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Inhibition of pyruvate:ferredoxin oxidoreductase from *Trichomonas vaginalis* by pyruvate and its analogues

Comparison with the pyruvate decarboxylase component of the pyruvate dehydrogenase complex

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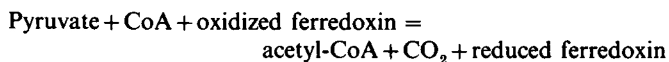
Pyruvate:ferredoxin oxidoreductase and the pyruvate dehydrogenase multi-enzyme complex both catalyse the CoA-dependent oxidative decarboxylation of pyruvate but differ in size, subunit composition and mechanism. Comparison of the pyruvate:ferredoxin oxidoreductase from the protozoon *Trichomonas vaginalis* and the pyruvate dehydrogenase component of the *Escherichia coli* pyruvate dehydrogenase complex shows that both are inactivated by incubation with pyruvate under aerobic conditions in the absence of co-substrates. However, only the former is irreversibly inhibited by incubation with hydroxypyruvate, and only the latter by incubation with bromopyruvate. Pyruvate:ferredoxin oxidoreductase activity is potently, but reversibly, inhibited by addition of bromopyruvate in the presence of CoA, and it is suggested that the mechanism involves formation of an adduct between CoA and bromopyruvate in the active site of the enzyme. It is proposed that both enzymes are inactivated by pyruvate through a mechanism involving oxidation of an enzyme-bound thiamin pyrophosphate/substrate adduct to form a tightly bound inhibitory species, possibly thiamin thiazolone pyrophosphate as hypothesized by Sumegi & Alkonyi [(1983) Arch. Biochem. Biophys. **223**, 417–424].

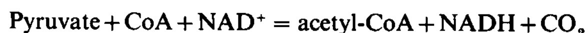
INTRODUCTION

In anaerobes, pyruvate:ferredoxin oxidoreductase (pyruvate synthase, EC 1.2.7.1) catalyses the CoA- and ferredoxin-dependent oxidative decarboxylation of pyruvate (Buchanan, 1972):



$$\text{Pyruvate} + \text{CoA} + \text{oxidized ferredoxin} = \text{acetyl-CoA} + CO_2 + \text{reduced ferredoxin}$$

In aerobic organisms, the oxidative decarboxylation of pyruvate is catalysed by the pyruvate dehydrogenase multienzyme complex, in a CoA- and NAD^+-dependent reaction:



$$\text{Pyruvate} + \text{CoA} + NAD^+ = \text{acetyl-CoA} + \text{NADH} + CO_2$$

This enzyme catalyses several partial reactions, the first of which is decarboxylation of pyruvate catalysed by the E1 (pyruvate decarboxylase, EC 1.2.4.1) component.

The pyruvate:ferredoxin oxidoreductase and pyruvate dehydrogenase complex differ in size, subunit composition and cofactor content (Reed, 1974; Kerscher & Oesterhelt, 1982; Perham *et al.*, 1987; Williams *et al.*, 1987). However, both pyruvate:ferredoxin oxidoreductase and E1 contain thiamin pyrophosphate (TPP) as the cofactor involved in pyruvate decarboxylation, suggesting similarities in the initial steps of the catalytic cycle.

The catalytic mechanism of pyruvate:ferredoxin oxidoreductase has not been unequivocally elucidated, and several alternative mechanisms have been proposed, depending on whether electrons are removed individually from the initial decarboxylation product (Kerscher & Oesterhelt, 1981*a,b*; Docampo *et al.*, 1987), or in one step (Wahl & Orme-Johnson, 1987), as in the pyruvate dehydrogenase complex.

In the important human protozoan parasite *Trichomonas vaginalis* pyruvate:ferredoxin oxidoreductase plays an essential role in anaerobic energy production from pyruvate (Muller, 1988). It is also the enzyme responsible for the activation of the widely used anti-trichomonal drug metronidazole (Chapman *et al.*, 1985). To aid rational design of drugs against the *T. vaginalis* pyruvate:ferredoxin oxidoreductase, we were interested in obtaining a better understanding of the active site of the enzyme and finding differences between it and its host counterpart, the pyruvate dehydrogenase complex.

To this end, we have utilized structural analogues of pyruvate, and pyruvate itself, to study the active site of the pyruvate:ferredoxin oxidoreductase from *T. vaginalis* and to compare it with that of the pyruvate decarboxylase component of the pyruvate dehydrogenase complex.

EXPERIMENTAL

Materials

2-Oxo acids, pyruvate analogues and coenzymes were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Pyruvate:ferredoxin oxidoreductase from *T. vaginalis* was purified as described by Williams *et al.* (1987). Pyruvate dehydrogenase multienzyme complex and its E1 component were isolated from *Escherichia coli* (Lowe & Perham, 1984). Tetrahydro-TPP was prepared as described by Lowe *et al.* (1983).

Enzyme assays

Oxidative decarboxylation of pyruvate by the pyruvate:ferredoxin oxidoreductase was measured spectrophotometrically with Methyl Viologen as electron acceptor, in the presence of an O_2-scavenging system to decrease the lag phase (Williams *et al.*, 1987). Enzyme activities were measured from the linear steady-state rates obtained after any lag phase.

Overall pyruvate dehydrogenase complex activity was measured in the direction of NADH formation in the presence of

Abbreviation used: TPP, thiamin pyrophosphate.

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pyruvate, CoA, NAD^+ and TPP as described by Lowe & Perham (1984). The enzymic activity of the E1 component was measured either by reconstitution with E2E3 subcomplex and measuring overall pyruvate dehydrogenase complex activity, or by assay of pyruvate decarboxylase activity after the enzyme-catalysed reduction of the dye 2,6-dichlorophenolindophenol by pyruvate in the absence of CoA (Lowe & Perham, 1984). The enzyme activity of the El component was dependent on the presence of TPP in the assay.

