Bromopyruvate as an Active-Site-Directed Inhibitor of the Pyruvate Dehydrogenase Multienzyme Complex from *Escherichia coli*†

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ABSTRACT: Bromopyruvate behaves as an active-site-directed inhibitor of the pyruvate decarboxylase (E1) component of the pyruvate dehydrogenase complex of *Escherichia coli*. It requires the cofactor thiamin pyrophosphate (TPP) and acts initially as an inhibitor competitive with pyruvate (K_i ca. 90 μM) but then proceeds to react irreversibly with the enzyme, probably with the thiol group of a cysteine residue. E1 catalyzes the decomposition of bromopyruvate, the enzyme becoming inactivated once every 40–60 turnovers. Bromopyruvate also inactivates the intact pyruvate dehydrogenase complex in a TPP-dependent process, but the inhibition is more rapid and is mechanistically different. Under these conditions, bromopyruvate is decarboxylated, and the lipoic acid residues in the lipoate acetyltransferase (E2) component become reductively bromoacetylated. Further bromopyruvate then reacts with the new thiol groups thus generated in the lipoic acid residues, inactivating the complex. If reaction with the lipoic acid residues is prevented by prior treatment of the complex with *N*-ethylmaleimide in the presence of pyruvate, the mode of inhibition reverts to irreversible reaction with the E1 component. In both types of inhibition of E1, reaction of 1 mol of bromopyruvate/mol of E1 chain is required for complete inactivation, and all the evidence is consistent with reaction taking place at or near the pyruvate binding site.

The pyruvate dehydrogenase multienzyme complex from *Escherichia coli* catalyzes the overall reaction

$$\text{pyruvate} + \text{NAD}^+ + \text{CoA} \rightarrow \text{acetyl-CoA} + \text{NADH} + \text{H}^+ + \text{CO}_2$$

The complex is composed of multiple copies of three different polypeptide chains, responsible for the three constituent enzyme activities: pyruvate decarboxylase (E1), lipoate acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3) [reviewed by Reed (1974)]. The E1 component catalyzes the decarboxylation of pyruvate, for which thiamin phosphate (TPP)[1] is an essential cofactor, and the subsequent reductive acetylation of lipoic acid residues covalently bound to lysine residues in E2. It has been shown that there is one binding site for TPP, or for some analogues of TPP, per E1 polypeptide chain (Moe & Hammes, 1974; Gutowski & Lienhard, 1976). Binding studies with pyruvate have only been performed in the absence of TPP or in the presence of the TPP analogue thiochrome pyrophosphate (Shepherd & Hammes, 1976; Bantel-Schaal & Bisswanger, 1980), since in the presence of TPP the substrate is decarboxylated and in the presence of E2 is then converted to *S*-acetyldihydrolipoic acid bound to E2. With intact pyruvate dehydrogenase complex in the absence of TPP, 1.75 pyruvate sites were found per TPP binding site, but in the presence of thiochrome pyrophosphate, this value fell to 1.2 (Moe & Hammes, 1974; Shepherd & Hammes, 1976).

Thus, little information is available about the number of catalytically active sites on E1 and even less about their location in the polypeptide chain. We have therefore performed experiments with an alkylating derivative of pyruvate, bromopyruvate, in an attempt to label pyruvate binding sites under catalytic conditions, i.e., in the presence of MgTPP.

Materials and Methods

Enzymes and Reagents. Pyruvate dehydrogenase complex was prepared from a constitutive mutant of *E. coli* as described by Danson et al. (1979) based on the method of Reed & Mukherjee (1969). The E2E3 subcomplex and the E1 component were prepared as described by Coggins et al. (1976). Pronase (protease from *Streptomyces griseus*) and bromopyruvic acid were obtained from Sigma, and sodium pyruvate was from BDH. [1-^{14}C]Pyruvate and [2-^{14}C]pyruvate were purchased from The Radiochemical Centre, Amersham.

Enzyme Assays. Pyruvate dehydrogenase complex activity and E3 activity were measured in the direction of NAD^+ reduction, as described by Danson et al. (1978). The activity of the E1 component was measured either at 30 °C by reconstitution with methyl acetimidate modified E2E3 subcomplex, as described by Hale & Perham (1979), or at 600 nm by following the reduction of DCPIP in the presence of pyruvate, as described by Lowe et al. (1983).

Preparation of N-Ethylmaleimide-Modified Pyruvate Dehydrogenase Complex. Pyruvate dehydrogenase complex (2 mg/mL) dissolved in 50 mM potassium phosphate, 0.2 mM TPP, and 1 mM $MgCl_2$, pH 7.0, in a volume of 200 μL was incubated with 4 μL of 15 mM *N*-ethylmaleimide dissolved in acetone and 2 μL of 100 mM sodium pyruvate at 0 °C under 1 atm of N_2 for 70 min. After this time, the NAD^+ reduction activity had fallen by 96–99% and the DCPIP reduction (E1) activity by 0–9%. The mixture was applied to a Sephadex G50 centrifuge column (volume 1 mL) as described by Penefsky (1977), and the effluent produced by centrifugation was collected. This was used within 2 h for an experiment.

Preparation of Bromo[^{14}C]pyruvate. Typically, [^{14}C]pyruvate (50 μCi, 16.6 Ci/mol) was dissolved in 200 μL of glacial acetic acid (redistilled from P_2O_5) and transferred to a siliconized, stoppered glass vial. Sodium pyruvate (20 μL, 0.6 M dissolved in glacial acetic acid) and concentrated H_2SO_4

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[1] Abbreviations: TPP, thiamin pyrophosphate; DCPIP, 2,6-dichlorophenolindophenol; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; CoA, coenzyme A.

