

# Acetylphosphinate is the most potent mechanism-based substrate-like inhibitor of both the human and *Escherichia coli* pyruvate dehydrogenase components of the pyruvate dehydrogenase complex

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Frank Jordan dedicates this paper to the memory of a valued collaborator and friend Miriam Hasson.

## Abstract

Two analogues of pyruvate, acetylphosphinate and acetylmethylphosphinate were tested as inhibitors of the E1 (pyruvate dehydrogenase) component of the human and *Escherichia coli* pyruvate dehydrogenase complexes. This is the first instance of such studies on the human enzyme. The acetylphosphinate is a stronger inhibitor of both enzymes ( $K_i < 1 \mu\text{M}$ ) than acetylmethylphosphinate. Both inhibitors are found to be reversible tight-binding inhibitors. With both inhibitors and with both enzymes, the inhibition apparently takes place by formation of a C2 $\alpha$ -phosphinolactylthiamin diphosphate derivative, a covalent adduct of the inhibitor and the coenzyme, mimicking the behavior of substrate and forming a stable analogue of the C2 $\alpha$ -lactylthiamin diphosphate. Formation of the intermediate analogue in each case is confirmed by the appearance of a positive circular dichroism band in the 305–306 nm range, attributed to the 1',4'-iminopyrimidine tautomeric form of the coenzyme. It is further shown that the  $\alpha\text{His63}$  residue of the human E1 has a role in the formation of

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C2 $\alpha$ -lactylthiamin diphosphate since the  $\alpha$ His63Ala variant is only modestly inhibited by either inhibitor, nor did either compound generate the circular dichroism bands assigned to different tautomeric forms of the 4'-aminopyrimidine ring of the coenzyme seen with the wild-type enzyme. Interestingly, opposite enantiomers of the carboligase side product acetoin are produced by the human and bacterial enzymes.

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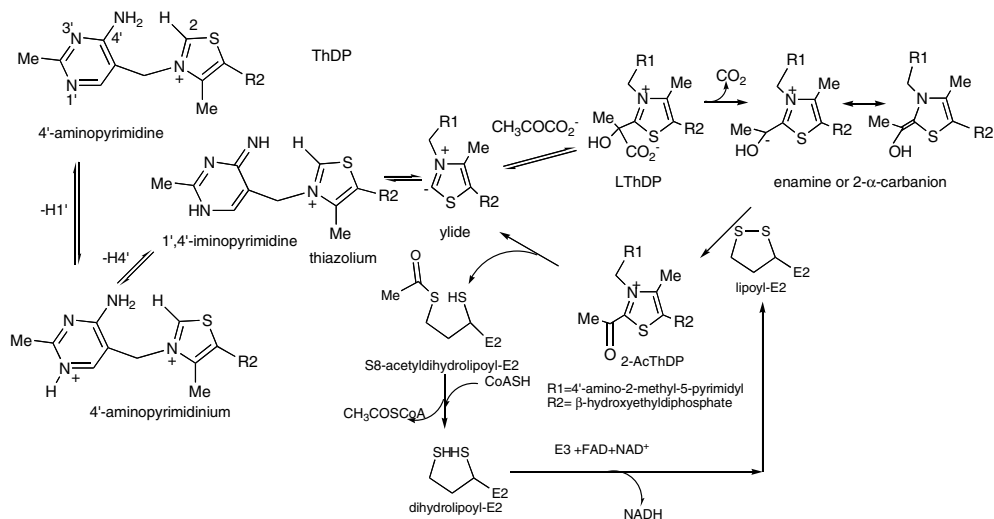
**Keywords:** Human pyruvate dehydrogenase complex; *Escherichia coli* pyruvate dehydrogenase complex; Acetylphosphinate; Acetylmethylphosphinate; Thiamin diphosphate; Thiamin 2-thiothiazolone diphosphate; Acetylphosphonate methyl ester; Circular dichroism; Mechanism-based inhibition; Acetoin

## 1. Introduction

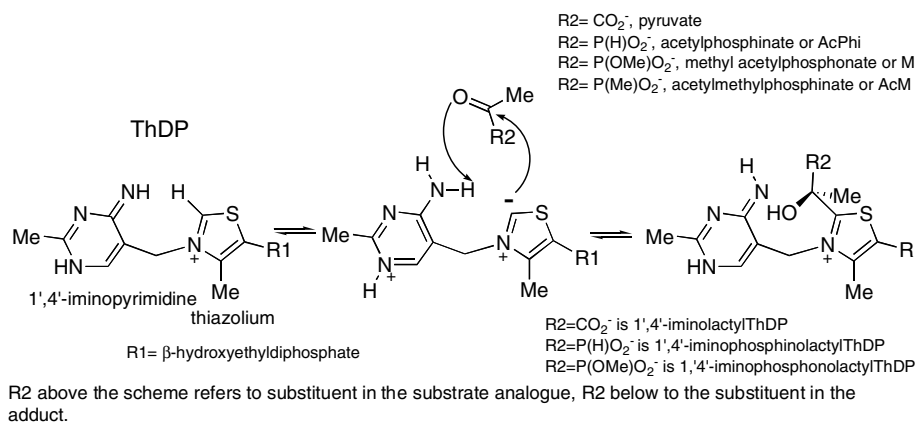
During the past few years, the Rutgers group has shown that the pyruvate analogue methyl acetylphosphonate sodium salt (MACPho), when added to the thiamin diphosphate (ThDP)-dependent enzymes yeast pyruvate decarboxylase (YPDC) and the E1 subunit (Elec) of the *Escherichia coli* pyruvate dehydrogenase complex (PDHc-ec)<sup>2</sup> generates a new UV–vis and a positive circular dichroism (CD) band in the wavelength range of 300–310 nm [1,2]. The same spectrum also results when the pre-assembled C2 $\alpha$ -phosphonolactylthiamin diphosphate (PLThDP, synthesized from thiamin diphosphate and MACPho) is added to the apo form of Elec (no ThDP-bound) [1]. This electronic transition was assigned to the 1',4'-iminopyrimidine tautomer of the ThDP coenzyme in the bound form on the basis of chemical model studies [3–5]. Concurrently, high-resolution X-ray structure determinations on Elec with the pre-assembled PLThDP [6], and on pyruvate oxidase from *Lactobacillus plantarum* with MACPho added [7] showed that a tetrahedral adduct was being formed between the keto carbon of the MACPho and the C2 thiazolium atom. In view of these complementary observations, we concluded that the PLThDP when enzyme-bound exists in the 1',4'-iminopyrimidine tautomeric form. The reaction scheme for PDHc is outlined in Scheme 1. Hence, MACPho can indeed act as a substrate analogue as shown in Scheme 2, but, once the reaction reaches the intermediate state corresponding to LThDP, by virtue of the non-cleavable C–P bond in place of the usual C–C bond, the adduct simply occupies the active center with very long lifetime. The UV and CD results inform us that a consequence of the presence of this stable pre-decarboxylation analogue at the active site is that with the PLThDP in place of ThDP, the 1',4'-iminopyrimidine tautomer of ThDP is the predominant tautomeric form rather than one of the other two forms on the left-hand side of Scheme 2.

