Slow-Binding Inhibition of the *Escherichia coli* Pyruvate Dehydrogenase Multienzyme Complex by Acetylphosphinate

Ernst Schönbrunn-Hanebeck,[‡] Bernd Laber,[§] and Nikolaus Amrhein*

Institut für Pflanzenwissenschaften, Biochemie und Physiologie der Pflanzen, ETH Zürich, Sonneggstrasse 5, CH-8092 Zürich, Switzerland

Received July 20, 1989; Revised Manuscript Received January 30, 1990

ABSTRACT: The pyruvate analogue acetylphosphinate (CH_3–CO–PO_2H_2) inhibits the pyruvate dehydrogenase component (E_1) of the *Escherichia coli* pyruvate dehydrogenase multienzyme complex in a time-dependent process with biphasic reaction kinetics. The formation of an initial, rapidly reversible enzyme–inhibitor complex (EI) with an apparent K_i of 0.12 ± 0.025 μM is followed by the conversion to a tighter complex (EI*) at a maximal rate of k_3 = 0.87 ± 0.34 min^{-1}. The inhibition is reversible (dissociation rate constant k_4 = 0.038 ± 0.002 min^{-1}), requires the presence of the cofactors thiamin pyrophosphate and Mg^{2+}, and is competitive with regard to pyruvate. The microscopic rate constants give a value of 5 nM for the overall dissociation constant [K_i^* = [E][I]/([EI] + [EI*]) = $K_ik_4/(k_3 + k_4)$] compared with values of 10 and 3.5 nM obtained by steady-state methods. Thus acetylphosphinate binds by 5 orders of magnitude more tightly to pyruvate dehydrogenase than does pyruvate (K_m = 0.35 mM). Acetylphosphinate also affects the pyruvate dehydrogenase complex fluorescence when excited at 290 nm in a time-dependent manner with a maximal rate constant of 0.99 min^{-1}, suggesting a conformational change in the enzyme complex as the slow step in conversion of EI to EI* (k_3). All these features taken together suggest that the interaction of the pyruvate dehydrogenase with acetylphosphinate involves the formation of a thiamin pyrophosphate–acetylphosphinate adduct that resembles the normal reaction intermediate, 2-(1-carboxy-1-hydroxyethyl)thiamin pyrophosphate (α-lactylthiamin pyrophosphate).

The pyruvate dehydrogenase multienzyme complex (PDC)[1] plays an essential role in the metabolism of aerobic organisms by connecting the glycolytic pathway with the citric acid cycle and thus with the energy metabolism of the cell. It is one of the largest and most highly organized enzymatically active protein aggregates known and catalyzes the oxidative decarboxylation of pyruvate by the pathway (Koike et al., 1963)

$$H^+ + CH_3COCO_2^- + E_1\text{–TPP} \rightarrow E_1\text{–hydroxyethylidene–TPP} + CO_2$$

$$E_1\text{–hydroxyethylidene–TPP} + E_2\text{–Lip}S_2 \rightarrow E_1\text{–TPP} + E_2\text{–Lip(SH)SCOCH}_3$$

$$E_2\text{–Lip(SH)SCOCH}_3 + \text{CoA–SH} \rightarrow E_2\text{–Lip(SH)}_2 + CH_3COS\text{–CoA}$$

$$E_2\text{–Lip(SH)}_2 + E_3\text{–FAD}_{ox} \rightarrow E_2\text{–Lip}S_2 + E_3\text{–FAD}_{red}$$

$$E_3\text{–FAD}_{red} + NAD^+ \rightarrow E_3\text{–FAD}_{ox} + NADH + H^+$$

$$\text{sum: } CH_3COCO_2^- + \text{CoA–SH} + NAD^+ \rightarrow CO_2 + CH_3COS\text{–CoA} + NADH + H^+$$

The *Escherichia coli* PDC is composed of multiple copies of three different enzyme components, which in the order of their participation in the reaction sequence are pyruvate dehydrogenase (E_1) (EC 1.2.4.1),[2] dihydrolipoamide transacetylase (E_2) (EC 2.3.1.12), and dihydrolipoamide dehydrogenase (E_3) (EC 1.8.1.4). A total of 24 identical E_2 polypeptide chains form the cubelike structural core of the complex, surrounded by 12 E_1 dimers and 6 E_3 dimers, which are bound in a noncovalent manner (Angelides et al., 1979; Yang et al., 1985).

The E_1 subunit is the primary target of most synthetic inhibitors of the enzyme, which can be roughly divided into analogues of the essential cofactor thiamin pyrophosphate (TPP) (Gutowski & Lienhard, 1976; Lowe et al., 1983; Buttler et al., 1977) and analogues of the substrate pyruvate. Pyruvate analogues act either as pure competitive inhibitors of substrate binding to the complex, such as fluoropyruvate (Bisswanger, 1980, 1981), or as enzyme-activated irreversible (suicide) inhibitors, such as bromopyruvate (Maldonado et al., 1972; Apfel et al., 1984; Lowe & Perham, 1984) and moniliformin (Gathercole et al., 1986), or, in the case of methyl acetylphosphonate, are transformed into an analogue of a reaction intermediate (Kluger & Pike, 1977).

In a previous paper (Laber & Amrhein, 1987) it was found that the novel pyruvate analogue acetylphosphinate inhibits the bovine heart PDC in a time-dependent reaction that followed pseudo-first-order and saturation kinetics and required the presence of TPP and Mg^{2+}. The inhibition was judged to be irreversible on the basis of dilution experiments, but extensive dialysis recovered more than 50% of the lost enzymic activity, indicating an essential reversibility of the inactivation.