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(3 μL) were added to the vial, and it was kept at 30 °C. A solution of Br_2 (0.65 mM) in glacial acetic acid was introduced in small portions (1–10 μL) over a period of 24 h. The vial was kept tightly closed between additions to prevent loss of Br_2 or HBr. The conversion of pyruvate to bromopyruvate was monitored by TLC on silica gel plates (Kieselgel 60 F_{254}, 0.25 mm thick, Merck), with acetonitrile/ethanol/glacial acetic acid (12:4:1 v/v) as solvent. Pyruvate and bromopyruvate were detected by exposure of the plates to I_2 vapor. When about 80–90% of the pyruvate had reacted, the contents of the vial were taken to dryness in vacuo over NaOH. During this process, unreacted pyruvate was selectively lost since pyruvic acid is volatile under these conditions but bromopyruvic acid is not. The contents of the vial were twice dissolved in 200 μL of acetic acid and dried down before the mixture was dissolved in 200 μL of water. The pH was adjusted to 2 with about 280 μL of 0.4 M NaOH, and the solution was stable for several months at −20 °C. Analysis by TLC demonstrated the complete absence of pyruvate and that 85–90% of the radioactivity was associated with a spot that had the mobility of authentic bromopyruvate (R_f = 0.52; R_f of pyruvate = 0.35). About 10% of the radioactivity ran just ahead of bromopyruvate (R_f = 0.60) and may be dibromopyruvate. On addition of more Br_2, an increased proportion of this product was obtained. The final yield of bromopyruvate was about 34 μCi with a specific radioactivity of 3.7 Ci/mol.

Estimation of Pyruvate Content. Samples containing pyruvate were added to 1 mL of a solution containing 0.3 mM NADH and 50 μg of lactic dehydrogenase (Sigma type II) dissolved in 100 mM sodium phosphate buffer, pH 7.0. The decrease in A_{340} was used to calculate the amount of pyruvate originally present, assuming ϵ_{340}^{NADH} = 6.22 L mol^{-1} cm^{-1}.

Estimation of Bromopyruvate Content. Samples containing bromopyruvate (90 μL) were added to 50 μL of 2.2 mM reduced glutathione dissolved in 0.1 M potassium phosphate, pH 7.0. The mixture was left at 22 °C for 30 min. A solution of 0.3 mM Nbs_2 in 0.1 M Tris-HCl, pH 8.4 (0.86 mL), was added, and after 2 min, the absorbance was measured at 412 nm. Controls lacking bromopyruvate or glutathione were also set up, and the amount of bromoacyl group present was calculated by assuming $\epsilon_{412}^{Nbs^-}$ = 13.6 L mol^{-1} cm^{-1}.

Estimation of the Number of Thiol Groups in E1. E1 (1.4 mg/mL) dissolved in 50 mM potassium phosphate, 0.2 mM TPP, and 1 mM $MgCl_2$, pH 7.0, was incubated for 2 h at 22 °C in the absence or presence of 0.6 mM bromopyruvate. 2-Mercaptoethanol (final concentration 15 mM) was then added to each incubation, and the mixtures were exhaustively dialyzed against 20 mM sodium phosphate and 2 mM EDTA, pH 8.0, at 4 °C before being diluted to a protein concentration of about 1 mg/mL with the same buffer. Samples of each protein (0.55 mL) were placed in a cuvette, and the protein concentration was estimated by measuring A_{280}. Sodium dodecyl sulfate (10% w/v; 0.1 mL) was added, followed by 20 μL of a solution of Nbs_2 (4 mg/mL) in 0.1 M Tris-HCl, pH 8.4. From the increase in A_{412}, the number of thiol groups was calculated by assuming $\epsilon_{412}^{Nbs^-}$ = 13.6 L mol^{-1} cm^{-1}. A control was also run containing no enzyme. Each determination was repeated several times.

Estimation of Binding of Bromopyruvate. This was performed in two ways: (1) precipitation of the treated enzyme with trichloroacetic acid followed by collection onto glass fiber filters for measurement of radioactivity, as described by Stanley et al. (1981), or (2) precipitation of the treated enzyme with trichloroacetic acid followed by protein assay and radioactivity measurement by using the procedure of Peterson (1977). The second method was found preferable for low amounts of enzyme or for subsequent application of samples to polyacrylamide gels.

Estimation of Protein Content. Protein content was normally estimated after precipitation with trichloroacetic acid by using the method of Peterson (1977) with bovine serum albumin as the standard. The assay was calibrated by amino acid analysis using the amino acid composition of Harrison (1974). Amino acid analysis of proteins was carried out on an LKB 4400 amino acid analyzer, using samples that had been hydrolyzed for 24 h at 105 °C in 6 M HCl. The molecular weights of the E1, E2, and E3 polypeptide chains were taken to be 100000, 66000, and 56000, respectively, and that of the intact complex to be 6.1 × 10^6 (Reed, 1974; Danson et al., 1979; Stephens et al., 1983). A polypeptide chain stoichiometry of 1.4 mol of E1:1 mol of E2:0.5 mol of E3 was recalculated from the data of Danson et al. (1979) in light of the amino acid compositions of the subunits inferred from the DNA sequence of their genes (Stephens et al., 1983), consistent with an octahedral E2 core containing 24 E2 polypeptide chains (Reed, 1974; Danson et al., 1979). One milligram of pyruvate dehydrogenase complex should thus contain 6.0 nmol of E1, 4.3 nmol of E2, and 2.1 nmol of E3 chains.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. This was performed in tube gels containing 7.5% polyacrylamide or in slab gels containing 5% polyacrylamide. In both cases, the continuous phosphate-buffered system incorporating 0.1% sodium dodecyl sulfate was used (Perham & Thomas, 1971). Unfixed and unstained gels were sliced and counted for radioactivity as described by Brown & Perham (1976).

Protein Chemical Techniques. Proteins were digested with *Streptomyces griseus* protease and hydrolyzed with 6 M HCl, and thin-layer electrophoresis and chromatography were performed, all as described by Perham (1978).