These results encouraged us to synthesize and test two further electrostatic mimics of pyruvate, sodium acetyl phosphinate (AcPhi) and sodium acetyl methylphosphinate (AcMPhi). These compounds possess the advantage over the phosphonate compound used previously in having no ester group present. In this fashion, the  $-\text{PO}_2^-$  group

<sup>2</sup> Abbreviations used: PDHc, pyruvate dehydrogenase complex; PDHc-h, human pyruvate dehydrogenase complex; PDHc-ec; *Escherichia coli* pyruvate dehydrogenase complex; E1h, E2h, E3h refer to the components of the human enzyme; E3BPh, E3h-binding protein formerly known as protein X; Elec, E2ec, E3ec refer to the subunits of the *Escherichia coli* enzyme; ThDP, thiamin diphosphate; MACPho, sodium salt of acetylphosphonic acid methyl ester; AcPhi, sodium salt of acetylphosphinic acid; AcMPhi, sodium salt of acetylmethylphosphinic acid; LThDP, C2 $\alpha$ -lactylThDP; PLThDP, C2 $\alpha$ -phosphonolactylThDP; ThTTDP, thiamin 2-thiathiazolone diphosphate.



Scheme 1. Reactions of pyruvate dehydrogenase complexes.



Scheme 2. Formation of LThDP and its phosphonate and phosphinate analogues.

becomes a better steric and electrostatic mimic of pyruvate's  $\text{CO}_2^-$  group. Similarly to MacPho, when bound to ThDP, AcMPhi and AcPhi can not be decarboxylated and form stable analogues of LThDP (Scheme 2). We here report on the kinetics of binding and inactivation of Elec (homodimer) and the human pyruvate dehydrogenase E1 ( $\alpha_2\beta_2$  heterotetramer) (E1h) by AcPhi and AcMPhi. Both substrate analogues behave as tight-binding reversible inhibitors with micromolar values of  $K_d$  and  $K_i$ . Inactivation of Elec and E1h resulted on addition of stoichiometric amounts of substrate analogues. The formation of stable analogues of LThDP could be monitored by CD spectroscopy providing strong evidence regarding the mechanism of inhibition. We wish to compare and contrast the inhibition experienced by the human and bacterial PDHc E1.

## 2. Materials and methods

### 2.1. Methods

#### 2.1.1. Chemical synthesis

*Synthesis of acetyl phosphinate* was carried out according to the reported procedure by Baillie et al. [8].

Methyl phosphonousdichloride was purchased from Aldrich and was used without further purification. NaI was dried at 70 °C under 0.1 mm/Hg overnight. Dry acetone was prepared with dry NaI according to literature procedures. NMR spectra were recorded in a 500 MHz Varian INOVA spectrometer NMR instrument.

*Synthesis of methyl (1,1-dimethoxyethyl)methylphosphinate.* Methyl phosphonousdichloride (5.0 g, 42.7 mmol) was added dropwise, with vigorous stirring to trimethyl orthoacetate (11.67 g, 97.2 mmol) at –30 °C under N<sub>2</sub>. After the addition was complete the temperature of the reaction mixture was maintained at –30 °C for one h, then the reaction mixture was allowed to warm to room temperature overnight. The excess trimethyl orthoacetate was removed under reduced pressure. The pure product (bp 103 °C, 3.2 mm/Hg) was obtained by fractional distillation of the residual reaction mixture. Yield 6.337 g (82%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>/TMS): δ 3.801 (d, 3H, *J* = 10.5 Hz), 3.405 (d, 6H, *J* = 8 Hz), 1.490 (d, 3H, *J* = 3.5 Hz), 1.465 (d, 3H, *J* = 1 Hz). MS (EI) 151, 109, 89, 77, 43.

*Synthesis of sodium acetylmethylphosphinate.* Methyl (1,1-dimethoxyethyl)methylphosphinate (500 mg, 2.75 mmol) was added to a solution of NaI (8.25 g, 55 mmol) in 30 mL dry acetone under N<sub>2</sub> in a sealed tube. The reaction mixture was heated at 70 °C overnight. After cooling to room temperature, the reaction mixture was evaporated to dryness and triturated with ethyl acetate. The crude product was recrystallized from absolute ethanol. Yield 134 mg (37.5%). <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O/DSS): δ 2.461 (d, 3H, *J* = 3.5 Hz), 1.406 (d, 3H, *J* = 14 Hz). MS (ESI) 153 (M+Na)<sup>+</sup>.

#### 2.1.2. Protein expression and purification

*Bacterial strains and plasmids.* *Escherichia coli* strain JRG 3456 and plasmid pGS878 with *aceE1* gene encoding the E1 $\alpha$  subunit of PDHc-ec were from J. Guest (University of Sheffield, UK). A co-expression vector for E1h (pET-28b-PDHA1/PDHB) harboring coding sequences of both human PDHc-E1 $\alpha$  and PDHc-E1 $\beta$  subunits was constructed as described previously [9].

The *E. coli* strain JRG 3456 transformed with pGS878 was used for overexpression of the *aceE* gene encoding E1 $\alpha$  subunit of PDHc-ec according to reference [10] and purification of E1 $\alpha$  followed the published procedure [11].

The E1h was overexpressed in *E. coli* BL21 cells harboring pET-28b-PDHA1/PDHB (a co-expression vector with coding sequences of both human E1 $\alpha$  and E1 $\beta$  subunits) and purified using Ni-NTA-agarose chromatography as described previously [9].

Recombinant human dihydrolipoamide acetyltransferase (E2h)-dihydrolipoamide dehydrogenase-binding protein (E3BPh) subcomplex and dihydrolipoamide dehydrogenase (E3h) were overexpressed in *E. coli* and purified as described elsewhere [12].

## 2.2. Activity measurements of E1ec

The E1ec activity was measured in the overall PDHc reaction. First E2ec and E3ec expressed independently were incubated for 1 h at a ratio of 1.0:1.0 (mg/mg) in 20 mM potassium phosphate buffer (pH 7.0) at 4 °C. The E1ec was reconstituted with E2ec–E3ec subcomplex at a ratio of 1:1:1 (mg/mg/mg) with an additional 10 min of incubation. The reaction medium contained (in 1 ml of test volume) 0.10 M Tris–HCl (pH 8.0), 1 mM MgCl<sub>2</sub>, 2 mM sodium pyruvate, 2.5 mM NAD<sup>+</sup>, 0.20 mM ThDP, and 2.6 mM dithiothreitol at 30 °C. The reaction was initiated by the addition of 0.20 mM CoA and PDHc-ec (1–1.5 µg of protein). Steady-state velocities were taken from the linear portion of the progress curve. One unit of activity is defined as the amount of NADH produced (µmol/min/mg of E1ec).

*Activity measurements of E1h.* Activity of E1h was determined by two assays: (i) PDHc assay, by the formation of NADH during overall PDHc reaction after reconstitution of E1h with E2h–E3BPh subcomplex and E3h into PDHc; and (ii) by DCPIP assay, by the reduction of 2,6-dichlorophenolindophenol (DCPIP), an artificial electron acceptor, to measure the first partial reaction of E1h, the decarboxylation of pyruvate in the absence of the second substrate, lipoyl moieties of E2h, as described previously [12].

Protein content was measured by the Bradford method using bovine serum albumin as a standard [13].