Irreversible inhibition was measured by preincubating enzyme and inhibitor, and then assaying samples at various time intervals by dilution into the standard assay mixtures. The preincubation reaction mixture for pyruvate:ferredoxin oxidoreductase activity consisted of 20 mM-Tris/HCl, pH 7.0, and 0.4 M-KCl. The preincubation reaction mixture for El consisted of 50 mM-potassium phosphate buffer, pH 7, 1 mM-$MgCl_2$ and 0.2 mM-TPP. Control experiments were carried out to check that no significant loss of activity of untreated enzyme occurred.

RESULTS

Inhibition by pyruvate

Preincubation of pyruvate:ferredoxin oxidoreductase with 1 mM-pyruvate at 4 °C resulted in a time-dependent inactivation of the enzyme activity as measured by extensive dilution into assay medium (Fig. 1). The inhibited assay traces were linear, suggesting that the inhibition was irreversible on the time scale of the assay.

The inactivation induced by pyruvate was partially prevented by prior addition of dithiothreitol to the enzyme, but dithiothreitol did not reverse inhibition by pyruvate that had already occurred (Fig. 1). Over 60 min incubation at 20 °C the enzyme loses 19% of activity (Table 1). This loss can be decreased by including dithiothreitol in the incubation medium. A slightly larger loss occurs in the presence of the oxygen-scavenging system, possibly owing to peroxide formation. In the absence of dithiothreitol and in the presence of oxygen-saturated buffer, 1 mM-pyruvate results in the almost complete loss of activity. This pyruvate-induced inhibition can be completely prevented by the inclusion of either dithiothreitol or the oxygen-scavenging system (Table 1). The inhibition caused by pyruvate could also be partially prevented by simply excluding air from the incubation buffer.

Table 1. Effect of dithiothreitol and exclusion of O_2 on the pyruvate-mediated inactivation of pyruvate:ferredoxin oxidoreductase

Each incubation contained pyruvate:ferredoxin oxidoreductase (0.12 unit) in a final volume of 100 μl. Before any additions, a 10 μl sample was removed from one of the incubations and assayed to give the initial enzyme activity. After the additions as detailed above, each sample was incubated for 60 min at 20 °C, and then a 10 μl sample was assayed for residual activity.

Additions to incubation	Inhibition (%)
None	19
Glucose oxidase/catalase/glucose*	38
1 mM-Pyruvate	91
Glucose oxidase/catalase/glucose* + 1 mM-pyruvate	32
2 mM-Dithiothreitol	12
2 mM-Dithiothreitol + 1 mM-pyruvate	14

* This O_2-scavenging system consisted of 12.5 units of glucose oxidase, 125 units of catalase and 1 μmol of glucose.

The inhibition was not reversed by treatment of inactivated enzyme with 0.2 M-hydroxylamine, pH 7, for 30 min at 20 °C, conditions which did not affect the activity of control enzyme untreated with pyruvate. This suggested that a thioester was not involved in the inhibition. Furthermore, incubation with 2 mM-acetoin for 60 min at 20 °C did not result in any inhibition, demonstrating that this potential enzymic product was also not the cause of the pyruvate-induced inhibition.

We found that the activity of pyruvate:ferredoxin oxidoreductase was not affected by incubation of the enzyme for 30 min at 4 °C with 1 mM-5,5′-dithiobis-(2-nitrobenzoic acid), 1 mM-iodoacetic acid, 1 mM-iodoacetamide, 1 mM-N-ethylmale-

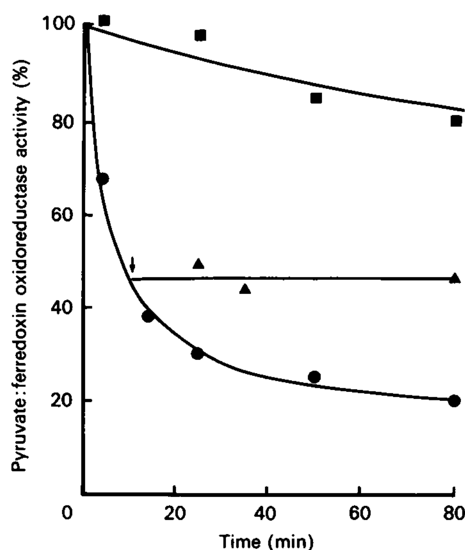


Fig. 1. Inactivation of pyruvate:ferredoxin oxidoreductase by pyruvate

Pyruvate:ferredoxin oxidoreductase (0.2 unit/ml) was incubated at 4 °C with 1 mM-pyruvate in the absence (●) or presence (■) of 2 mM-dithiothreitol. After 10 min (indicated by the arrow), the incubation with pyruvate alone was divided into two portions. One was untreated, and to the other was added 2 mM-dithiothreitol (▲). Samples (20 μl) were removed at time intervals shown and assayed for enzyme activity.

Table 2. Effect of pyruvate concentration on pyruvate-mediated inactivation of pyruvate ferredoxin oxidoreductase

Each incubation contained pyruvate:ferredoxin oxidoreductase (0.2 unit) in a final volume of 200 μl. Before the addition of pyruvate, a 10 μl sample was removed from one of the incubations and assayed to give the initial enzyme activity. Pyruvate was then added to each incubation to give the concentrations detailed above. The samples were incubated for 15 min at 20 °C, and then a 10 μl sample was assayed for residual activity.