Results

Inhibition of E1 Component and of Pyruvate Dehydrogenase Complex by Bromopyruvate. Bromopyruvate can act as a reversible competitive inhibitor of the isolated pyruvate decarboxylase (E1) component of the *E. coli* pyruvate dehydrogenase complex (Figure 1). Using the DCPIP assay for E1, we found K_i(bromopyruvate) to be 90 ± 15 μM and K_m(pyruvate) to be 10 μM. This experiment could not be performed by using the assay for the overall complex reaction since bromopyruvate reacts with the thiol groups of CoA and cysteine present in the assay mixture.

On longer incubation of isolated E1 with bromopyruvate, an irreversible inactivation occurred (Figures 2 and 3). The inhibition was irreversible, as indicated by the linearity of the assay traces with partly inactivated enzyme. Bromoacetate (1 mM) and hydroxypyruvate (1 mM) did not cause any inhibition, demonstrating that the bromoacyl group was essential for the inactivation. Addition of 2-mercaptoethanol prevented inhibition by bromopyruvate (Figure 2), presumably by reaction with the bromoacyl moiety, but it did not reverse inhibition that had already taken place.

The inhibition caused by bromopyruvate was dependent on the presence of added TPP and was prevented by the inclusion of pyruvate (Figure 2). Acetyl phosphonate and bromoacetyl phosphonate, which are believed to bind to TPP in place of pyruvate (Kluger & Pike, 1977), also reduced the rate of inhibition by bromopyruvate. These features all suggest that bromopyruvate is acting at or near the pyruvate binding site.

The kinetics of inhibition showed an unusual dependence on the concentration of inhibitor (Figure 3). With low con-

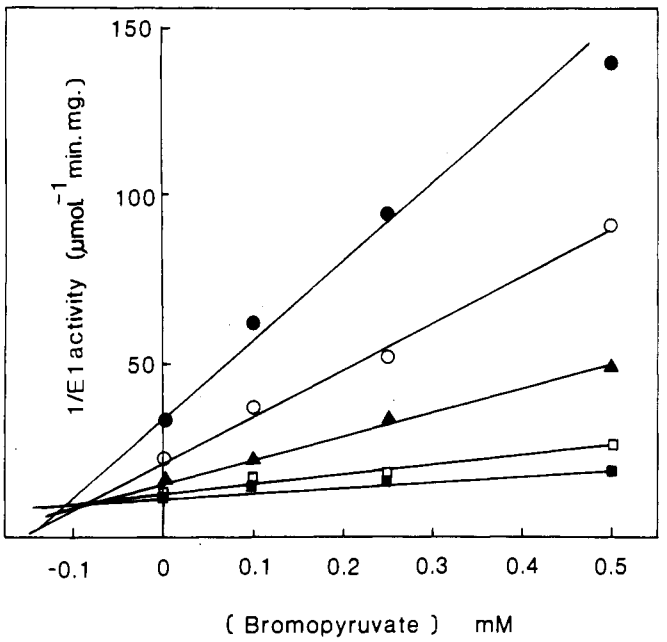


FIGURE 1: Dixon plot of reversible inhibition of E1 activity by bromopyruvate. E1 (33 μg) was added to 1 mL of assay medium containing 70 μM DCPIP. The rate of reduction of the dye was measured at 600 nm by using 5 (●), 10 (○), 20 (▲), 50 (□), and 100 (■) μM pyruvate as substrate. Rates were measured in the absence or presence of bromopyruvate (0.1, 0.25, and 0.5 mM). Bromopyruvate, if present, was added just before the assay was started with pyruvate.

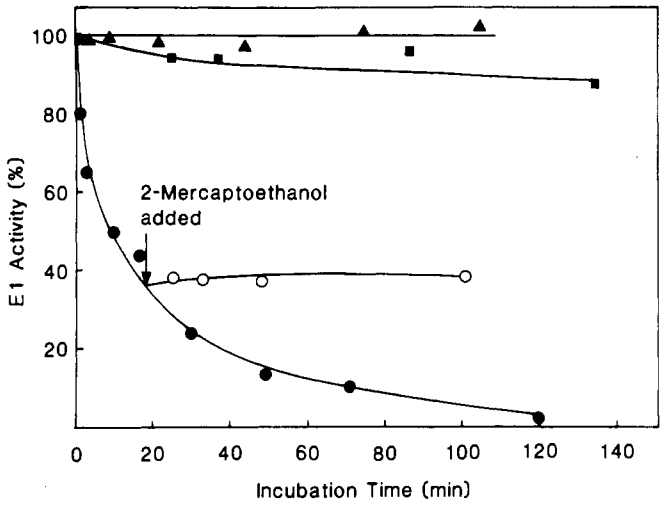


FIGURE 2: Inactivation of E1 activity by bromopyruvate; effects of TPP, pyruvate, and 2-mercaptoethanol. E1 (0.26 mg/mL) in 50 mM potassium phosphate buffer, pH 7, containing 1 mM $MgCl_2$ and 0.2 mM TPP was incubated at 22 °C with 0.52 mM bromopyruvate (●) or with 0.52 mM bromopyruvate plus 3 mM pyruvate (▲). At intervals, 2-μL samples were tested in the NAD^+ reduction assay after reconstitution with 4.6 μg of E2E3 subcomplex. After 20 min, the incubation treated with bromopyruvate alone was divided into two equal portions. One was kept, and to the other was added (○) 2-mercaptoethanol (5 mM). E1 (0.26 mg/mL) was also incubated in the absence of TPP in 50 mM potassium phosphate buffer, pH 7, containing 1 mM $MgCl_2$ and 0.52 mM bromopyruvate (■) and the activity followed as above.