* Address correspondence to this author at the Institut für Pflanzenwissenschaften, Biochemie und Physiologie der Pflanzen, ETH Zürich.

‡ Present address: Max Planck Arbeitsgruppen für strukturelle Molekularbiologie, c/o DESY, D-2000 Hamburg 52, FRG.

§ Present address: Max-Planck-Institut für Biochemie, D-8033 Martinsried, FRG.

[1] Abbreviations: CoA–SH, coenzyme A; DCPIP, 2,6-dichlorophenolindophenol; E_1, pyruvate dehydrogenase; E_2, dihydrolipoamide transacetylase; E_3, dihydrolipoamide dehydrogenase; EDTA, ethylenediaminetetraacetate; LDH, lactate dehydrogenase; $Lip(S)_2$, lipoamide; $Lip(SH)_2$, dihydrolipoamide; PDC, pyruvate dehydrogenase complex; TPP, thiamin pyrophosphate.

[2] Enzymes: pyruvate dehydrogenase (EC 1.2.4.1), dihydrolipoamide transacetylase (EC 2.3.1.12), dihydrolipoamide dehydrogenase (EC 1.8.1.4), lactate dehydrogenase (EC 1.1.1.27), and phosphotransacetylase (EC 2.3.1.8).

0006-2960/90/0429-4880$02.50/0

On the basis of the experimental criteria available at that time, it had been concluded that acetylphosphinate is an enzyme-activated inhibitor of the PDC. Further investigations of the interaction of acetylphosphinate with the purified *E. coli* PDC have now provided direct evidence that acetylphosphinate fulfils the criteria for a slow-binding inhibitor (Morrison, 1982) of the pyruvate dehydrogenase complex.

Experimental Procedures

Enzymes and Reagents. Acetylphosphinate, synthesized by the method of Baillie et al. (1980, 1988), which involved treating triethyl orthoacetate with H_3PO_2 in acid conditions to obtain CH_3–$C(OEt)_2$–$P(O)(H)$–OEt_1 and hydrolyzing this with aqueous alkali followed by aqueous acid, was provided by Ciba-Geigy AG, Basel, Switzerland. Dihydrolipoamide was prepared from lipoamide as described by Reed et al. (1958). Lactate dehydrogenase from bovine heart was supplied by Sigma Chemie, Deisenhofen, FRG. Biochemical reagents were obtained from Boehringer, Mannheim, FRG, and Sigma Chemie.

Preparation of Enzyme. PDC was purified from an overproducing mutant of *E. coli* K12, kindly provided by Dr. L. Packman, University of Cambridge, England, by the procedure of Reed and Mukherjee (1969) as modified by Speckhard and Frey (1975) and Danson et al. (1979). The enzyme was stored at −70 °C in 100 mM potassium phosphate buffer, pH 7.0, containing 2 mM EDTA. The specific overall activity of the purified PDC was 35 μmol of NADH formed min^{-1} (mg of protein)$^{-1}$, comparable to the highest specific activities reported in the literature (Maldonado et al., 1972; Graupe et al., 1982). The specific E_1 activity was 300 nmol of DCPIP reduced min^{-1} (mg of protein)$^{-1}$. K_m values of 350 μM and 1.5 μM (data not shown) were determined for pyruvate and TPP, respectively [literature values: 300 μM for pyruvate (Bisswanger, 1980) and 1.15 μM for TPP (Horn & Bisswanger, 1983)]. The PDC showed no activity in the absence of TPP or Mg^{2+} or CoA-SH.

The E_2–E_3 subcomplex and the E_1 component were prepared as described by Coggins et al. (1976) and separated by fast protein liquid chromatography (FPLC) on Superose 12 (Pharmacia, Freiburg, FRG).

Enzyme Assays. Overall PDC activity was assayed by following NADH formation at 340 nm and 30 °C according to Reed and Willms (1966) as modified by Danson et al. (1978). Assay mixtures contained 50 mM potassium phosphate buffer, pH 8.0, 2.5 mM NAD, 0.2 mM TPP, 10 mM $MgCl_2$, 0.13 mM CoA-SH, 2 mM sodium pyruvate, and enzyme complex in a final volume of 1 mL. The reaction was started by the addition of pyruvate or enzyme or cofactors (as indicated). The increase in absorbance at 340 nm was followed with a Uvikon 810 spectrophotometer (Kontron Analytic GmbH, Eching, Switzerland). Enzyme activity is expressed as micromoles of NADH produced per minute.

The activity of the pyruvate dehydrogenase component (E_1) of the PDC was measured at 30 °C by following the reduction of DCPIP at 600 nm in the presence of pyruvate as described by Packman et al. (1982) and Lowe et al. (1983). The assay mixture contained 100 mM potassium phosphate buffer, pH 7.0, 10 mM $MgCl_2$, 0.2 mM TPP, 0.1 mM DCPIP, 2 mM sodium pyruvate, and 24 μg of PDC in 1 mL.

Dihydrolipoamide transacetylase (E_2) actvity was determined according to Schwartz and Reed (1969). Assay mixtures contained 100 mM Tris-HCl, pH 7.4, 1 mM acetyl phosphate, 5 units of phophotransacetylase, 2 mM DL-dihydrolipoamide, 0.13 mM CoA-SH, and 6 μg of PDC in 1 mL.