## 2.3. Kinetic analysis of inhibition of E1h by AcPhi and AcMPhi

To study the inhibition of E1h by AcPhi and AcMPhi, E1h (5 µg) was incubated at room temperature in 50 µl of 50 mM potassium phosphate buffer (pH 7.0) with 2 mM MgCl<sub>2</sub>, 0.2 mM ThDP and the concentration of substrate analogues indicated. Three–four aliquots (1 µg of E1h each) were withdrawn after 30 min and mixed into a cuvette containing 3 µg of E2h–E3BPh subcomplex, 3 µg of E3h and all reagents necessary to measure PDHc activity except pyruvate. The entire complex was reconstituted for 1 min and the reaction was started by addition of pyruvate and was monitored for 30 s to record the initial velocity. To determine the inactivation of E1h and its αH63A variant by substrate analogues, 200 µg of E1h or E1h αH63A was incubated at room temperature in 200 µl of 50 mM potassium phosphate buffer (pH 7.0) with 2 mM MgCl<sub>2</sub>, 0.2 mM ThDP, and the indicated concentrations of substrate analogues. Three aliquots (25 µg for wild-type E1h and 50 µg for E1h αH63A) were taken after 30 min to measure the activity of the E1h *per se* according to the DCPIP assay [12].

## 2.4. Reaction progress curve analysis

*E1ec.* The progress of the overall PDHc reaction was monitored via the formation of NADH at 340 nm as a function of time (the reaction time was approximately 18 min). The reaction was started by the addition of PDHc after the E1ec was reconstituted with E2–E3 subcomplex (total protein about 0.60 µg) and CoA. A family of progress curves was recorded in the presence of 0.50 mM pyruvate and AcMPhi (0.1–4.5 µM) or AcPhi (0.05–1.2 µM) and the values of  $k_{app}$  were calculated as reported earlier [10]. The values

of  $K_d$  were calculated from the plot of  $k_{app}$  vs. concentration of substrate analogue according to Kitz and Wilson [14].

*E1h*. Progress curve analysis was used to determine the  $K_i$  for inhibition of wild-type *E1h* by *AcPhi* and *AcMPhi* [10,15]. The PDHc was reconstituted in the cuvette by mixing 0.3  $\mu$ g of *E1h*, 1  $\mu$ g of *E2h*-*E3BP* and 1  $\mu$ g of *E3h*. The reaction was initiated by addition of pyruvate (300  $\mu$ M) along with different concentrations of *AcPhi* (0.4–1.8  $\mu$ M) or *AcMPhi* (200–600  $\mu$ M) as indicated in the figure legends. The progress of the reaction was monitored for 5 min. Less than 5% of pyruvate was consumed under these conditions. The progress curves were analyzed according to Eq. (1):

$$P = v_s t + (v_o - v_s)/k(1 - e^{-kt}) \quad (1)$$

where  $P$  is the product concentration at time  $t$ ,  $v_s$  is the steady-state reaction velocity,  $v_o$  is the initial velocity and  $k$  is the apparent pseudo-first-order rate constant. The dependence of  $k_{app}$  on substrate analogue concentration was linear for both compounds, enabling calculation of  $k_1$  and  $k_{-1}$ , the rate constants for binding and dissociation of substrate analogue, respectively, were calculated from Eq. (2):

$$k_{app} = k_1 I_0 / (1 + S_0 / K_m) + k_{-1} \quad (2)$$

where  $I_0$  is the concentration of substrate analogue,  $S_0$  is the concentration of pyruvate used and  $K_m$  is the value of  $K_m$  for pyruvate. The values of  $K_i$  were calculated from:  $K_i = k_{-1}/k_1$ . Regression coefficients were  $>0.95$ .

## 2.5. Circular dichroism spectroscopy

*Circular dichroism titration of E1ec with sodium acetylphosphinate and sodium acetylmethylphosphinate*. The *E1ec* (1.50 mg/ml, concentration of active centers = 15.0  $\mu$ M) in 20 mM potassium phosphate buffer (pH 7.0) containing 2 mM  $MgCl_2$  and 0.20 mM ThDP was titrated with 1–100  $\mu$ M of *AcPhi*. For experiments with *AcMPhi*, *E1ec* (1.63 mg/ml, concentration of active centers = 16.2  $\mu$ M) in 20 mM potassium phosphate buffer (pH 7.0) containing 0.20 mM ThDP and 2.0 mM  $MgCl_2$  was titrated with 1–150  $\mu$ M of *AcMPhi*. The difference CD spectra with both substrate analogues were obtained on subtraction of the CD spectrum of *E1ec* in the presence of ThDP and  $MgCl_2$ . The  $K_d$  for *AcPhi* or *AcMPhi* were calculated from a plot of ellipticity at 317 nm vs. concentration of *AcPhi* or *AcMPhi* using a quadratic equation [10]. CD spectra were recorded on an Aviv Model 202 CD or an Applied Photophysics Chirascan spectrometer at 30 °C.

*Circular dichroism titration of E1h with sodium acetylphosphinate and sodium acetylmethylphosphinate*. The *E1h* (1.50 mg/ml, concentration of active centers = 19.6  $\mu$ M) in 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM  $MgCl_2$  and 0.20 mM ThDP was titrated with 1–150  $\mu$ M of *AcPhi*. For experiments with *AcMPhi*, *E1h* (1.06 mg/ml, concentration of active centers = 13.8  $\mu$ M) in 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM  $MgCl_2$  and 0.20 mM ThDP was titrated with 1–150  $\mu$ M of *AcMPhi*. A plot of ellipticity at 305 nm vs.  $[AcMPhi]$  was constructed by subtracting the ellipticity of the enzyme at the same wavelength but recorded in the absence of *AcMPhi*. The values of  $K_d$  were calculated using a quadratic equation [10].

### 3. Results

#### 3.1. Kinetic analysis of the inhibition of E1ec by sodium acetyl phosphinate

As reported previously, the AcPhi behaves as a tight-binding reversible inhibitor of the PDHc-ec ( $K_i = 0.15 \mu\text{M}$ ) and of the E1ec resolved from the complex ( $K_i = 0.21 \mu\text{M}$ ) [16]. We studied inhibition of the recombinant E1ec (concentration of active centers =  $0.50 \mu\text{M}$ ) by preincubating it with  $0.50$ – $1000 \mu\text{M}$  AcPhi for 20 min at  $25^\circ\text{C}$  and then measuring the activity after reconstitution with its E2ec–E3ec subcomplex. The overall PDHc reaction was triggered by adding CoA and PDHc to the reaction mixture. More than 90% of E1ec activity was lost at a  $[\text{AcPhi}]/[\text{E1ec}]$  ratio of  $1.4/1.0$  (data not shown). These kinetic data are in good agreement with a CD titration experiment. On addition of AcPhi to the E1ec, a broad CD band with maximum at  $317 \text{ nm}$  was observed (Fig. 1A). We assigned this band to the  $1',4'$ -iminopyrimidine tautomer of phosphinolactyl-ThDP