centrations of bromopyruvate (<50-fold molar excess over enzyme), the rate of inhibition decreased with time, and a maximum inhibition was achieved. With higher concentrations of bromopyruvate (e.g., 250-fold molar excess), the inhibition of E1 activity was pseudo first order with respect to the remaining enzyme activity (Figure 3). As the bromopyruvate concentration was increased still further, the rate of inhibition also increased, but at very high concentrations of bromo-

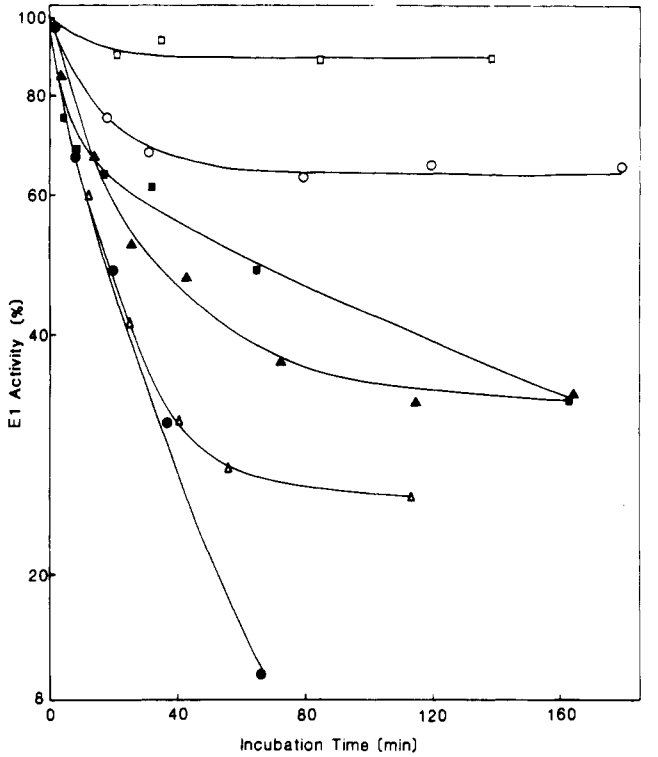


FIGURE 3: Irreversible inactivation of E1 activity by bromopyruvate; effects of concentrations of bromopyruvate of E1. E1 (0.25 or 1 mg/mL) was incubated in 50 mM potassium phosphate buffer, pH 7, containing 1 mM $MgCl_2$ and 0.2 mM TPP at 22 °C with various concentrations of bromopyruvate. At intervals, samples containing 1 μg of E1 were assayed after reconstitution with 3 μg of E2E3 subcomplex in the NAD^+ reduction assay. [E1] = 0.25 mg/mL; [bromopyruvate] = 0.011 (□), 0.055 (○), 0.11 (▲), 0.52 (●), and 9 mM (■); [E1] = 1 mg/mL; [bromopyruvate] = 0.52 mM (△).

pyruvate (>2 mM), the rate of inhibition fell again (Figure 3). This could be due to reaction of excess bromopyruvate with a TPP–bromopyruvate adduct so as to form a condensation product (cf. the known reaction of excess pyruvate to produce acetolactic acid or acetoin). It was also noted that with a fixed concentration of bromopyruvate, the maximum level of inhibition was not independent of enyzme concentration but decreased with increasing E1 concentration (Figure 3; also see next section). The rate of inhibition showed a high temperature dependence, increasing about 20-fold as the temperature was raised from 0 to 22 °C.

Incubation of bromopyruvate with intact pyruvate dehydrogenase complex caused an irreversible inhibition of overall complex activity (Figure 4), but E3 activity was unaffected. The loss of overall complex activity was dependent on the presence of TPP (Figure 4), suggesting that the inhibition is related to the normal catalytic cycle. If the inhibition was caused by bromopyruvate reacting at the pyruvate binding site(s), one might except that the inclusion of pyruvate in the incubation mixture would lower the rate of inhibition induced by bromopyruvate. However, the rate of inhibition was greatly increased by the inclusion of pyruvate (Figure 4). This suggests that under these conditions bromopyruvate was reacting with the *S*-acetyldihydrolipoic acid residues being generated on the E2 component by the inclusion of substrate, as in the pyruvate-dependent modification of lipoic acid residues on E2 by *N*-ethylmaleimide (Brown & Perham, 1976). This explanation was confirmed by the experiments utilizing radiolabeled bromopyruvate described below.

E1-Catalyzed Decomposition of Bromopyruvate. Incubation of bromopyruvate with isolated E1 caused a loss of the

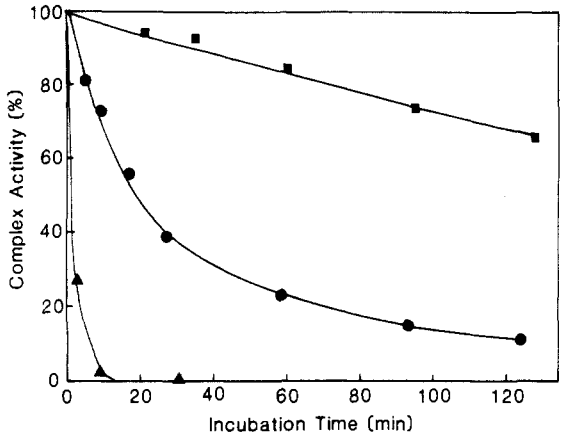


FIGURE 4: Inactivation of pyruvate dehydrogenase complex by bromopyruvate; effects of TPP and pyruvate. Pyruvate dehydrogenase complex (1 mg/mL) in 50 mM potassium phosphate buffer, pH 7, containing 1 mM $MgCl_2$ was incubated at 22 °C in the presence or absence of 0.2 mM TPP for 30 min. Bromopyruvate (0.52 mM) or pyruvate (3 mM) or bromopyruvate (0.52 mM) plus pyruvate (3 mM) was added to the enzyme, and samples (4 μL) were assayed in the NAD^+ reduction assay at various times thereafter. Bromopyruvate plus TPP (●); bromopyruvate plus pyruvate plus TPP (▲); bromopyruvate alone (■). This experiment was performed under N_2 to prevent any pyruvate-induced inhibition. Under these conditions, no change was found in the activity of enzyme samples incubated without bromopyruvate.