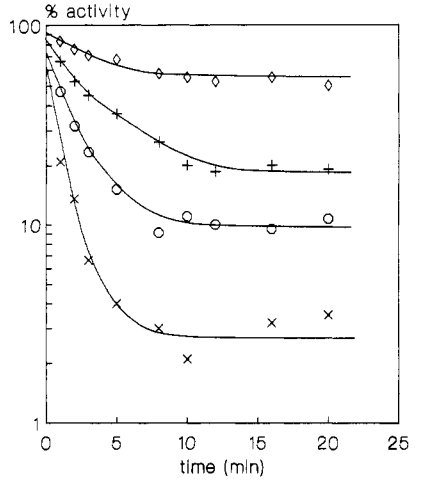


FIGURE 1: Time-dependent inhibition of the overall activity of the PDC by acetylphosphinate. The PDC (2.4 μg/mL) was incubated at 30 °C in 0.99 mL of 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM $MgCl_2$, 0.2 mM TPP, 2.5 mM NAD^+, 0.13 mM CoA-SH, and 0.1 (◊), 0.25 (+), 0.5 (○), or 1 μM (×) acetylphosphinate. At intervals the enzymic reaction was started by the addition of 10 μL of 0.2 M pyruvate and initial velocities were measured.

Dihydrolipoamide dehydrogenase (E_3) activity of the PDC was assayed at 30 °C in 50 mM potassium phosphate buffer, pH 8.0, containing 2.5 mM NADH, 0.4 mM DL-dihydrolipoamide, 10 mM $MgCl_2$, and 0.6 μg of PDC according to Brown and Perham (1976) and Danson et al. (1978).

Determination of Acetylphosphinate. Quantitative determination of acetylphosphinate was based on its NADH-dependent reduction to (hydroxyethyl)phosphinate, catalyzed by lactate dehydrogenase (Laber & Amrhein, 1987). The assay mixture (1 mL) contained 300 mM triethanolamine, pH 7.6, 3 mM EDTA, 0.2 mM NADH, 30 units of lactate dehydrogenase, and acetylphosphinate to be determined. After a 1-h incubation at room temperature, the difference in E_{340} between the sample and a blank without acetylphosphinate was used to calculate the amount of acetylphosphinate.

Protein Determination. Protein was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

Fluorescence Measurements. Fluorescence measurements were performed with a Shimadzu spectrofluorometer. The excitation wavelength was 290 nm, and emission spectra were recorded in the range of 300–500 nm. The measurements were done with PDC solutions of 100 μg/mL in 50 mM potassium phosphate buffer, pH 7.4, at room temperature.

Results

Time-Dependent Inhibition of the PDC Overall Activity. When the PDC is incubated with increasing concentrations of acetylphosphinate in the presence of saturating concentrations of $MgCl_2$ and TPP, the overall activity decreases as a function of time and of acetylphosphinate concentration (Figure 1).

Loss of activity follows biphasic pseudo-first-order kinetics: an initial rapid phase is terminated within less than 30 s and cannot be resolved in time by the spectrophotometric assay used here. The rapid phase is followed by a slower phase terminated by a steady-state level at which residual enzymic activity is stable for more than 4 h. Further addition of enzyme to the incubation mixture at times when maximal inhibition has been achieved (i.e., after approximately 20 min) leads to further inhibition of the overall activity with the same inhibition pattern as that shown in Figure 1 (data not shown). This

pattern excludes the possibility that the observed steady-state levels are due to the disappearance of acetylphosphinate during the incubation with the PDC (e.g., decomposition in solution or enzyme-catalyzed modification). Therefore, an explanation of the biphasic reaction kinetics must account for at least two steps in which an initial, reversible complex (EI) isomerizes to a form (EI*) from which the inhibitor is released only slowly (Fleck, 1971; Kluger, et al., 1984), as shown in Scheme I.

Scheme I

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^*$$

Assuming that establishment between free inhibitor (I), enzyme (E), and transient enzyme–inhibitor complex (EI) is not controlled by a rate-limiting step, data may be fitted to the single exponential equation (Schloss, 1989)

$$A/A_0 = F_2 + (F_1 - F_2)e^{-k_{obs}} \quad (1)$$

A and A_0 are the activities at time t and zero time, respectively, F_1 equals the fractional activity after establishment of equilibrium between E, I, and EI, and F_2 represents the residual activity at infinite time. k_{obs} is the apparent first-order rate constant for the slow phase of inhibition.

The initial level of inhibition (F_1) depends on the concentrations of substrate ([S]) and inhibitor ([I']) in the assay mixture

$$F_1 = \frac{1 + [S]/K_m}{1 + [I']/K_i + [S]/K_m} \quad (2)$$

whereas the final steady-state level (F_2) varies as a function not only of the concentrations of substrate ([S]) and inhibitor ([I']) in the final assay mixture but also of the inhibitor concentration in the preincubation mixture prior to assay ([I]) as discussed by Schloss (1989):

$$F_2 = \frac{1 + [S]/K_m}{(1 + [I']/K_i + [S]/K_m)(1 + [I]/K_i^{**})} \quad (3)$$

K_i is the concentration of the competitive inhibitor that gives 50% initial inhibition at low substrate concentration ([S] $\ll$ K_m) and is defined by

$$k_2/k_1 = [E][I]/[EI] = K_i \quad (4)$$

while the concentration of inhibitor that gives an equal amount of free enzyme (E) and the final enzyme–inhibitor complex (EI*) is designated by K_i^{**} (Schloss, 1989):

$$k_2k_4/k_1k_3 = [E][I]/[EI^*] = K_i^{**} \quad (5)$$

The rate of the loss of activity in the slower phase of acetylphosphinate-mediated PDC inactivation is characterized by the observed first-order rate constant

$$k_{obs} = k_4 + k_3[[I]/(K_i + [I])] \quad (6)$$

or simply

$$k_{obs} = k_3[[I]/(K_i + [I])] \quad (7)$$

(Aldridge & Reiner, 1972), provided that dissociation of the EI* complex is negligible (i.e., k_3/k_4 is much larger than unity).