[Pyruvate] (μM)	Inhibition (%)
10	31
50	51
100	68
200	86
400	97
1000	80
2500	23
5000	6

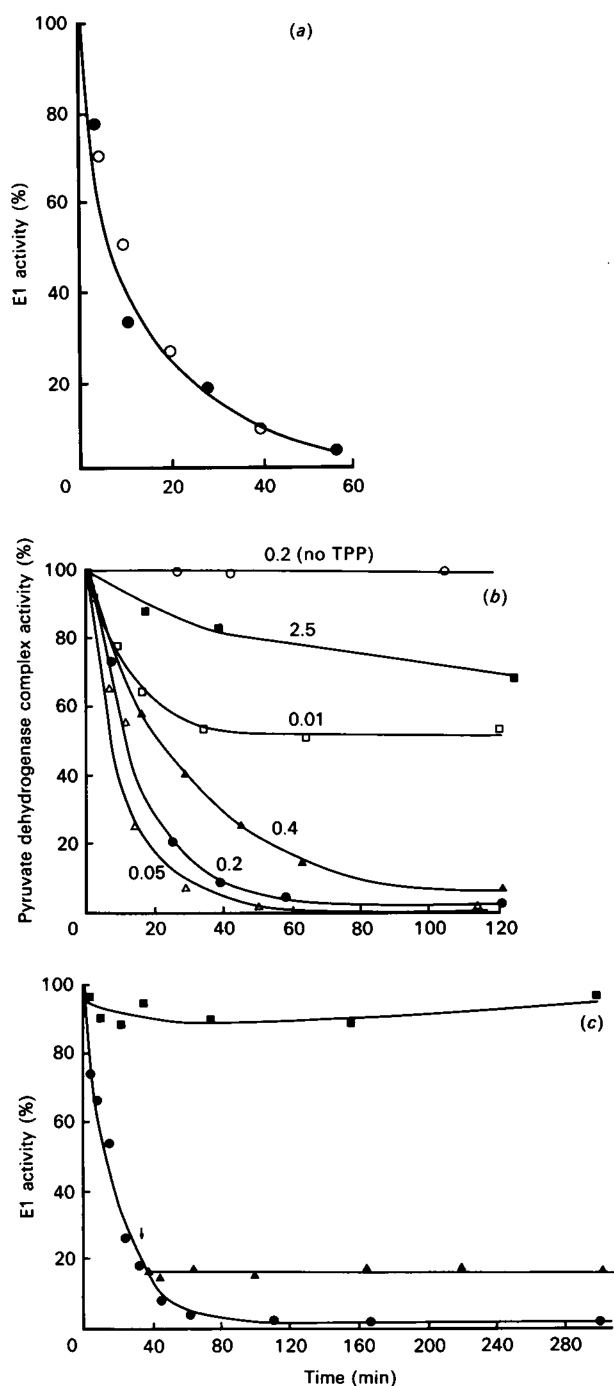


Fig. 2. Inactivation of E1 component of the pyruvate dehydrogenase complex by pyruvate

(*a*) E1 (0.25 mg/ml) was incubated with 0.1 mM-pyruvate at 23 °C. At intervals, samples were assayed for E1 activity either by reconstitution with E2E3 subcomplex and measuring overall pyruvate dehydrogenase complex activity (○) or by the dichlorophenol-indophenol-reduction assay (●). In either case activity is expressed relative to a control incubation in the absence of pyruvate, which did not change in activity during the incubation period. (*b*) E1 (0.25 mg/ml) was incubated with various concentrations of pyruvate and assayed by reconstitution with E2E3 subcomplex as in (*a*) above. Additionally, E1 was incubated in the absence of TPP in the presence of 0.2 mM-pyruvate (○). The concentration of pyruvate (mM) is indicated next to each curve. (*c*) As in (*a*), E1 was incubated in the absence (●) or presence (■) of 5 mM-mercaptoethanol. At the time indicated by the arrow, 5 mM-mercaptoethanol was added to a portion of the incubation in the absence of mercaptoethanol (▲).

imide, or 1 mM-methyl methanethiosulphonate in the presence or absence of 5 mM-pyruvate, strongly suggesting that thiol groups are not essential for its activity.

The rate of pyruvate-induced inhibition displayed an unusual dependence on the concentration of pyruvate (Table 2). The rate of inhibition increased as the pyruvate concentration was increased in the range 10–400 μM, but with higher concentrations of pyruvate the rate of inhibition decreased.

Similar experiments were performed with *E. coli* pyruvate dehydrogenase complex and with its isolated E1 component. Both the overall pyruvate dehydrogenase complex activity (results not shown) and its E1 activity were inactivated by preincubation with pyruvate (Fig. 2*a*). The inhibition of E1 activity was not related to reductive acetylation of lipoate residues, since a similar rate of inhibition was seen with intact complex and with its isolated E1 component (results not shown), and the inhibition of the activity of isolated E1 was the same whether inhibition was measured by reconstitution with E2/E3 subcomplex or by dichlorophenolindophenol reduction (Fig. 2*a*). The inhibition was completely dependent on the inclusion of TPP in the preincubation medium (Fig. 2*b*).

As with pyruvate:ferredoxin oxidoreductase, the inhibition of E1 activity could be prevented by exclusion of O_2 from the medium, or by the presence of mercaptoethanol, and was not reversed by addition of mercaptoethanol to pyruvate-inhibited enzyme (Fig. 2*c*). A further similarity was the unusual dependence on pyruvate concentration (Fig. 2*b*), which was such that at high concentrations of pyruvate no inactivation occurred. Once inhibition had occurred, high concentrations of mercaptoethanol (up to 50 mM) did not re-activate the enzyme.