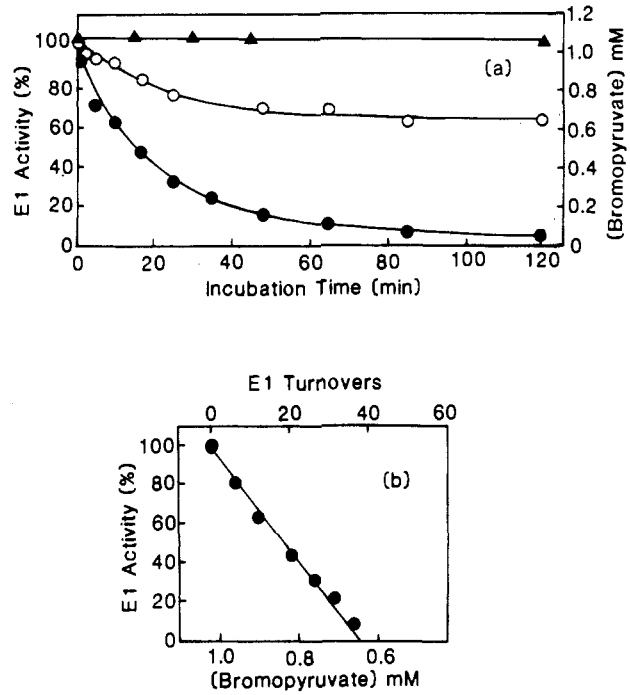


FIGURE 5: Catalytic destruction of bromopyruvate by E1. (a) Isolated E1 (1 mg/mL) in 50 mM potassium phosphate buffer, pH 7, containing 1 mM $MgCl_2$ and 0.2 mM TPP was incubated with bromopyruvate (1 mM) at 22 °C. At intervals, samples of 2 μL were assayed in the NAD^+ reduction assay after reconstitution with 5 μg of E2E3 subcomplex, and samples of 90 μL were assayed for bromopyruvate (see Materials and Methods). E1 activity in the presence of bromopyruvate plus TPP (●); bromopyruvate in the presence (○) and absence (▲) of TPP. (b) The concentration of bromopyruvate is plotted against the residual E1 activity by using the experimental results from (a).

bromoacyl group of the reagent and a simultaneous inhibition of E1 activity (Figure 5a). The two processes were directly proportional to each other, although the enzyme was present only in a catalytic amount (Figure 5b). No decomposition of bromopyruvate occurred in the absence of E1, and addition of TPP was essential for loss of the bromoacyl group (Figure 5a). These results suggest that E1 promotes a decomposition of bromopyruvate. The kinetics are consistent with bromopyruvate acting as a suicide inhibitor (Waley, 1981), the enzyme becoming inactivated once every 40–60 turnovers, a turnover being defined as the number of bromopyruvate molecules broken down per E1 chain. This could explain why the inactivation is not pseudo first order at low inhibitor concentrations (Figure 3) since the concentration of inhibitor would constantly be falling. Similarly, the maximum extent of inhibition would decrease with higher enyzme concentrations (Figure 3), since at higher enzyme concentrations, fewer enzyme turnovers would be possible with a fixed initial concentration of bromopyruvate.

Incorporation of Bromo[^{14}C]pyruvate. When bromo[2-^{14}C]pyruvate was incubated with the isolated E1 component as in Figure 5, radioactivity was found in the protein after its precipitation with trichloroacetic acid. The incorporation of radioactivity was directly proportional to the degree of inhibition induced (data not shown). Completely inhibited enzyme contained 1.0 mol of inhibitor bound/mol of E1 polypeptide chain. The incorporation was prevented by the presence of pyruvate and much diminished in the absence of added TPP. The small incorporation in the absence of added TPP may be due to the presence of TPP tightly bound to the enzyme as isolated.

When this experiment was repeated with intact pyruvate dehydrogenase complex, a much larger amount of bromo[2-^{14}C]pyruvate was incorporated before the enzyme complex was completely inhibited. Completely inhibited enzyme contained 11–15 nmol of ^{14}C per mg of complex protein in the absence of pyruvate, and 6–7 nmol of ^{14}C per mg in its presence. It appeared likely that bromopyruvate was being decarboxylated by the enzyme and that fresh bromo[2-^{14}C]pyruvate was reacting with the free thiol groups of the *S*-(bromoacetyl)dihydrolipoic acid residues that were generated on the E2 component. In support of this idea, it was found that preincubation of the enzyme complex with thiamin thiothiazolone pyrophosphate, which caused 98% inhibition of overall complex activity by inhibiting the E1 component (Gutowski & Lienhard, 1976), lowered the incorporation of radioactivity from 15 to 1.6 nmol/mg of protein under otherwise identical conditions.

In order to prevent reaction of bromopyruvate with the lipoyl groups, a sample of pyruvate dehydrogenase complex was treated with *N*-ethylmaleimide in the presence of pyruvate (see Materials and Methods). Such treatment selectively modifies the *S*-acetyldihydrolipoic acid residues (Brown & Perham, 1976). When this enzyme complex was used, bromo[2-^{14}C]pyruvate caused an inhibition of its E1 activity (DCPIP assay) at a rate similar to that observed with the isolated E1 component. At levels of inhibition up to 70%, the inhibition of E1 was directly proportional to the amount of radioactivity incorporated into the enzyme complex (Figure 6). Above 70% inhibition, the incorporation departed from linearity, reflecting some nonspecific labeling. *N*-Ethylmaleimide-modified pyruvate dehydrogenase complex whose E1 activity was abolished by subsequent incubation with bromo[2-^{14}C]pyruvate contained by extrapolation (Figure 6) approximately 7 nmol of ^{14}C/mg of enzyme, equivalent to 1.1 mol of bromopyruvate/mol of E1 chain. The presence of pyruvate completely prevented the inhibition of, and the incorporation of bromo-[2-^{14}C]pyruvate into, *N*-ethylmaleimide-modified pyruvate dehydrogenase complex. The titration curve for the TPP analogue, thiamin thiothiazolone pyrophosphate (Figure 6), was similar to that for bromopyruvate, suggesting that an equal