According to eq 1 the numerical value of k_{obs} for any given inhibitor concentration can be obtained from a semilogarithmic plot of (percent activity remaining $- F_2$) versus incubation time. The resulting straight line has a slope of $-k_{obs}$. Values for k_{obs} obtained with a total of 13 concentrations of acetylphosphinate revealed that the rate of inhibition was saturable with a maximal value of $k_3 = 0.6$ min^{-1}. The concentration of acetylphosphinate giving a half-maximal rate of inhibition was $K_i = 0.15$ μM, and the rate constant for inactivation at low inhibitor concentration (in the absence of substrate) was 6.7×10^4 M^{-1} s^{-1}.

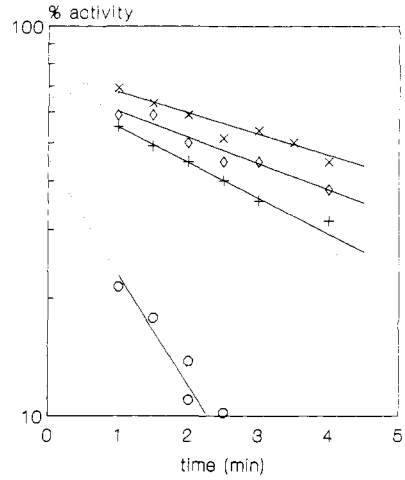
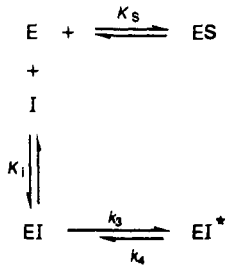


FIGURE 2: Effect of pyruvate on the inhibition of the PDC by acetylphosphinate. The PDC (2.4 μg/mL) was incubated at 30 °C in 0.97 mL of 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM $MgCl_2$, 0.2 mM TPP, 1 μM acetylphosphinate, and 0 (○), 0.1 (+), 0.2 (◇), or 0.4 mM (×) pyruvate. At intervals the enzymic reaction was started by the addition of CoA–SH (final concentration 0.13 mM), NAD^+ (final concentration 2.5 mM), and pyruvate (final concentration 2.2 mM). Initial velocities were measured.

Scheme II



The time-dependent inhibition was also studied in the presence of pyruvate. As illustrated in Figure 2, a protective effect by pyruvate in both phases of the inhibition is observed. The simplest scheme for the interpretation of this effect is one in which the inhibitor (I) is able to combine with enzyme (E) only when pyruvate is not bound, presumably because of competition for the same site (Scheme II). Because of the apparently low value of k_4/k_3, it is possible to calculate the equilibrium dissociation constant for pyruvate (K_s) by plotting the reciprocal of the observed pseudo-first-order rate constant for inhibition (k_{obs}^{-1}) versus the substrate concentration in the preincubation mixture before assay according to the equation (Aldridge & Reiner, 1972)

$$k_{obs} = \frac{k_3[I]}{K_i(1 + [S]/K_s) + [I]} \quad (8)$$

or

$$\frac{1}{k_{obs}} = \frac{K_i + [I]}{k_3[I]} \frac{K_i[S]}{k_3[I]K_s} \quad (9)$$

The ratio of the intercept to the slope [intercept/slope = $K_s(1 + [I]/K_i)$] of the resulting straight line yielded a K_s value of 20 μM, which is about 18 times lower than the K_m for pyruvate (0.35 mM). As NAD^+ was omitted from the protection experiment, turnover did not occur, and the K_s value need not be equivalent to K_m as it includes only the reversible binding

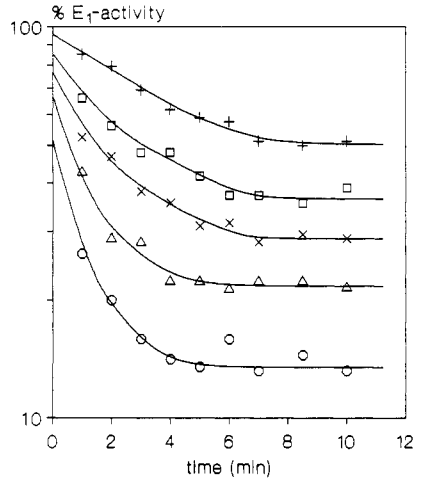


FIGURE 3: Time-dependent inhibition of the E_1 activity of the PDC by acetylphosphinate. The PDC (24 μg/mL) was incubated at 30 °C in 0.99 mL of 100 mM potassium phosphate buffer, pH 7, containing 1 mM $MgCl_2$, 0.2 mM TPP, 0.1 mM DCPIP, and 0.25 (+), 0.5 (□), 1 (×), 2 (△), or 4 μM (○) acetylphosphinate. At intervals the enzymic reaction was started by the addition of 10 μL of 0.2 M pyruvate and initial velocities were measured.