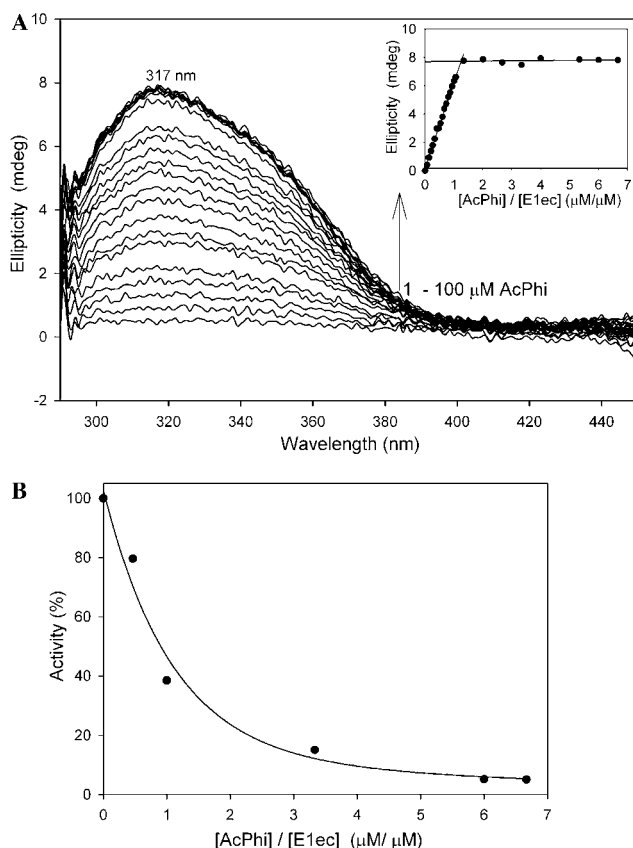


Fig. 1. CD titration of E1ec by AcPhi: (A) difference spectra of E1ec on addition of AcPhi. Inset, dependence of the ellipticity at  $317 \text{ nm}$  on the  $[\text{AcPhi}]/[\text{E1ec}]$  active centers in homodimer] ratio. (B) E1ec activity remaining with increasing  $[\text{AcPhi}]/[\text{E1ec}]$  ratio. Conditions under Section 2.

(iminophosphinolactylThDP), a stable analogue of LThDP, on the basis of similar results obtained with MAcPho (Fig. 2) and PLThDP [1]. The CD band at 317 nm reached saturation at a [AcPhi]/[Elec] ratio of 1.3/1.0 which correlates with the loss of 85% of Elec activity measured in a parallel experiment (Fig. 1B). A  $K_d = 0.062 \mu\text{M}$  was calculated for AcPhi. Inhibition by AcPhi was reversible according to the following results: (i) approximately 50% of activity was recovered after overnight dialysis against 20 mM potassium phosphate buffer (pH 7.0) from a sample that had first been inhibited to 95%; (ii) the amplitude of the CD band at 317 nm was reduced by 80% after overnight dialysis (20% of the CD signal was retained on Elec). These results suggest that AcPhi behaves as a stoichiometric reversible inhibitor of the Elec. The binding of AcPhi leads to the formation of 1',4'-iminophosphinolactyl-ThDP, a covalent analogue of the LThDP intermediate clearly indicating mechanism-based inhibition.

### 3.2. Circular dichroism analysis of binding of sodium acetylmethylphosphinate to Elec

Addition of AcMPhi to the Elec also resulted in a CD band with maximum at 317 nm suggesting formation of 1',4'-imino,methylphosphinolactyl-ThDP. The maximum at 317 nm was reached at a 1.0/1.0 ratio of [Elec]/[AcMPhi] indicating stoichiometric binding of AcMPhi ( $K_d = 0.10 \mu\text{M}$ ) (Fig. 3). After overnight dialysis approximately 80% of the CD intensity at 317 nm was retained on the enzyme indicating that binding is partially reversible. However, on addition of 2 mM pyruvate, the AcMPhi was displaced from the Elec, as indicated by the formation of (*S*)-acetoin according to the CD spectra after removal of protein (Fig. 4).

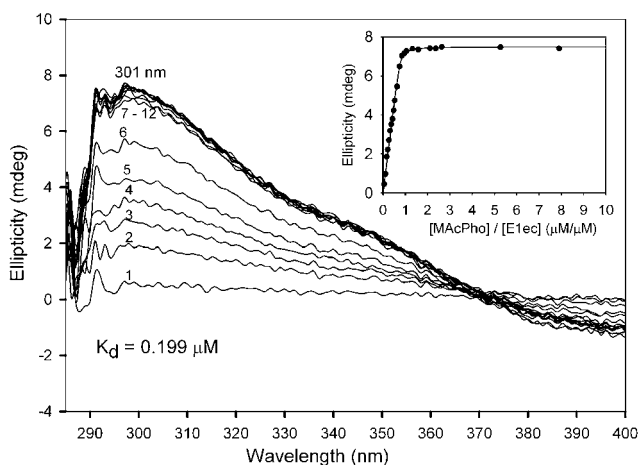


Fig. 2. Difference CD spectra of Elec on addition of MAcPho. Elec (1.90 mg/ml, concentration of active centers =  $19 \mu\text{M}$ ) in 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM  $\text{MgCl}_2$  and 0.20 mM ThDP was titrated by MAcPho ( $\mu\text{M}$ ): spectrum 1 (1.0); 2 (3.0); 3 (5.0); 4 (7.0); 5 (9.0); 6 (12.0); 7 (16.0); 8 (18.0); 9 (20.0); 10 (40.0); 11 (50.0); 12 (150.0). The difference CD spectra were obtained on subtraction of the spectrum of Elec in the presence of  $\text{MgCl}_2$  and ThDP. Inset, dependence of the ellipticity at 301 nm on [MAcPho]/[Elec] ratio.



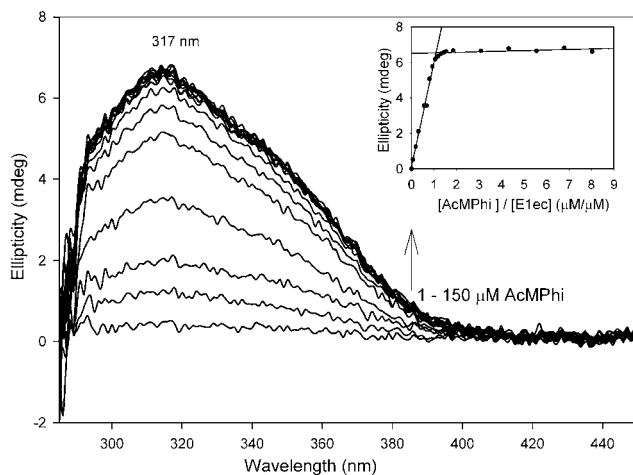


Fig. 3. Difference spectra of Elec on addition of AcMPhi. Inset, dependence of the ellipticity at 317 nm on  $[AcMPhi]/[Elec]$  ratio. Conditions under Section 2.

