze the deuteration of the 4 position of the neutral ester, ethyl acetoacetate. Since the carbon–carbon double bond of the enamine resulting from the enzymic decarboxylation of acetoacetate must form between the carbon atoms that were originally C_2 and C_3 of acetoacetate, the general acid–base moiety which catalyzes tautomerization to imine (Tagaki and Westheimer, 1968) must operate on the same portion of the molecule which eventually becomes acetone. The "wrong" side of ethyl acetoacetate can come into the vicinity of the catalytic group whereas the negative charge on the other molecules directs binding to occur only in the "useful" manner. The observation of acetoacetate decarboxy-

lase catalyzed deuteration of only the 2 position of ethyl acetonylphosphonate conforms to the predictions of this model. The fact that deuteration of acetonylphosphonate is not enhanced in the presence of acetoacetate decarboxylase is consistent with a major function of acetoacetate decarboxylase in the decarboxylation process to be one of fixing proper orbital alignment (Guthrie and Jordan, 1972). The σ bond which is broken in the decarboxylation step for optimal catalysis should be restricted to a plane which causes overlap with the π system of the imine. If this occurs, and the binding is rigid, we would expect that the protons of the 2 position are no longer able to assume the proper conformation to facilitate removal by a base (Corey and Sneen, 1956). If acetonylphosphonate binds in a geometric manner which closely resembles that of acetoacetate, then its protons should not be exchangeable while the molecule is enzymically bound. Methyl and ethyl acetonylphosphonate, which bind much more poorly, may be unable to bind at the site in the exact manner of the substrate because of the bulk of the alkyl group; thus a proton in the vicinity of the catalytic group can be lost.

The structural and dynamic properties of ketophosphonates have provided us with a means of probing the active site of acetoacetate decarboxylase in a manner that has not been feasible with other inhibitors. We are in the process of extending these procedures to the study of other enzymes which utilize suitable substrates and which bind these subtrate analogs.

Acknowledgment

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