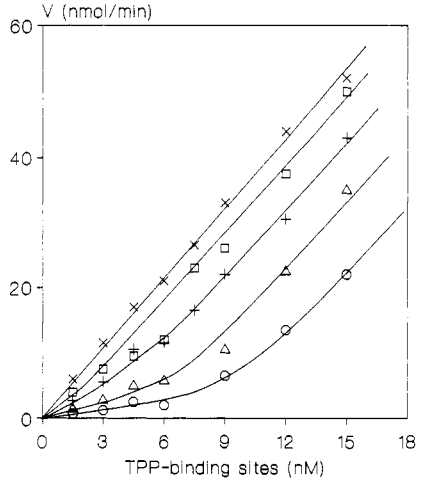


FIGURE 4: PDC activity as a function of enzyme concentration during a preincubation with increasing acetylphosphinate concentrations (Ackermann–Potter plot). Various amounts of the PDC were incubated at room temperature in 0.97 mL of 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM $MgCl_2$, 0.2 mM TPP, and 0 (×), 10 (□), 25 (+), 50 (△), or 100 nM (○) acetylphosphinate. After 50 min, the enzymic reaction was started by the addition of CoA–SH (final concentration 0.13 mM), NAD^+ (final concentration 2.5 mM), and pyruvate (final concentration 2.2 mM).

of pyruvate up to, but not including, the decarboxylation step (Reed & Willms, 1966; Hucho, 1975; Bisswanger, 1984).

Inhibition of the Pyruvate Dehydrogenase (E_1) Activity of the PDC. In order to investigate the effect of acetylphosphinate on the activity of the individual components of the PDC, the intact multienzyme complex was incubated with 2 μM acetylphosphinate in the presence of $MgCl_2$ and TPP for 5 min, and then pyruvate dehydrogenase (E_1), dihydrolipoamide transacetylase (E_2) and dihydrolipoamide dehydrogenase (E_3) activities were measured as described under Experimental Procedures. The results (data not shown) indicated that acetylphosphinate had no effect on the dihydrolipoamide transacetylase (E_2) and dihydrolipoamide dehydrogenase (E_3) activities, whereas pyruvate dehydrogenase activity decreased by 75% with respect to the uninhibited activity.

Consistent with this result, incubation of the isolated pyruvate dehydrogenase subunit (see Experimental Procedures) in the presence of 2 μM acetylphosphinate for 5 min led to 70% loss of activity, but no inhibition of the activity of the isolated E_2–E_3 subcomplex could be observed (data not shown). Moreover, upon incubation with acetylphosphinate, the pyruvate dehydrogenase (E_1) of the intact PDC displays a time-dependent decrease in activity similar to the time-dependent inhibition of the overall activity (Figure 3).

Graphical estimation of the apparent first-order rate constants (k_{obs}) was performed as described above, and a replot of k_{obs}^{-1} versus the reciprocal of the corresponding inhibitor concentration gave $k_3 = 0.4$ min^{-1} and $K_i = 0.21$ μM. The pyruvate dehydrogenase component of the PDC may therefore be considered the target of the inhibition of the PDC by acetylphosphinate.

Tight-Binding Behavior of Acetylphosphinate. An experiment was carried out in which the velocity obtained after a 50-min preincubation in the presence of acetylphosphinate was studied as a function of the total TPP binding site concentration. The concentration of TPP binding sites was calculated by assuming a M_r of the PDC of 4.6×10^6 (Eley et al., 1972) and 24 TPP binding sites per complex (Moe & Hammes, 1974). Concentrations of inhibitor used were such that equilibrium between enzyme, inhibitor, and enzyme–inhibitor complexes was achieved within the incubation time. Under these conditions, the velocity obtained shortly after addition of substrate was equal to the steady-state velocity, since the already established equilibrium between enzyme, inhibitor, and enzyme–inhibitor complexes is not expected to be shifted significantly because of substrate binding.

Plots of velocity against enzyme concentration at different inhibitor concentrations have proven to be a useful method for characterizing tight-binding inhibition (Ackermann & Potter, 1949; Morrison, 1969; Cha, 1975; Agarwal et al., 1977; Williams & Morrison, 1979). As shown in Figure 4, acetylphosphinate appears to act as a "stoichiometric inhibitor"; i.e., the activity increases linearly with increasing TPP binding site concentration only after the enzyme concentration exceeds that of the inhibitor. As pointed out by Williams and Morrison (1979), the asymptotes of curves such as the ones presented in Figure 4 will intersect the abscissa at $E_t = I_t$. Thus, a plot of this type allows the determination of the ratio of bound inhibitor to free inhibitor, which should be unity in the case of tight-binding and slowly reversible inhibition (provided that inhibition takes place with every catalytic cycle) but was found to be 1:10 in the case of PDC inactivation by acetylphosphinate.

Since the data of Figure 4 were obtained under equilibrium conditions, it is possible to determine the overall dissociation constant (K_i^*), in which all the rate constants of Scheme I are expressed as

$$K_i^* = K_i \frac{k_4}{k_3 + k_4} = \frac{[E][I]}{[EI] + [EI^*]} \qquad (10)$$

The graphical procedure for the determination of K_i^* as described by Cha (1975) gave a value of 10 nM for K_i^* compared to a value of 3.5 nM obtained under conditions where the concentration of substrate (pyruvate) in the final assay mixture was varied (Henderson, 1972) (data not shown).