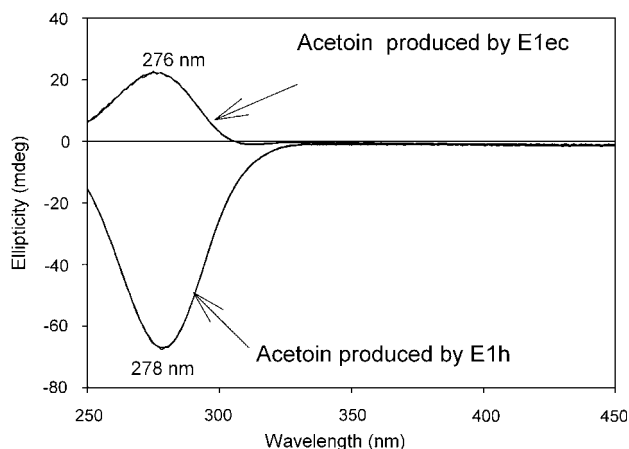


Fig. 4. Acetoin production by Elec and E1h. Elec titrated with AcMPhi (as in Fig. 3) was dialyzed overnight against 20 mM potassium phosphate buffer (pH 7.0). Then 2 mM  $MgCl_2$ , 0.20 mM ThDP, and 2 mM pyruvate were added to the dialyzed Elec and CD spectra were recorded at different times. After 50 min of incubation of Elec with 2 mM pyruvate the protein was removed by centrifugation using a Centricon 30 and the spectrum of the reaction mixture was recorded. A similar experiment was conducted with E1h.

### 3.3. Progress curve analysis of the inhibition of Elec by sodium acetylphosphinate and sodium acetyl methylphosphinate

The inhibition by AcPhi and AcMPhi was studied using reaction progress curve analysis as we reported earlier [10]. The dependence of the pseudo first-order rate constant ( $k_{app}$ ) on the concentration of AcPhi or AcMPhi was hyperbolic, indicating saturation (Fig. 5A and B) leading to values of  $K_i$  of 0.76  $\mu M$  for AcPhi and 3.33  $\mu M$  for AcMPhi according to a Kitz–Wilson treatment (Ref. [14]); Table 1]. The data suggest that

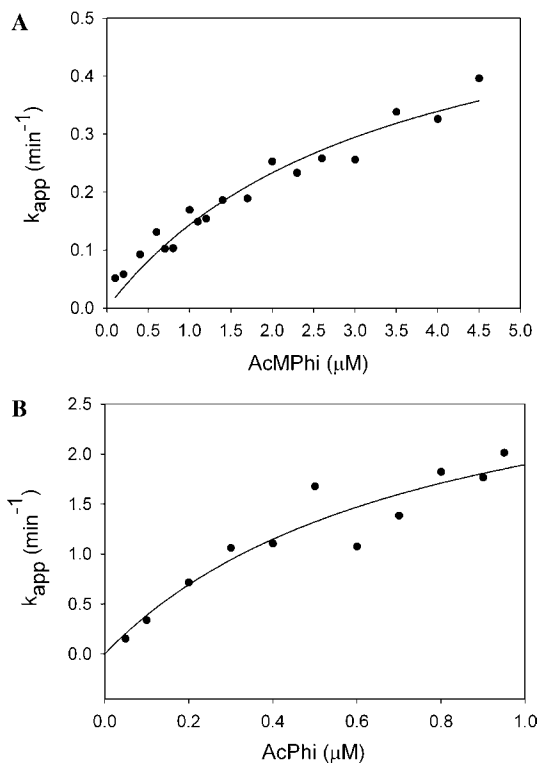


Fig. 5. Determination of  $K_i$  for inhibition of Elec by AcMPhi and AcPhi from progress curve analysis. Dependence of  $k_{app}$  of inhibition of Elec by different concentrations of AcMPhi (A) and AcPhi (B). Conditions in Section 2.

Table 1

Kinetic parameters for the inhibition of Elec by substrate analogues

Compound	$K_i$ ( $\mu\text{M}$ )	$k_{inact}$ ( $\text{min}^{-1}$ )	$k_{inact}/K_i$ ( $\mu\text{M min}^{-1}$ ) <sup>-1</sup>	$K_d$ ( $\mu\text{M}$ )
Acetylphosphinate	0.76	3.34	4.40	0.060 <sup>a</sup>
Acetylphosphinate <sup>b</sup>	0.12	1.30	10.83	0.005
Acetylphosphinate <sup>d</sup>	0.33	0.55	1.67	
Acetylmethylphosphinate	3.33	0.62	0.19	0.10 <sup>a</sup>
Methyl acetylphosphonate				0.199 <sup>a</sup>
2-Oxo-3-butynoic acid <sup>c</sup>	8.5	1.79	0.21	0.20

$K_i$  is the dissociation constant for the initial reversible complex (from kinetic experiment) obtained in the presence of 0.50 mM pyruvate in the incubation mixture.

$k_{inact}$  is the rate constant for conversion of the reversible complex to irreversibly inactivated enzyme.

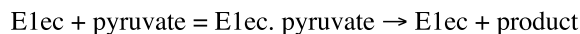
$k_{inact}/K_i$  is the second-order rate constant for inactivation.

<sup>a</sup>  $K_d$  is the dissociation constant obtained from CD analysis.

<sup>b</sup> Determined from kinetic analysis of inhibition for *E. coli* PDHc from Ref. [16].

<sup>c</sup> Determined from kinetic analysis of inhibition for *E. coli* PDHc from Refs. [11,22].

<sup>d</sup> Determined from kinetic analysis of inhibition for PDHc from bovine heart from Ref. [19].



Scheme 3. Mechanism of inhibition of Elec by AcPhi.

inhibition of Elec by AcPhi or AcMPhi follows the mechanism presented in Scheme 3 for competitive tight-binding inhibitors [17]. The kinetic parameters for inhibition of Elec by AcPhi and AcMPhi are presented in Table 1.

### 3.4. Kinetic analysis of inhibition of E1h by acetylphosphinate and acetylmethylphosphinate

The following results were obtained for inhibition of E1h by AcPhi: (i) full inhibition was achieved with stoichiometric concentrations of AcPhi (Fig. 6); (ii) inhibition by AcPhi displayed time dependence, reaching its maximum at approximately 20 min (data not shown); and (iii) inhibition by AcPhi was partially reversible, judging by the partial restoration of activity when an aliquot of inactivated E1h was used in the overall PDHc assay and the reaction was allowed to proceed for longer than 1 min. Kinetic analysis indicated that AcPhi is a tight slow-binding inhibitor of E1h [15]. Analysis of the progress curves of the overall PDHc reaction (E1h was reconstituted with E2h-E3BPh and E3h) in the presence of pyruvate and AcPhi enabled calculation of  $K_i = 0.014 \mu\text{M}$ ,  $k_{-1} = 0.0305 \text{ min}^{-1}$ , and  $k_1 = 2.18 \mu\text{M}^{-1} \text{ min}^{-1}$  (Fig. 7; Table 2).