With the E1 component of the *E. coli* complex, after inactivation by incubation with [2-^{14}C]pyruvate less than 0.1 mol of pyruvate-derived radioactivity per mol of E1 was bound to trichloroacetic acid-precipitated protein. Furthermore, when the native sample was gel-filtered, enzyme could be obtained that was still inhibited but did not have radiolabel associated with it. This suggested that the formation of a covalent adduct between enzyme and pyruvate was not necessary for inactivation.

Inhibition by 3-bromopyruvate

Bromopyruvate is a potent active-site-directed alkylating agent of the E1 component of the pyruvate dehydrogenase complex (Lowe & Perham, 1984; Apfel *et al.*, 1984), though the high reactivity of the α-haloketone group makes it a candidate as a non-selective alkylating agent.

However, preincubation of pyruvate:ferredoxin oxidoreductase with 1 mM-bromopyruvate for 60 min did not result in any time-dependent irreversible inhibition, although it did have an inhibitory effect. When samples were assayed by dilution into assay medium which did not contain dithiothreitol, a considerable lag period was observed before a linear, yet inhibited, assay rate was seen. This lag could be many minutes at high bromopyruvate concentrations (Fig. 3 and Table 3). If, however, the samples were assayed in the presence of dithiothreitol, the observed rate was linear and uninhibited as soon as the short lag phase was over, i.e. the progress curve was the same as that for an incubation which did not contain bromopyruvate. Assays in which bromopyruvate was added directly to assays and then the reaction was initiated by adding enzyme (Fig. 3) indicated that the extended lag phase and inhibited rate was due to carry-over of bromopyruvate present in the preincubation. Again, both the inhibition of the linear rate and the extended lag phase were prevented by the presence of dithiothreitol in the assay, presumably owing to destruction of the bromoacyl moiety by reaction with the thiol groups of dithiothreitol. Thus this inhibition was of a reversible rather than an irreversible nature.

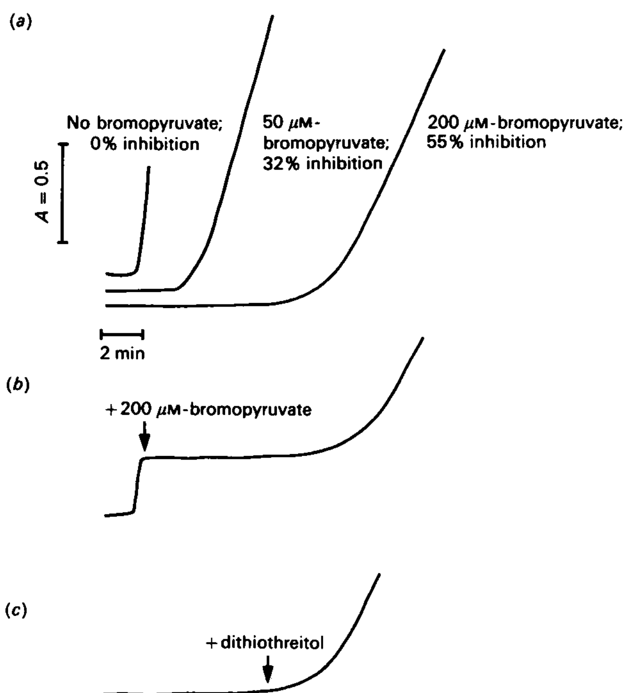


Fig. 3. Effect of bromopyruvate on the time course of the pyruvate:ferredoxin oxidoreductase-catalysed reaction

The Figure shows assay traces for the pyruvate:ferredoxin oxidoreductase reaction. In all cases, the reaction was initiated by the addition of enzyme to reaction mixture, without dithiothreitol, but otherwise complete. In (*a*), bromopyruvate was added at either 50 μM or 200 μM to assays just before initiation of the reaction. In (*b*), 200 μM-bromopyruvate was added during the linear phase 90 s after the initiation of the reaction. In (*c*), 20 mM-dithiothreitol was added to any assay, which initially contained 200 μM-bromopyruvate, near the end of the extended lag phase (approx. 8 min).

In control assays, without bromopyruvate, a lag is seen, followed by a linear reaction rate. The length of the lag phase is inversely related to the amount of enzyme activity present in the assay. This lag can be accounted for by the time taken to remove the residual oxygen in the cuvette by its reaction with the reduced Methyl Viologen formed in the reaction. In the presence of oxygen the coloured reduced Methyl Viologen is oxidized back to colourless oxidized Methyl Viologen, and thus no colour formation occurs and a lag phase is seen. The length of the lag phase caused by bromopyruvate increased with increasing concentrations of bromopyruvate added (Table 3). It could be argued that the extended lag induced by bromopyruvate was due to inhibition of enzyme activity resulting in a longer time before residual oxygen was removed. However, we do not think this explanation is correct, because the lag phase observed with 25 μM-bromopyruvate was 690s when the enzyme activity was decreased by 68 % relative to a control without bromopyruvate, but was only 190 s when less enzyme was added to the assay (in the absence of bromopyruvate), such that the amount of enzyme activity present was decreased by 77 % (Table 3). Increased concentrations of pyruvate in the assay decreased the inhibition. Thus at 20 μM-bromopyruvate in the presence of 200 μM-pyruvate the enzyme was inhibited 96 %, whereas at 2 mM-pyruvate the inhibition was only 18 % (Table 3). This suggested that the inhibition was reversible and at or near the pyruvate-binding site.

Since bromopyruvate can react rapidly with the thiol groups of CoA, which is present as substrate in the assay mixture, experiments were carried out to establish the nature of the inhibitory species.

Table 3. Effect of 3-bromopyruvate on the length of lag phase and the subsequent linear rate of the reaction catalysed by pyruvate:ferredoxin oxidoreductase

The inhibition of pyruvate:ferredoxin oxidoreductase activity is based on the linear assay rate after the lag phase. Assay medium did not contain dithiothreitol. For comparison, an assay containing 2000 μM-pyruvate and only one-quarter of the amount of enzyme used for the assays described in the Table, gave a linear assay rate, which was 23 % of that seen with the standard amount of enzyme, and a lag phase of 190 s.