Reversibility of PDC Inhibition by Acetylphosphinate. The reversibility of binding of acetylphosphinate to the PDC was tested by extensive dialysis of the inhibited complex as well as by successive gel filtration and dilution. The half-time of recovery of enzyme activity in the dialysis experiment was determined to be 18 min. Since the dissociation of the PDC–acetylphosphinate (EI*) complex is rate determining for the recovery of PDC activity, the half-time of recovery of enzymic

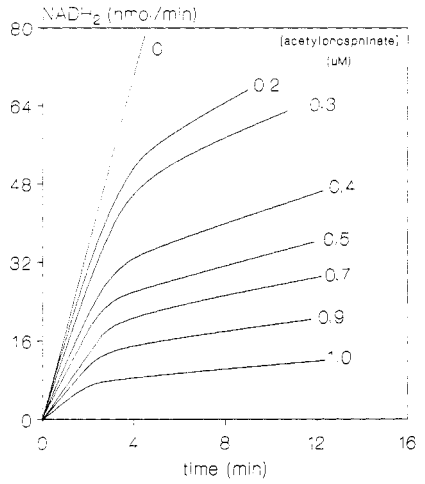


FIGURE 5: Assay progress curves in the presence of acetylphosphinate. Assays were initiated by the addition of 0.6 μg of PDC to 0.99 mL of 50 mM potassium phosphate buffer, pH 7.4, containing all cofactors, 2 mM pyruvate, and increasing concentrations of acetylphosphinate.

activity (0.04 min^{-1}) equals the rate constant k_4 of Scheme I. The possible contribution of diffusion-controlled rates during dialysis was neglected in this calculation.

Analysis of Progress Curves. When the overall PDC activity was continuously assayed after addition of enzyme to assay mixtures containing both acetylphosphinate and pyruvate, the resulting progress curves displayed a time-dependent decrease in the reaction rate, which varied as a function of the inhibitor concentration (Figure 5). By contrast, when an aliquot of a preincubation mixture of acetylphosphinate and PDC was added to the assay mixture such that enzyme and inhibitor were diluted 10-fold, a lag phase prior to the final steady-state velocity was observed (data not shown). Since the slow onset of inhibition is clearly not due to irreversible enzyme inactivation and since the experimental conditions chosen exclude the possibility of inhibitor or substrate depletion while the final steady-state rate was achieved, the curves of Figure 5 reflect the slow establishment of the equilibrium between enzyme, inhibitor, and enzyme–inhibitor complexes. The analysis of a total of 30 assay progress curves (1.5, 3, and 4.5 nM TPP-binding site concentration) by the equations of Cha (1975, 1976) and Morrison and Walsh (1987) gave values of k_3 = 1.3 min^{-1}, k_4 = 0.036 min^{-1}, and K_i = 0.12 μM (data not shown).

Acetylphosphinate-Induced Fluorescence Quenching. The fluorescence emission spectrum of the PDC in potassium phosphate buffer, when excited at 290 nm, reveals a maximum at 333 nm (Figure 6, curve A), which is characteristic of the indole moiety of tryptophan [for comparison, Horn and Bisswanger (1983) report a maximum at 330 nm]. Addition of 1 mM $MgCl_2$ to the enzyme complex has negligible effects on the emission spectrum (quenching = approximately 3%), but addition of 0.2 mM TPP to the PDC/$MgCl_2$ mixture reduces the fluorescence intensity at 333 nm by approximately 30% (Figure 6, curve B).

Upon addition of 0.2 mM acetylphosphinate to a mixture of PDC, TPP, and $MgCl_2$ the maximum of curve B in Figure 6 decreases by a factor of $^1/_3$ in a time-dependent manner (Figure 6 insert). By contrast, addition of 2 mM pyruvate to the PDC/TPP/$MgCl_2$ mixture reduces the maximum of curve B by approximately 6% but no time-dependent loss of fluorescence intensity was observed.

The curve in the insert of Figure 6 can be described by the single exponential equation

$$y_t = y_f + Ae^{-k_{obs}t} \quad (11)$$

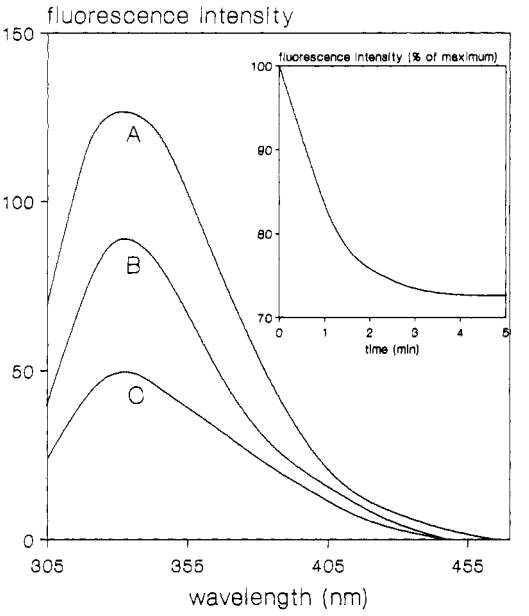


FIGURE 6: Effect of acetylphosphinate on the fluorescence emission spectrum of the PDC. Fluorescence emission spectra of 300 μg of PDC in 3 mL of 50 mM potassium phosphate buffer, pH 7.4 (curve A), after addition of $MgCl_2$ (final concentration 1 mM) and TPP (final concentration 0.2 mM) (curve B) and 10 min after addition of acetylphosphinate (final concentration 0.2 mM) to the PDC/$MgCl_2$/TPP mixture (curve C). Excitation was at 290 nm. The insert shows the time course of the acetylphosphinate-induced fluorescence quenching. The reaction mixture contained 300 μg of PDC in 3 mL of 50 mM potassium phosphate buffer, pH 7.4, 0.2 mM TPP, 1 mM $MgCl_2$ and 200 μM acetylphosphinate. Excitation was at 290 nm, and tryptophan fluorescence was recorded at 333 nm.