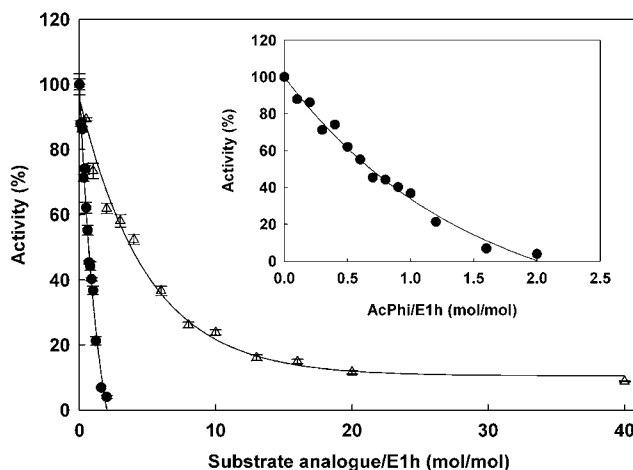


Fig. 6. Inhibition of E1h by AcPhi (●) and AcMPhi (△). E1h ( $0.649 \mu\text{M}$ ) was incubated in 50 mM potassium phosphate buffer (pH 7.0), 2 mM  $\text{MgCl}_2$ , and 0.2 mM ThDP with different concentrations of AcPhi or AcMPhi for 30 min. Aliquots were taken to determine activity of E1h in the PDHc assay as described in Section 2. One hundred percentage of activity was determined in the absence of inhibitors in the incubation mixture and corresponds to 32 U/mg of E1h protein. Data points are means  $\pm$  SE ( $n = 3-4$ ). Inset shows inhibition of E1h by AcPhi at lower concentrations.

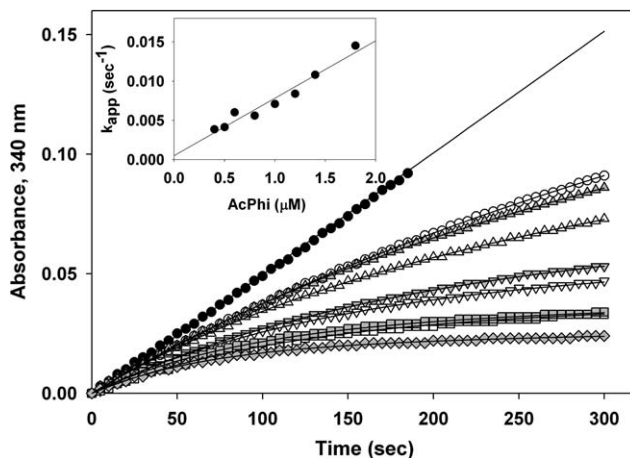


Fig. 7. Progress curve analysis of E1h inhibition by AcPhi. E1h (0.3  $\mu\text{g}$ , 1.95 nM) was reconstituted to PDHc-h with 1  $\mu\text{g}$  of E2h-E3BPh and 1  $\mu\text{g}$  of E3h and the reactions were started by the addition of 300  $\mu\text{M}$  pyruvate and varying concentrations of AcPhi (in  $\mu\text{M}$ ): 0 ( $\bullet$ ); 0.4 ( $\circ$ ); 0.5 ( $\blacktriangle$ ); 0.6 ( $\triangle$ ); 0.8 ( $\blacktriangledown$ ); 1.0 ( $\nabla$ ); 1.2 ( $\blacksquare$ ); 1.4 ( $\square$ ); 1.8 ( $\blacklozenge$ ). Lines represent the regression curves generated by SigmaPlot 2001 based on the equation [1] (see Section 2). Inset shows the dependence of  $k_{\text{app}}$  on the concentration of APhi.

Table 2

Kinetic parameters for the inhibition of E1h by substrate analogue inhibitors

Compound	$K_i$ ( $\mu\text{M}$ )	$k_1$ ( $\mu\text{M}^{-1} \text{min}^{-1}$ )	$k_{-1}$ ( $\text{min}^{-1}$ )	$K_d$ ( $\mu\text{M}$ )
Acetylphosphinate	0.014	2.18	0.0305	N/A
Acetylmethylphosphinate	1.46	0.0101	0.0148	0.45

$K_d$  is measured from CD titration where experimentally accessible. N/A, not available.

The AcMPhi is a weaker inhibitor than AcPhi of E1h (Fig. 6). Progress curve analysis indicated inhibition of E1h at higher concentrations of AcMPhi with  $K_i = 1.46 \mu\text{M}$ ,  $k_{-1} = 0.0148 \text{ min}^{-1}$ ; and  $k_1 = 0.0101 \mu\text{M}^{-1} \text{ min}^{-1}$  (Fig. 8; Table 2).

Kinetic analysis of inhibition by AcPhi and AcMPhi according to the DCPIP assay (measuring the rate through the decarboxylation step) revealed similar patterns of inhibition: AcPhi was a stronger inhibitor than AcMPhi (Fig. 9). The stoichiometry of inhibition of E1h by AcPhi and AcMPhi when determined with the DCPIP assay was similar to the one determined with the PDHc-h assay (compare Fig. 6 to Fig. 9 inset).

Both AcPhi and AcMPhi are much stronger inhibitors of E1h compared to MAcPho. MAcPho did not result in more than 20% reduction in E1h activity even at 500-fold molar excess (data not shown).

The E1h  $\alpha\text{H63A}$  variant has undetectable activity when measured in the overall PDHc assay and about 17% when measured with the DCPIP assay. For that reason, inhibition of E1h  $\alpha\text{H63A}$  could only be studied using the DCPIP assay. Only modest inhibition of E1h  $\alpha\text{H63A}$  is detected with both AcPhi and AcMPhi (Fig. 9), indicating that E1h  $\alpha\text{H63}$  is necessary for the interaction with both substrate analogues and is probably involved in the interaction with pyruvate during the decarboxylation reaction.

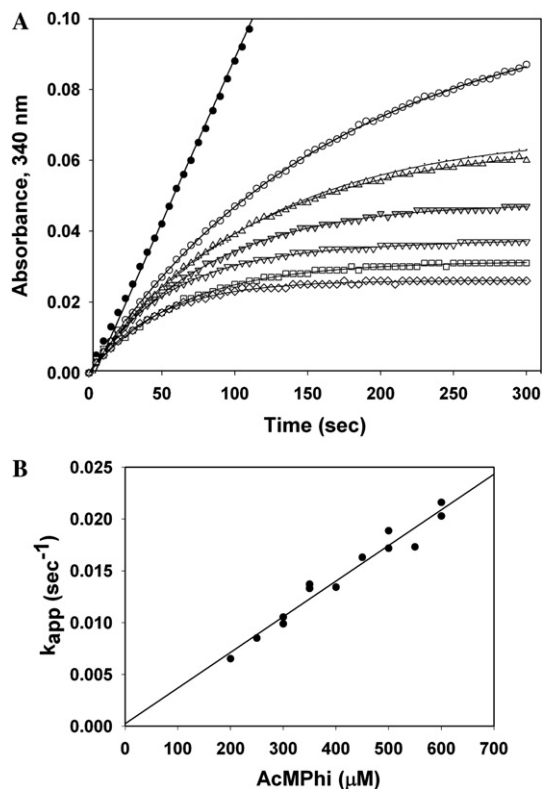


Fig. 8. Progress curve analysis of E1h inhibition by AcMPhi. (A) E1h (0.3  $\mu$ g, 1.95 nM) was reconstituted to PDHc-h with 1  $\mu$ g of E2h-E3BP and 1  $\mu$ g of E3h and the reactions were started by the addition of 300  $\mu$ M pyruvate and varying concentrations of AcMPhi (top in  $\mu$ M): 0 ( $\bullet$ ); 200 ( $\circ$ ); 250 ( $\blacktriangle$ ); 300 ( $\triangle$ ); 400 ( $\blacktriangledown$ ); 450 ( $\triangledown$ ); 500 ( $\square$ ); 600 ( $\diamond$ ). Lines represent the regression curves generated by SigmaPlot 2001 based on the equation [1] (see Section 2). Several curves used for calculations of  $k_{app}$  are omitted for clarity. (B) Dependence of  $k_{app}$  on the concentration of AcMPhi.