Final [3-bromopyruvate] in assay (μM)	[Pyruvate] in assay (μM)	Inhibition (%)	Lag phase time (s)
0	2000	0	100
20	2000	18	160
50	2000	32	230
100	2000	45	390
200	2000	55	510
250	2000	68	690
0	200	0	260
0.25	200	12	300
1	200	67	450
5	200	75	500
20	200	96	615

Prior addition of dithiothreitol prevented any effect of bromopyruvate on the lag phase or any inhibition of enzyme activity, presumably by covalent reaction with bromopyruvate (results not shown). However, if dithiothreitol was added to an assay during the extended lag phase caused by bromopyruvate, the progress curve was the same as if dithiothreitol had not been added. In particular, the linear reaction rate seen after the lag was inhibited to the same extent as if dithiothreitol had not been added (Fig. 3). These data suggest that neither bromopyruvate itself nor an adduct between dithiothreitol and bromopyruvate is the cause of the inhibition by bromopyruvate of the linear reaction rate (see the Discussion section).

Bromopyruvate (100 μM or 1 mM) was allowed to react completely with 1 mM-CoA or 100 μM-CoA respectively, so that two mixtures were obtained both containing 100 μM of a CoA adduct with bromopyruvate but with one containing additionally 900 μM-bromopyruvate and the other 900 μM-CoA. A 100 μl sample of each was then added to an assay containing enzyme, 2 mM-pyruvate and either 56 μM-CoA or 146 μM-CoA. For both assays the same lag phase and inhibited rate was seen, strongly suggesting that the inhibitory species is the adduct of CoA and bromopyruvate.

Inhibition by hydroxypyruvate

Surprisingly, hydroxypyruvate was a potent time-dependent irreversible inhibitor of pyruvate:ferredoxin oxidoreductase activity (Fig. 4). In contrast, preincubation of either *E. coli* pyruvate dehydrogenase complex El component (Lowe & Perham, 1984) or yeast pyruvate decarboxylase with 1 mM-hydroxypyruvate in incubation buffer containing 0.2 mM-TPP and 1 mM-$MgCl_2$ for up to 2 h at 20 °C did not result in any irreversible inhibition.

With pyruvate:ferredoxin oxidoreductase, 25 μM-hydroxypyruvate caused 50 % inactivation within 3 min, and increasing the inhibitor concentration resulted in an increase in the rate of inhibition, but saturation was seen at high concentrations (> 250 μM). Since the inhibition was measured after dilution, and little or no increase in catalytic activity occurred during the

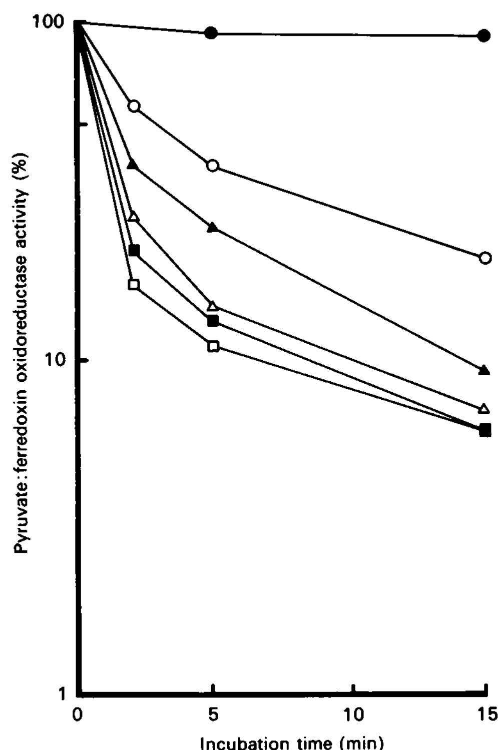


Fig. 4. Inactivation of pyruvate:ferredoxin oxidoreductase by 3-hydroxypyruvate

Pyruvate:ferredoxin oxidoreductase (2.7 units/ml) was incubated at 20 °C with various concentrations of hydroxypyruvate: 0 (●), 25 μM (○), 100 μM (▲), 250 μM (△), 500 μM (■); 1000 μM (□). At intervals, 20 μl samples were assayed for remaining enzyme activity.

assay, we conclude that the inhibition is irreversible on the time-scale of an assay.

The kinetics of inhibition were not pseudo-first-order with respect to remaining enzyme activity, and so a detailed kinetic analysis was not performed. Unlike inhibition by pyruvate, inhibition was not prevented by inclusion of 1 mM-dithiothreitol in the preincubation mixture, and it was not reversed by addition of 20 mM-dithiothreitol in the assay medium. Inclusion of 5 mM-pyruvate in a preincubation with 1 mM-hydroxypyruvate (in the presence of 1 mM-dithiothreitol to prevent pyruvate-induced inhibition) totally prevented inactivation by 1 mM-hydroxypyruvate over 60 min.

In the absence of pyruvate, but in the presence of CoA, hydroxypyruvate was a substrate for pyruvate:ferredoxin oxidoreductase, with a K_m of 0.45 mM and a $V_{max.}$ 0.16 that with pyruvate. At 1 mM-hydroxypyruvate, after a standard (1 min) lag time, a linear rate of Methyl Viologen reduction was seen for over 15 min, suggesting that under these conditions hydroxypyruvate was not acting as an irreversible inhibitor.