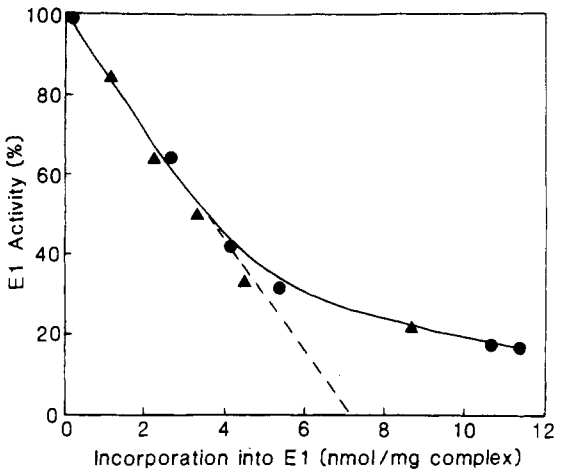


FIGURE 6: Incorporation of bromo[^{14}C]pyruvate into the E1 component of the pyruvate dehydrogenase complex. Pyruvate dehydrogenase complex modified by *N*-ethylmaleimide (NAD^+ reduction activity inhibited by 96%; 0.46 mg/mL) was incubated at 22 °C under N_2 with various concentrations (0.5, 1, 1.5, 2, 3, or 4 mM) of bromo-[2-^{14}C]pyruvate (6.1 Ci/mol) in 50 mM potassium phosphate buffer, pH 7, containing 1 mM $MgCl_2$ and 0.2 mM TPP. After 135 min, samples were assayed for NAD^+ reduction and E1 activities, for protein, and for radioactivity precipitable with trichloroacetic acid, as described under Materials and Methods. The radioactivity was corrected (●) by subtracting the amount of radiolabel bound to E2, assuming that each E2 chain can in principle bind four molecules of bromopyruvate through two lipoic acid residues but that 96% of the lipoic acid residues were modified with *N*-ethylmaleimide and hence incapable of reacting with bromopyruvate. In a parallel experiment, unmodified pyruvate dehydrogenase complex was incubated with a range of concentrations of thiamin thiothiazolone pyrophosphate. After 4 h at 22 °C, the residual E1 activity was measured and is plotted against the amount of inhibitor added expressed as nanomoles of inhibitor per milligram of pyruvate dehydrogenase complex (▲).

number of TPP and pyruvate binding sites were present.

Some experiments were performed with bromo[1-^{14}C]pyruvate in order to establish whether decarboxylation of the covalently bound bromopyruvate was taking place. If decarboxylation precedes irreversible binding, no radiolabel should be precipitated with the protein by trichloroacetic acid. In a number of experiments with isolated E1, it was found that between 0.4 and 0.7 mol of radiolabel had been bound per mol of E1 chain when the catalytic activity was completely inhibited. Unlike the results obtained with bromo[2-^{14}C]pyruvate, the incorporation of radioactivity varied from experiment to experiment, indicating that the fraction decarboxylated was dependent upon the preparation of E1 or some other experimental conditions. However, it was clear that a significant proportion (30–60%) of the bound bromopyruvate had lost its carboxyl group.

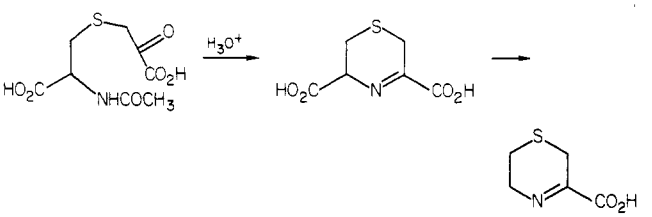
Site of Reaction of Bromopyruvate with Pyruvate Dehydrogenase Complex. Isolated E1 was treated with bromo[2-^{14}C]pyruvate in the presence of TPP, precipitated with trichloroacetic acid, and examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. All the radioactivity applied to the gel was recoverable in the protein band corresponding to E1.

Similar experiments with intact pyruvate dehydrogenase complex showed that enzyme that had been incubated with bromo[2-^{14}C]pyruvate for just sufficient time to inhibit completely the overall activity of the complex had about 75% of the incorporated radiolabel associated with the E2 chain, 17% with E1, and 8% with E3. On prolonged incubation with inhibitor, during which time the E1 activity declined and the E3 activity was unaffected, incorporation of radioactivity into the E1 chain increased. Inclusion of pyruvate in the incubation mixture caused the amount of radioactivity associated with the E2 chain to fall by 50% and almost completely prevented reaction of the inhibitor with E1. The small amount of reaction with E3 was unaffected.

When *N*-ethylmaleimide-modified pyruvate dehydrogenase complex was treated with bromo[2-^{14}C]pyruvate, a very small incorporation of radioactivity into the E2 chain was found, which corresponded with the amount of residual enzymic activity of the starting complex. However, the major radiolabeling was of the E1 component and reflected the inhibition of E1 activity: it could be prevented by adding pyruvate. The small amount of reaction with E3 still occurred.