### 3.5. Circular dichroism titration of E1h with sodium acetylmethylphoshinate

On addition of 1–150  $\mu$ M of AcMPhi to the E1h the positive band at 305 nm developed and reached the maximum intensity at a molar ratio of [AcMPhi]/[E1h active centers] equal to 2.0/1.0 (Fig. 10). Similar spectra with CD maximum at 306 nm were obtained when AcPhi was used with molar ratio of [AcMPhi]/[E1h] equal to 1.0/1.0 (data not shown). A  $K_d = 0.45$   $\mu$ M was calculated for AcMPhi binding (the  $K_d$  for AcPhi is not available). The CD spectra of E1h treated by AcMPhi and AcPhi were not affected by overnight dialysis indicating tight binding. On addition of 5 mM pyruvate to either the E1h.AcPhi or E1h.AcMPhi complex, the CD band at 305–306 nm disappeared suggesting that pyruvate competes with the substrate analogues at the active centers of the E1h, similarly to that seen with E1ec (data not shown). Removal of the enzyme from the incubation mixture revealed the presence of a species with a negative CD band with maximum at 278 nm corresponding to (*R*)-acetoin. This is in interesting contrast to the (*S*)-acetoin produced by E1ec (Fig. 4) [18]. The data represent the first evidence for the formation of the chiral acetoin by the E1h.

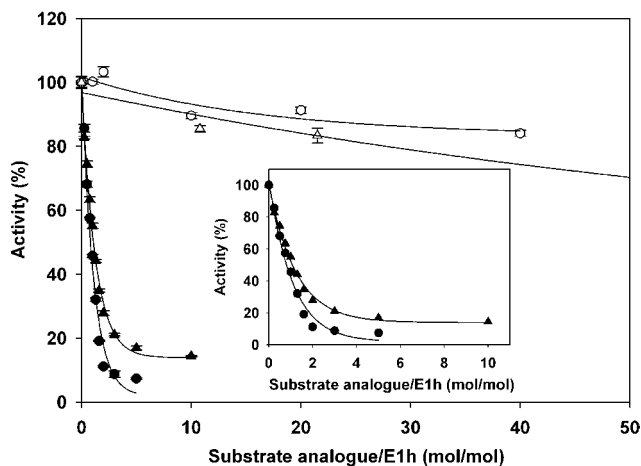


Fig. 9. Inhibition of wild-type E1h and E1h  $\alpha$ H63A by AcPhi and AcMPhi. Symbols are: wild-type E1h inhibited by AcPhi (●) and AcMPhi (▲);  $\alpha$ H63A E1h inhibited by AcPhi (○) and AcMPhi (△). E1h (6.49  $\mu$ M) or its  $\alpha$ H63A variant was incubated in 50 mM  $\text{KH}_2\text{PO}_4$  (pH 7.0), 2 mM  $\text{MgCl}_2$ , 0.2 mM ThDP and different concentrations of AcPhi or AcMPhi for 30 min. Aliquots were taken to determine the E1-specific activity according to the DCPIP assay as described in Methods. 100% of the activity was determined in the absence of inhibitors in the incubation mixture and corresponds to 231 mU/mg of E1 protein. Data points are means  $\pm$  SE ( $n = 3$ ). Inset shows inhibition of the wild-type E1h by AcPhi (●) and AcMPhi (▲) at lower concentrations.

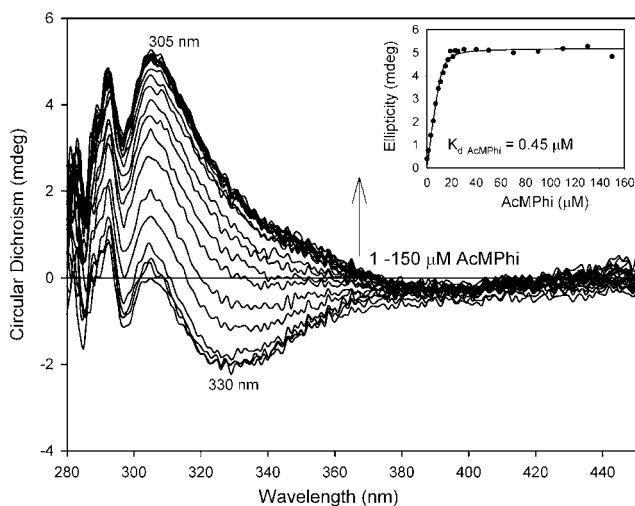


Fig. 10. CD titration of E1h with AcMPhi. The E1h was titrated with the indicated concentrations of AcMPhi, and led to the  $K_d$  of 0.45  $\mu$ M (see inset for ellipticity at 305 nm vs. inhibitor concentration). For conditions see Section 2.

Addition of 1–150  $\mu$ M of AcPhi to the  $\alpha$ H63A E1h variant in the presence of 0.60 mM ThDP did not produce the positive CD band with maximum at 305 nm (Fig. 11A) indicating that E1h  $\alpha$ His63A is important in the reaction(s) leading to formation of LThDP. Significantly, nor did the E1h  $\alpha$ H63A variant produce the negative CD band with maximum

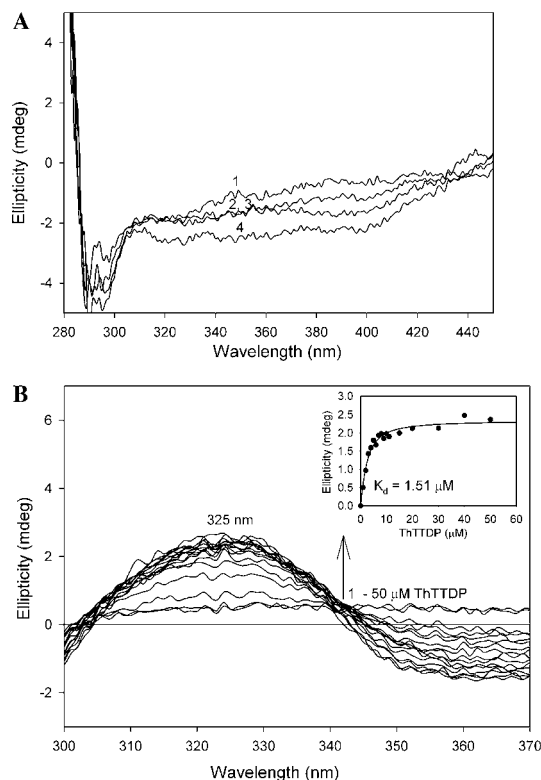


Fig. 11. CD spectra of E1h  $\alpha$ H63A titrated by AcPhi. (A) E1h  $\alpha$ H63A (concentration of active centers = 16.4  $\mu$ M) in 20 mM potassium phosphate buffer (pH 7.0) containing 0.60 mM ThDP and 1.0 mM  $\text{MgCl}_2$  (spectrum 1) was titrated with 5  $\mu$ M (spectrum 2), 50  $\mu$ M (spectrum 3) and 150  $\mu$ M (spectrum 4) of AcPhi. (B) CD spectra of E1h  $\alpha$ H63A titrated by thiamin 2-thiothiazolone diphosphate (ThTTDP). E1h  $\alpha$ H63A (concentration of active centers = 10.2  $\mu$ M) in 20 mM potassium phosphate buffer (pH 7.0) was titrated by 1–50  $\mu$ M ThTTDP. Inset, dependence of the ellipticity at 325 nm on concentration of ThTTDP.