Inhibition by TPP analogues

Preincubation of 10 μM-thiamin thiothiazolone pyrophosphate at 0 °C for 30 min resulted in no inhibition of *T. vaginalis* pyruvate:ferredoxin oxidoreductase activity but almost complete inhibition of *E. coli* pyruvate dehydrogenase complex activity, as have previously been reported (Gutowski & Lienhard, 1976). No inhibition of oxidoreductase activity by this compound was observed with concentrations up to 1 mM and with incubation times up to 60 min at 20 °C. Similarly, preincubation of 336 μM-tetrahydro-TPP with pyruvate:ferredoxin oxidoreductase resulted in no significant inhibition of activity, whereas in an equivalent experiment using *E. coli* pyruvate dehydrogenase complex complete inhibition was obtained. With the latter enzyme 4.5 μM-tetrahydro-TPP gave 95% inhibition, as previously reported (Lowe *et al.*, 1983).

DISCUSSION

The results described in this paper demonstrate some interesting similarities and differences between the active sites for pyruvate decarboxylation in pyruvate:ferredoxin oxidoreductase and the pyruvate dehydrogenase complex. Both enzymes are inactivated by preincubation with one of their substrates, pyruvate, and both enzymes displayed a similar unusual dependence on the concentration of pyruvate. However, only the pyruvate dehydrogenase complex is irreversibly inhibited by bromopyruvate, whereas only the pyruvate: ferredoxin oxidoreductase is irreversibly inhibited by hydroxypyruvate.

Pyruvate-induced inactivation of the pyruvate dehydrogenase complex has been reported previously from the enzyme isolated from a number of sources, including pigeon breast muscle (Khailova *et al.*, 1983, 1985), pig heart (Sumegi & Alkonyi, 1983) and *Bacillus stearothermophilus* (Henderson, 1979). However, different mechanisms have been proposed to account for the inactivation.

Using pigeon muscle pyruvate dehydrogenase complex, Khailova *et al.* (1983) found a similar concentration-dependence of inhibition on pyruvate concentration, as is reported here with bacterial complex and pyruvate:ferredoxin oxidoreductase. They proposed that pyruvate bound to TPP on the enzyme was decarboxylated in the normal way, but in the presence of low pyruvate concentrations underwent a side reaction to cause inactivation. However, at higher pyruvate concentrations, inactivation is prevented, because the intermediate reacts with a second molecule of pyruvate to produce acetolactic acid and then acetoin, faster than it undergoes the inactivating side reaction. We consider that this is a good explanation of the effects that we too have described. A similar concentration-dependence for inhibition by bromopyruvate has been reported and a similar explanation proposed for it (Lowe & Perham, 1984).

The protective effect of thiol-containing compounds was also reported by Henderson (1979) and by Khailova *et al.* (1983). However, Khailova *et al.* (1983) did not comment on any protective effect of excluding oxygen, and we assume that their experiments were conducted in air-saturated buffers.

Khailova *et al.* (1985) reported that inactivation of pyruvate dehydrogenase complex by [^{14}C]pyruvate was accompanied by incorporation of radioactivity into the protein fraction and the appearance of a new peak in the absorption spectrum of the enzyme at 235 nm. This peak was attributed to the formation of a thioester bond. They demonstrated that inactivation resulted in the covalent modification of two thiol groups per El molecule. Khailova *et al.* (1985) proposed that inactivation was the result of the hydroxyethyl-TPP carbanion intermediate reacting with functionally essential thiol groups to give an acetylated form of the enzyme, and dithiothreitol prevented the inactivation by acting as an alternative acetyl acceptor. No explanation was offered as to how hydroxyethyl-TPP or the acetaldehyde/thiol adduct is oxidized to acetyl-TPP or acetyl-enzyme.

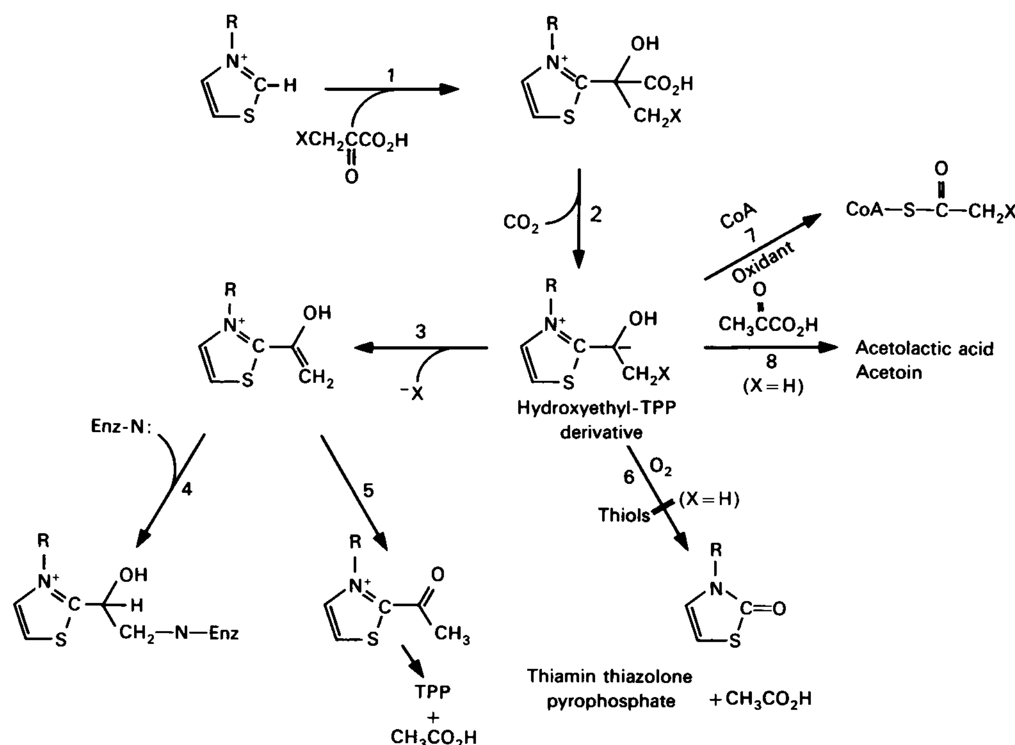
There are thus substantial indications from our results that the mechanism proposed by Khailova *et al.* (1985) may be incorrect. Firstly there is no evidence that thiol groups play an essential role in the TPP-dependent decarboxylation of pyruvate catalysed by the El component of the pyruvate dehydrogenase complex (Lowe & Perham, 1984; Henderson *et al.*, 1979) or for the functioning of the pyruvate:ferredoxin oxidoreductase enzyme. Secondly,