The stoichiometry of the reaction of bromopyruvate with isolated E1 (1.0 mol/mol of E1 chain for complete inhibition) suggested that bromopyruvate might be reacting with a specific site in the protein, probably alkylating an amino acid side chain. Inhibition of E1 was rapid at pH 7.0. In model reactions at this pH, the reaction of bromopyruvate with free histidine, methionine, and *N*-acetyllysine was slow, whereas that with the thiol group of cysteine or *N*-acetylcysteine was fast. After denaturation with sodium dodecyl sulfate or urea and exposure to Nbs_2, the polypeptide chain of E1 was calculated to contain 5.6 ± 0.3 thiol groups. When this measurement was repeated with E1 that had been 90% inhibited by treatment with bromopyruvate, the number of thiol groups per chain fell to 4.5 ± 0.3, approximately one less than before.

Various attempts were made to identify the site of reaction directly. An obvious model compound, *N*-acetylcysteine, was treated with bromo[1-^{14}C]- and bromo[2-^{14}C]pyruvate. After acid hydrolysis, 84% of the radioactivity of the 1-^{14}C-labeled derivative had been lost, indicative of a facile decarboxylation reaction, whereas more than 60% of the radioactivity in the 2-^{14}C-labeled derivative was retained. The hydrolysis products were examined by thin-layer electrophoresis at pH 3.5 and chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:12:10 v/v). The major radioactive product remaining from the adduct with bromo[2-^{14}C]pyruvate did not stain with ninhydrin, was negatively charged at pH 3.5, and had a high mobility (R_f = 0.79) in the chromatographic solvent. Its identify is unknown, but it might have arisen as follows [cf. Streichman & Avi-Dor (1966)]:



There were in addition many other radioactive products visible in the autoradiograph, some of which at least were ninhydrin positive.

E1, labeled with bromo[2-^{14}C]pyruvate, was digested with Pronase (1% w/w, 37 °C for 20 h) in 0.5% ammonium bicarbonate at pH 8.0, and the products were examined by thin-layer electrophoresis and chromatography as above. Three radioactive compounds were found in the radioautograph, most of the radioactivity (67%) being associated with a compound negatively charged at pH 3.5 and having a moderate mobility (R_f = 0.46) in the chromatographic solvent. Acid hydrolysis of the Pronase digest greatly changed the pattern, the major radioactive compound being negatively charged at pH 3.5 but having a low chromatographic mobility (R_f = 0.24). Many other minor radioactive spots were detected, but there was no obvious coincidence after acid hydrolysis between any of the

major products of the reaction of bromo[2-^{14}C]pyruvate with E1 or with *N*-acetylcysteine.

Discussion

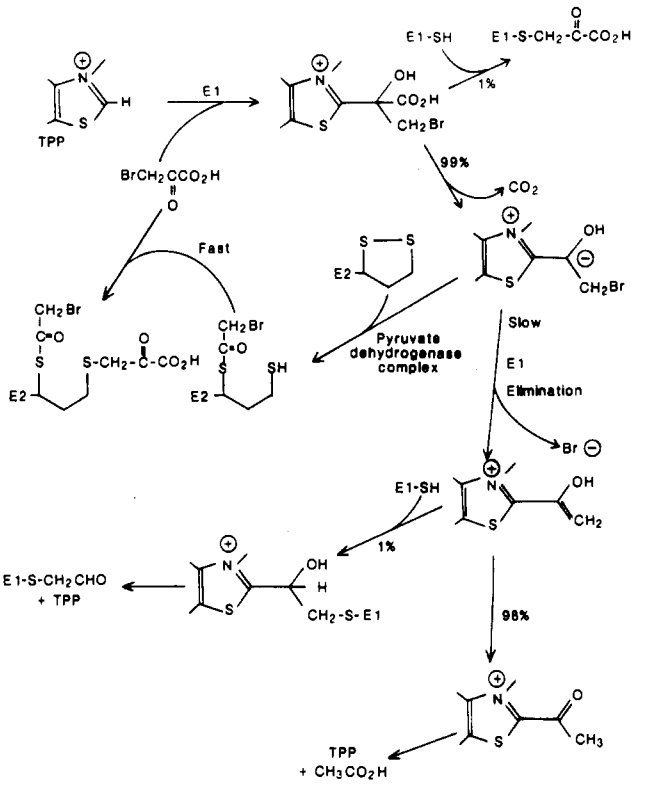
Bromopyruvate is an obvious substrate analogue to test as an active-site-directed inhibitor of the pyruvate dehydrogenase complex. It has been reported that modification of the methyl group in pyruvate to fluoromethyl, bromomethyl, and hydroxymethyl, among others, produces compounds that behave initially as strong competitive inhibitors of the pyruvate dehydrogenase complex of *E. coli* (Bisswanger, 1981), and bromopyruvate was known to inactivate the complex irreversibly, possibly by interference with its lipoic acid residues, in a TPP-dependent process (Maldonado et al., 1972). Our experiments demonstrate that bromopyruvate is an effective inhibitor of the intact complex and of the isolated E1 component but illustrate some interesting differences in its mode of action on the two forms of enzyme.

Bromopyruvate acted initially as a competitive inhibitor of the isolated E1 component (Figure 1), but prolonged incubation of the enzyme with bromopyruvate caused an irreversible inhibition which required the presence of TPP, and against which the substrate, pyruvate, protected (Figure 2). Bromopyruvate also caused an irreversible inhibition of the intact pyruvate dehydrogenase complex which was promoted by adding TPP and which was further accelerated by adding pyruvate (Figure 4). When enzyme complex was used in which the lipoic acid residues were modified by prior treatment with *N*-ethylmaleimide in the presence of pyruvate (Brown & Perham, 1976), bromopyruvate caused an inactivation of its E1 component at a rate similar to that observed with the isolated E1. Thus, in the native complex, it would appear that bromopyruvate can be decarboxylated by the enzyme to generate *S*-(bromoacetyl)dihydrolipoic residues in the E2 component and that further bromopyruvate then reacts preferentially with their free thiol groups. In the presence of pyruvate, which presumably acts as a better substrate than bromopyruvate, reductive acetylation of lipoic acid residues is favored over reductive bromoacetylation, but the free thiol groups so generated can still react rapidly with the bromopyruvate in solution.