at 330 nm on addition of 0.20–0.60 mM ThDP, a signature of ThDP binding (curve 1 in Fig. 11A presents the CD spectrum of E1h  $\alpha$ H63A in the presence of 0.60 mM ThDP). To confirm that ThDP did bind in the active centers of E1h  $\alpha$ H63A, the variant was titrated by thiamin 2-thiothiazolone diphosphate (ThTTDP), a transition state analogue (Fig. 11B) that gives a positive CD band near 330 nm, as reported earlier for the Elec [10]. The data presented in Fig. 11B indicate binding of the ThTTDP in the active centers of E1h  $\alpha$ H63A. A  $K_d$  of 1.51  $\mu$ M was obtained for binding ThTTDP to the E1h  $\alpha$ H63A, as compared with the value of  $K_d = 0.59 \mu$ M obtained for the wild-type Elec (Korotchkina, unpublished results).

#### 4. Discussion

One of our goals in designing and testing inhibitors for ThDP enzymes is to achieve enzyme-selective inhibition. In this paper, we wish to compare and contrast inhibition of the bacterial (Elec) and human (E1h) pyruvate dehydrogenase components by the same

substrate analogue-type inhibitor. While inhibition of Elec by AcPhi and AcMPhi was studied in several laboratories, we here present the first inhibitory analysis of the E1h by such compounds. In an earlier study Laber and Amrhein [19] demonstrated that the compound 1-aminoethylphosphinate [ $\text{H}_3\text{C}-\text{CH}(\text{NH}_2)-\text{PO}_2\text{H}_2$ ] inhibited the PDHc from bovine heart in the presence of alanine aminotransferase and a similar inhibitory effect was observed when synthetic AcPhi was used (see Table 1, Ref. [19]). Since those early results, inhibition by AcPhi of several ThDP-dependent enzymes was reported, including Elec [16], pyruvate decarboxylase (YPDC) from brewer's yeast [19], and both YPDC and pyruvate oxidase (POX) from *L. plantarum* [20]. A comparison of kinetic constants with ours on the recombinant Elec (Table 1), confirms that AcPhi is a very powerful inhibitor of Elec, as is to a lesser extent AcMPhi.

The positive broad unsymmetrical band CD band at 317 nm resulting from mixing Elec and AcPhi signaled formation of 1',4'-iminophosphinolactyl-ThDP, since this band is a reporter of the tetrahedral intermediates in ThDP enzymes [1,2]. The broad CD band in Fig. 1 could be deconvoluted into two bands [21], one of which has a maximum at 309 nm, the other at 343 nm. The CD evidence elucidates the mechanism of inhibition, consistent with data published at Rutgers and elsewhere for interaction of phosphonates and phosphinates with ThDP enzymes (Scheme 2) [1–7].

Regarding the kinetic behavior of E1h with the substrate analogues (Table 2), progress curve analysis revealed that the inhibition involves a single step since there is no evidence for the presence of an  $E \cdot I^*$  intermediate in Scheme 2 [17]. The analysis yields a second-order rate constant for substrate analogue binding and a first-order 'off' rate constant for the dissociation of substrate analogue. A comparison of the second-order rate constants for the human and bacterial enzyme indicates that AcPhi is a strong inhibitor of both Elec and E1h. Also, the value of  $k_{\text{inact}}/K_i = 1.67 \text{ (}\mu\text{M min)}^{-1}$  for inhibition of PDHc from bovine heart by AcPhi, is similar to that for Elec and E1h in Table 1 [19]. For the interaction of YPDC with AcPhi mixed type of inhibition was observed, with  $K_i = 0.47 \text{ mM}$  for competitive inhibition and  $K_i = 7.14 \text{ mM}$  for the non-competitive inhibition [20]. A value of  $K_i = 50 \text{ }\mu\text{M}$  was calculated from the kinetics of inhibition of POX from *L. plantarum* by acetylphosphinate [20].

Among classes of inhibitors studied in our laboratories, the acetylphosphinate appears to be the strongest inhibitor of Elec and of E1h. This includes the three compounds mentioned in this paper, thiamin 2-thiothiazolone diphosphate (ThTTDP) and thiamin 2-thiazolonediphosphate (ThTDP), and potential Michael acceptor 2-oxo-3-butynoic acid. The 2-oxo-3-butynoic acid is an irreversible inhibitor and activity could not be regained by dilution of inhibitor [22]; its site of reactivity is still not known, but we do know that it does not react with any of the six cysteines of Elec [11]. The compounds ThTTDP and ThTDP are sometimes referred to as transition state analogues since the  $K_i$  values obtained are much lower than the  $K_d$  for ThDP [10,23]. The nature of inhibition by these compounds has been clarified with the publication of a crystal structure of its complex with Elec [24].

The phosphonate/phosphinate class mimics the substrate and the cumulative evidence indicates that they inhibit via enzymatic synthesis of LThDP-like adducts (Scheme 2). While these adducts certainly must have significant lifetime, the chemical bond between the keto carbon of the inhibitor and the C2 atom of thiazolium is formed reversibly and excess pyruvate or overnight dialysis (i.e., dilution of the inhibitor concentration) can reverse the inhibition. The  $k_{\text{off}}$  appears to be slow for both enzymes.



At the same time, we point out that these phosphonate and phosphinate compounds have tuned out to be excellent mechanistic tools since their ThDP-bound adducts (Scheme 2) all give evidence of the positive CD band in the 300–310 nm region (as well as in the UV spectrum) [21].

Another notable finding of our studies is that the two species of pyruvate dehydrogenase studied E1h and E1ec lead to the formation of the opposite enantiomer of acetoin, a carboligase side product of ThDP enzymes (Fig. 4). This makes the E1ec truly unusual [produces the (*S*) enantiomer], since all other ThDP enzymes appear to produce (*R*)-acetoin as does E1h. As was reasoned elsewhere, this implies that the pyruvate entering the enzyme presents a different face (*re* or *si*) to the enamine [18,25].

Finally, the results provide very strong support for the function of residue  $\alpha$ H63 in E1h. It is suggested by the absence of the negative 330 CD band for the E1h  $\alpha$ H63.ThDP complex, unlike with the wild-type E1h, that the ThDP binding site is perturbed. However, the experiments with ThTTDP indicate that this ThDP analogue can still bind to the active center. The absence of the positive CD band at 305 nm on adding AcPhi to the E1h  $\alpha$ H63.ThDP complex and the low level of inhibition of E1h  $\alpha$ H63A by AcPhi and AcMPhi confirm that the variant can no longer form the C2 $\alpha$ -phosphinolactylThDP (see Scheme 2), nor can it presumably form LThDP. The presence or absence of these CD bands provides a straightforward experimental method to determine whether or not a particular residue is involved in the formation of LThDP, a common intermediate in all ThDP-dependent decarboxylases.

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