the oxidation state of hydroxyethyl-TPP is wrong in order to produce acetyl-enzyme; an added oxidant is required. Thirdly, we found that, with the E1 component of the *E. coli* complex, after inactivation by incubation with [2-^{14}C]pyruvate the derived radioactivity was not bound to trichloroacetic acid-precipitated protein. Indeed, enzyme could be obtained that was still inhibited but did not have radiolabel associated with it. This suggested that the formation of a covalent adduct between enzyme and pyruvate was not necessary for inactivation. Fourthly, inactivation could be prevented by excluding oxygen, and Khailova *et al.* (1985) did not propose a role for oxygen in their mechanism.

A satisfactory explanation for the pyruvate-mediated inhibition seen both in our experiments and in those of Khailova *et al.* (1983, 1985) comes from the work of Sumegi & Alkonyi (1983) on pig heart pyruvate dehydrogenase complex. They found that inactivation of this complex by pyruvate was only observed in the presence of an oxidant such as dichlorophenolindophenol. The inhibition of overall complex activity was due to both inhibition of the pyruvate decarboxylase (E1) and transacetylase (E2) components. These components were still inhibited after gel filtration. In contrast with the results of Khailova *et al.* (1983), activity was not restored by mercaptoethanol or hydroxylamine. Although Sumegi & Alkonyi (1983) found that radioactivity from [2-^{14}C]pyruvate was covalently incorporated into the enzyme, most of it was bound to the E2 component (at sites other than lipoic acid) and only a minor part was incorporated into E1. When the inactivated complex was deproteinized, a new compound absorbing at 232 nm, which potently inhibited the activity of the E1 component, was found in the supernatant. This compound was proposed to be thiamin thiazolone pyrophosphate, a known potent inhibitor of E1 (Gutowski & Lienhard, 1976). Sumegi & Alkonyi (1983) proposed a plausible mechanism to account for the pyruvate-induced inhibition. The enzyme-bound hydroxyethyl-TPP carbanion can be oxidized by extrinsic oxidants such as dichlorophenolindophenol or oxygen to give a reactive intermediate, possibly acetyl-TPP, most of which is transformed to acetate and enzyme-bound TPP. However, once every 300 turnovers inactivation occurs as the oxidized intermediate is transformed into the inhibitor, thiamin thiazolone pyrophosphate, tightly bound at the active site of E1. They further proposed that the acetyl-TPP (or equivalent) generated at the active site acetylated amino acid residues on E2, and to a lesser extent on E1, accounting for an irreversible inhibition of E2 activity that they also had observed.

Very similar observations were made by Cogoli-Greuter *et al.* (1979), who observed a pyruvate-induced inactivation of yeast pyruvate decarboxylase only in the presence of dichlorophenolindophenol. This inactivation was also not reversed by gel filtration or by the addition of mercaptoethanol or hydroxylamine. They also observed that a novel TPP derivative had been produced which appeared to be more tightly bound than TPP.

From these results on pyruvate:ferredoxin oxidoreductase, pyruvate dehydrogenase complex and its E1 component and on yeast pyruvate decarboxylase, a common mechanism can be proposed (Scheme 1). In general, inactivation of the pyruvate-decarboxylating activity is not associated with covalent incorporation of portions of the pyruvate molecule (though incorporation can occur), and it is not reversed by hydroxylamine or dithiothreitol, suggesting that the inactivation is not due to acetylation of essential groups, as proposed by Khailova *et al.* (1985). The increase in A_{235} observed by Khailova *et al.* (1985) and which was attributed to formation of a thioester bond can be



Scheme 1. Interaction of enzyme-bound TPP with 2-oxo acids

This Scheme summarizes interactions between pyruvate and its analogues with enzyme-bound TPP and the possible resulting products of the reactions. X = H (pyruvate), OH (hydroxypyruvate), F (fluoropyruvate) or Br (bromopyruvate). The substituents of the thiazole moiety of TPP, other than R (= aminodimethylpyrimidine), have been omitted for clarity. Enz-N: represents a nucleophilic side chain of an enzyme amino acid residue.

better explained as being due to conversion of TPP into thiamin thiazolone pyrophosphate (reaction 6, Scheme 1), which has an absorbance maximum at 234 nm (Gutowski & Lienhard, 1976), or an equivalent tightly bound inhibitory species. Modification of thiols would then be a secondary reaction independent of the inactivation. Dithiothreitol presumably prevents inactivation either by preventing oxidation of hydroxyethyl-TPP or by reacting with the reactive oxidized species. The exact mechanism by which thiamin thiazolone pyrophosphate is formed is unclear, but could involve attack of peroxide.