This conclusion is strongly supported by the experiments with bromo[2-^{14}C]pyruvate. Completely inhibited enzyme complex contained 11–15 nmol of inhibitor/mg of complex in the absence of pyruvate, in good agreement with the value of 14 nmol/mg obtained by Maldonado et al. (1972). Inhibition in the presence of pyruvate led to roughly half this incorporation (6–7 nmol/mg of protein), a value that is consistent with the incorporation (6.9 nmol/mg of protein) of *N*-ethyl[2,3-^{14}C]maleimide measured in the presence of pyruvate (Danson & Perham, 1976). The latter reaction is known to be specifically with the *S*-acetyldihydrolipoic acid residues of the E2 component. Moreover, the incorporation of bromo[^{14}C]pyruvate was found by means of sodium dodecyl sulfate–polyacrylamide gel electrophoresis to be principally into the E2 component, which carries the lipoyl groups.

The isolated E1 component catalyzed the breakdown of bromopyruvate in a TPP-dependent process, the enzyme itself becoming inactivated once every 40–60 turnovers (Figure 5). We have not investigated the mechanism further, but it is almost certain to be similar to that proposed for the interaction between E1 and fluoropyruvate in which fluoride ions are eliminated (Leung & Frey, 1978). This is sketched as part of Scheme I but may not be a complete explanation since, if it were, one might expect all of the covalently bound product to be decarboxylated. However, we found that 40–70% of the

Scheme I



product retained its carboxyl group. Therefore, E1 may be able to react directly with bromopyruvate in a TPP-dependent process or with the 1-carboxy-1-hydroxybromoethyl-TPP derivative.

With the isolated E1 component, 1 mol of bromo[2-^{14}C]-pyruvate covalently bound per mol of E1 polypeptide chain caused complete inhibition of the E1 activity. Similarly, with the pyruvate dehydrogenase complex in which the lipoic acid residues were modified by prior treatment with *N*-ethylmaleimide in the presence of pyruvate, complete inactivation of its E1 activity required the reaction of approximately 1.1 mol of bromopyruvate per mol of E1 chain (Figure 6), identical with the number of binding sites for the TPP analogue, thiamin thiothiazolone pyrophosphate (Gutowski & Lienhard, 1976). This incorporation of bromo[2-^{14}C]pyruvate was dependent on TPP and prevented by pyruvate or thiamin thiothiazolone pyrophosphate. It appears that bromopyruvate can only bind covalently and specifically at the active site of E1 if it can interact with prebound TPP. Because it acts initially as a reversible inhibitor competitive with pyruvate, bromopyruvate must first bind noncovalently to E1, presumably at the same site as pyruvate. The techniques we have used would not have detected noncovalent binding in the absence of TPP, but Shepherd & Hammes (1976) and Bantel-Schaal & Bisswanger (1980) have demonstrated that pyruvate can bind to the complex in the absence of TPP. The stoichiometry of pyruvate binding differed from the number of TPP binding sites (Shepherd & Hammes, 1976; Moe & Hammes, 1974), and it remains possible that pyruvate binds at sites other than the catalytic sites.

Bromopyruvate fulfills the normal criteria of an active-site-directed inhibitor of the pyruvate dehydrogenase complex of *E. coli*, but the course of its reaction depends very much on whether or not the E1 component, its primary binding site, is part of the assembled enzyme complex. The various reactions we have documented are summarized in Scheme I. If bromopyruvate turns out also to be a selective inhibitor of the

pyruvate dehydrogenase complexes of mitochondria (Reed, 1974) or *Bacillus stearothermophilus* (Henderson et al., 1979) and *Bacillus subtilis* (Hodgson et al., 1983), in all of which the E1 component is divided into E1α and E1β chains, it should prove especially helpful in localizing the pyruvate binding site within the structure.

Registry No. Pyruvate decarboxylase, 9001-04-1; lipoate acetyltransferase, 9032-29-5; pyruvate dehydrogenase, 9014-20-4; bromopyruvate, 1113-59-3.

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Structural and Functional Influence of Enzyme–Antibody Interactions: Effects of Eight Different Monoclonal Antibodies on the Enzymatic Activity of *Escherichia coli* Tryptophan Synthase†

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ABSTRACT: Twelve monoclonal antibodies directed against the β_2 subunit of *Escherichia coli* tryptophan synthase (EC 4.2.1.20) were produced from hybridoma clones. These monoclonal antibodies are found to recognize at least eight different epitopes on β_2, and eight classes of monoclonal antibodies are thus defined. The effects of these monoclonal antibodies on the enzymatic activities of β_2 are studied. The monoclonal antibodies from three classes rapidly inhibit the serine deaminase activity catalyzed by the β_2 subunit alone; two of them lead to an inhibition plateau under stoichiometric conditions, and their inhibitory effects are cumulative. With the antibodies from two of these three classes, the tryptophan synthase activity of the $\alpha_2\beta_2$ complex is recovered, through a competition between the α subunit and the monoclonal antibody. On the contrary, the antibody from the third class is inhibitory even in the presence of an excess of α subunit. The antibodies from the five other classes, though binding easily to the coated antigen in the enzyme-linked immunosorbent assay, react only very slowly with β_2 in solution and, only after a long time of incubation, inhibit the enzymatic activity at different levels.

The tryptophan synthase from *Escherichia coli* has been shown to contain two types of subunits, α and β_2,[1] normally associated in an $\alpha_2\beta_2$ active complex (Yanofsky & Crawford, 1972). The formation of the complex, as well as the folding of both the α and β_2 subunits, has been the subject of extensive

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[1] Abbreviations: β_2, holo-β_2 (the β_2 subunit of tryptophan synthase saturated with pyridoxal 5'-phosphate); EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; PMSF, phenylmethanesulfonyl fluoride.

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