where y_t is the signal at time t, y_f is the final value of fluorescence, A is an amplitude parameter, and k_{obs} is the apparent first-order rate constant for fluorescence decrease. According to this equation, the numerical value for k_{obs} can be determined from a semilogarithmic plot of $(y_t - y_f)$ versus time and was calculated to be 0.99 min^{-1}.

An increase in the concentration of either acetylphosphinate, TPP, or $MgCl_2$ did not affect the rate of fluorescence quenching. Thus, the estimated k_{obs} value equals the maximum rate constant for conversion of the transient EI complex into the slowly reversible EI* complex (k_3 in Scheme I). The acetylphosphinate-induced time-dependent decrease of the tryptophan fluorescence is dependent on the presence of both TPP and Mg^{2+}.

Discussion

Several phosphonate and phosphinate analogues of pyruvate have been designed as potential mechanism-based inhibitors of pyruvate dehydrogenase (Kluger & Pike, 1977; O'Brien et al., 1980; Baillie et al., 1988). While methyl acetylphosphonate was found to be a competitive inhibitor with a K_i of 50 nM, acetylphosphinate and acetyl methylphosphinate were both reported to inhibit pyruvate dehydrogenase in a time-dependent and essentially irreversible manner (Laber & Amrhein, 1987; Baillie et al., 1988).

The data presented here clearly demonstrate that inactivation of the pyruvate dehydrogenase complex of *E. coli* by acetylphosphinate represents slow-binding inhibition, a type of inhibition that is currently receiving considerable attention (Morrison & Walsh, 1987; Schloss, 1988). Scheme I represents the most probable sequence of the interaction between pyruvate dehydrogenase and acetylphosphinate. With an overall dissociation constant of K_i^* = 5 nM, acetylphosphinate is one of the most potent inhibitors of the pyruvate de-

hydrogenase complex hitherto described. The low K_i* value as well as the slow onset of inhibition is characteristic of reaction intermediate analogues (Westerik & Wolfenden, 1972, 1974; Wolfenden, 1972; Lienhard, 1973; Schloss, 1988). Moreover, the acetylphosphinate-induced time-dependent fluorescence quenching (Figure 6) indicates a conformational change in the enzyme as the slow step in the conversion of EI to EI* (k_3 in Scheme I). Since both the inhibition of the enzymatic reaction and the fluorescence quenching strictly depend on the presence of TPP and Mg^{2+}, we propose that pyruvate dehydrogenase catalyzes the formation of an acetylphosphinate–TPP adduct, a close analogue of α-lactyl-TPP [2-(1-carboxy-1-hydroxyethyl)-TPP], which is the first intermediate of the normal catalytic process [see Kluger (1987) for review]. In consequence, subsequent interactions mimic those associated with the formation of the transition state but are slow because not all of the structural features required for this process are provided by the analogue of the intermediate. The reverse isomerization reaction, however, is even slower because dephosphinylation (i.e., cleavage of the C–P bond) cannot take place, with the result of a "dead-end complex" between enzyme and analogue. A similar mechanism has been proposed for the inhibition of the pyruvate dehydrogenase complex by methyl acetylphosphonate (Kluger & Pike, 1977). Chemical synthesis of the postulated acetylphosphinate–TPP adduct is clearly required to test this hypothesis.

ACKNOWLEDGMENTS

We thank Dr. L. Maier, Ciba-Geigy AG, Basel, for the sample of acetylphosphinate and Dr. L. Packman, University of Cambridge, for the strain of *E. coli*. We greatly appreciate suggestions for the improvement of the manuscript by Dr. J. V. Schloss, Du Pont, Wilmington, DE, as well as by one of the reviewers.

REFERENCES

Ackerman, W. W., & Potter, V. R. (1949) *Proc. Soc. Exp. Biol. Med. 72*, 1–9.

Agarwal, R. P, Spector, T., & Parks, R. E., Jr. (1977) *Biochem. Pharmacol. 26*, 359–367.

Aldridge, W. N., & Reiner, E. (1972) in *Frontiers of Biology* (Neuberger, A., & Tatum, E. L., Eds.) Vol. 26, North Holland Publishing Company, Amsterdam.

Angelides, K. J., Akiyama, S. K., & Hammes, G. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3279–3283.

Apfel, M. A., Ikeda, B. H., Speckhard, D. C., & Frey, P. A. (1984) *J. Biol. Chem. 259*, 2905–2909.

Baillie, A. C., Wright, B. J., & Wright, K. (1980) Eur. Pat. Appl. 9348.

Baillie, A. C., Wright, K., Wright, B. J., & Earnshaw, C. G. (1988) *Pestic. Biochem. Physiol. 30*, 103–112.