A simple explanation for the inability of the pyruvate:ferredoxin oxidoreductase to be inhibited by bromopyruvate might be that no suitably placed nucleophile is available, whereas in the pyruvate dehydrogenase complex a thiol (albeit not essential for the mechanism) is present (Lowe & Perham, 1984). As yet, we have no definitive explanation for the lag phase observed after preincubation of pyruvate:ferredoxin oxidoreductase with bromopyruvate, but possibly bromopyruvate binds at the active site and reacts *in situ* with CoA to form an adduct which is a potent inhibitor, thus giving stoicheiometric inhibition of enzyme activity. However, the inhibitor could be slowly displaced by the combination of CoA and pyruvate present in the medium, and a steady-state linear inhibited rate would then be obtained. However, this would not explain satisfactorily why addition of pre-formed (unpurified) adduct should also give a lag phase, unless there was sufficient free bromopyruvate still present in the adduct-containing mixture to allow rapid reaction at the active site. In view of the extreme potency of inhibition by bromopyruvate (Table 3), this possibility is quite likely, and indeed we would have been unable to detect such low amounts of bromopyruvate in the mixture.

Hydroxypyruvate was not an irreversible inhibitor of the E1 component of pyruvate dehydrogenase complex (Lowe & Perham, 1984), but was a potent inhibitor of pyruvate:ferredoxin oxidoreductase. Bisswanger (1981) reported irreversible inhibition of *E. coli* complex by hydroxypyruvate. Possibly this apparent discrepancy is due to a reaction with the lipoic acid groups of the complex, rather than an effect on the E1 component itself (cf. bromopyruvate; Lowe & Perham, 1984).

We presume that in the presence of CoA the carbanion intermediate produced after decarboxylation of hydroxypyruvate by pyruvate:ferredoxin oxidoreductase reacts rapidly with CoA and is released from the enzyme (reaction 7, Scheme 1). We propose that, in the absence of CoA, two competing reactions, inactivation or turnover can occur (reactions 4 or 5, Scheme 1). Turnover could result in a decrease in the concentration of the inhibitor, and might account for the curvature of the semi-log plots (Fig. 4). Protonation of the hydroxyl group of a dihydroxyethyl-TPP anion intermediate by an enzyme-bound group would make it a potential leaving group, generating a site susceptible to attack by an enzyme–nucleophile resulting in inactivation (reactions 3 and 4). If this is correct, one would conclude that the E1 component of the pyruvate dehydrogenase complex is unable to protonate the equivalent intermediate, and hence hydroxypyruvate does not inactivate this enzyme. Alternatively, the different inhibitory effects might be related to the proposed radical mechanism of pyruvate:ferredoxin oxidoreductase (Kerscher & Oesterhelt, 1981*b*).

We believe that the reason for the lack of effect on pyruvate:ferredoxin oxidoreductase by TPP analogues might be due to the extremely low off-rate of TPP from the enzyme, as witnessed by the observation that this enzyme retains TPP during the purification procedure (Williams *et al.*, 1987), which is performed in the absence of TPP. In contrast, pyruvate dehydrogenase complex does not retain stoicheiometric amounts of TPP. The extremely tight binding of TPP to the oxidoreductase suggests an alternative hypothesis for the different effects of hydroxypyruvate and bromopyruvate. Possibly hydroxypyruvate is not a truly irreversible inhibitor, but forms a tightly bound dihydroxyethyl-TPP adduct *in situ* in the active site. Elimination of hydroxide is slow, and hence the adduct is stable. Inhibition of pyruvate dehydrogenase complex does not occur, since the TPP adduct is not so tightly bound. In contrast, bromopyruvate eliminates bromide more readily, and turnover via acetyl-TPP to regenerate TPP occurs efficiently on pyruvate:ferredoxin oxidoreductase. The partitioning to form an enzyme–nucleophile adduct is low on this enzyme, whereas on pyruvate dehydrogenase complex irreversible inhibition, by reaction with an enzyme thiol, occurs once in every 100 turnovers (Lowe & Perham, 1984).

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REFERENCES

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

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

Buchanan, B. B. (1972) Enzymes 3rd Ed. **6**, 193–216

Chapman, A., Cammack, R., Linstead, D. J. & Lloyd, D. (1985) J. Gen. Microbiol. **131**, 2141–2144

Cogoli-Greuter, M., Hausner, U. & Christen, P. (1979) Eur. J. Biochem. **100**, 295–300

Docampo, R., Moreno, S. N. J. & Mason, R. P. (1987) J. Biol. Chem. **262**, 12417–12420

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

Henderson, C. E. (1979) Ph.D. Thesis, University of Cambridge

Henderson, C. E., Perham, R. N. & Finch, J. T. (1979) Cell **17**, 85–93

Kerscher, L. & Oesterhelt, D. (1981*a*) Eur. J. Biochem. **116**, 587–594

Kerscher, L. & Oesterhelt, D. (1981*b*) Eur. J. Biochem. **116**, 595–600

Kerscher, L. & Oesterhelt, D. (1982) Trends Biochem. Sci. **7**, 371–374

Khailova, L. S., Nemerya, N. S. & Severin, S. E. (1983) Biochem. Int. **7**, 423–432

Khailova, L. S., Alexandrovitch, O. V. & Severin, S. E. (1985) Biochem. Int. **10**, 291–300

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

Muller, M. (1988) Annu. Rev. Microbiol. **42**, 465–488

Perham, R. N., Packman, L. C. & Radford, S. E. (1987) Biochem. Soc. Symp. **54**, 67–81

Reed, L. J. (1974) Acc. Chem. Res. **7**, 40–46

Sumegi, B. & Alkonyi, I. (1983) Arch. Biochem. Biophys. **223**, 417–424

Wahl, R. C. & Orme-Johnson, W. H. (1987) J. Biol. Chem. **262**, 10489–10496

Williams, K. P., Lowe, P. N. & Leadlay, P. F. (1987) Biochem. J. **246**, 529–536

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