Bisswanger, H. (1980) *Biochem. Biophys. Res. Commun. 95*, 513–519.

Bisswanger, H. (1981) *J. Biol. Chem. 256*, 815–822.

Bisswanger, H. (1984) *J. Biol. Chem. 259*, 2457–2465.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Brown, J. P., & Perham, R. N. (1976) *Biochem. J. 155*, 419–427.

Buttler, J. R., Pettit, F. H., Davis, P. F., & Reed, L. J. (1977) *Biochem. Biophys. Res. Commun. 74*, 1667–1674.

Cha, S. (1975) *Biochem. Pharmacol. 24*, 2177–2185.

Cha, S. (1976) *Biochem. Pharmacol. 25*, 2695–2707.

Coggins, J. R., Hooper, E. A., & Perham, R. N. (1976) *Biochemistry 15*, 2527–2533.

Danson, M. J., Hooper, E. A., & Perham, R. N. (1978) *Biochem. J. 175*, 193–198.

Danson, M. J., Hale, G., Johnson, P., & Perham, R. N. (1979) *J. Mol. Biol. 129*, 603–617.

Eley, M. H., Namihara, G., Hamilton, L., Mank, P., & Reed, L. J. (1972) *Arch. Biochem. Biophys. 152*, 655–669.

Fleck, G. M. (1971) *Chemical Reaction Mechanisms*, pp 158–181, Holt, Rinehart & Winston, New York.

Gathercole, P. S., Thiel, P. G., & Hofmeyr, J. H. S. (1986) *Biochem. J. 233*, 719–723.

Graupe, K., Abusaud, M., Karfunkel, H., & Bisswanger, H. (1982) *Biochemistry 21*, 1386–1394.

Gutowski, J. A., & Lienhard, G. E. (1976) *J. Biol. Chem. 251*, 2863–2866.

Henderson, P. J. F. (1972) *Biochem. J. 127*, 321–333.

Horn, F., & Bisswanger, H. (1983) *J. Biol. Chem. 258*, 6912–6919.

Hucho, F. (1975) *Angew. Chem., Int. Ed. Engl. 14*, 591–601.

Kluger, R. (1987) *Chem. Rev. 87*, 863–876.

Kluger, R., & Pike, D. C. (1977) *J. Am. Chem. Soc. 99*, 4504–4506.

Kluger, R., Gish, G., & Kauffmann, G. (1984) *J. Biol. Chem. 259*, 8960–8965.

Koike, M., Reed, L. J., & Carroll, W. R. (1963) *J. Biol. Chem. 238*, 30–39.

Laber, B., & Amrhein, N. (1987) *Biochem. J. 248*, 351–358.

Lienhard, G. E. (1973) *Science 180*, 149–154.

Lowe, P. N., & Perham, R. N. (1984) *Biochemistry 23*, 91–97.

Lowe, P. N., Leeper, F. J., & Perham, R. N. (1983) *Biochemistry 22*, 150–157.

Maldonado, M. E., Oh, K. J., & Frey, P. A. (1972) *J. Biol. Chem. 247*, 2711–2716.

Moe, O. A., Jr., & Hammes, G. G. (1974) *Biochemistry 13*, 2547–2551.

Morrison, J. F. (1969) *Biochim. Biophys. Acta 185*, 269–286.

Morrison, J. F. (1982) *Trends Biochem. Sci. 7*, 102–105.

Morrison, J. F., & Stone, S. R. (1985) *Comments Mol. Cell. Biophys. 2*, 347–368.

Morrison, J. F., & Walsh, C. T. (1987) *Adv. Enzymol. Relat. Areas Mol. Biol. 61*, 201–301.

O'Brien, T. A., Kluger, R., Pike, D. C., & Gennis, R. B. (1980) *Biochim. Biophys. Acta 613*, 10–17.

Packman, L. L., Perham, R. N., & Roberts, G. C. K. (1982) *Biochem. J. 205*, 389–396.

Reed, L. J., & Willms, C. R. (1966) *Methods Enzymol. 9*, 247–265.

Reed, L. J., & Mukherjee, B. B. (1969) *Methods Enzymol. 12*, 55–61.

Reed, L. J., Koike, M., Levitch, M. E., & Leach, F. R. (1958) *J. Biol. Chem. 232*, 143–158.

Schloss, J. V. (1988) *Acc. Chem. Res. 21*, 348–353.

Schloss, J. V. (1989) in *Target Sites of Herbicide Action* (Böger, P., & Sandmann, G., Eds.) CRC Press, Inc., Boca Raton, FL (in press).

Schwartz, E. R., & Reed, L. J. (1969) *J. Biol. Chem. 244*, 6074–6079.

Speckhard, D. C., & Frey, P. A. (1975) *Biochem. Biophys. Res. Commun. 62*, 614–620.

Westerik, J. O'C., & Wolfenden, R. (1972) *J. Biol. Chem. 247*, 8195–8197.

Westerik, J. O'C., & Wolfenden, R. (1974) *J. Biol. Chem. 249*, 6351–6353.

Williams, J. W., & Morrison, J. F. (1979) *Methods Enzymol. 63*, 437–467.

Wolfenden, R. (1972) *Acc. Chem. Res. 5*, 10–18.

Yang, H., Hainfield, J. F., Wall, J. S., & Frey, P. A. (1985) *J. Biol. Chem. 260*, 16049–16051.