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INACTIVATION AND MODIFICATION OF PHOSPHO*ENOL*PYRUVATE CARBOXYKINASE DIFFERENTIALLY LABELED WITH BROMOPYRUVATE

RICHARD SILVERSTEIN, CHENG-CHUNG LIN *, KYLE W. FANNING and BAO-SHIN T. HUNG

Department of Biochemistry, Kansas University Medical Center, Kansas City, KS 66103 (U.S.A.)

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Summary

Phospho*enol*pyruvate carboxykinase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) is inactivated by bromopyruvate with specific substrate protection against the inactivation. Despite the fact that the enzyme also is known to possess oxalacetate decarboxylase activity, the modification does not appear to be directed toward a pyruvate or *enol*pyruvate binding site, as evident from the kinetics of the inactivation and from protection studies. Thus, the reactivity of bromopyruvate is different than toward several other enzymes where pyruvate is a substrate or product. Acetopyruvate and oxalate inhibit carboxykinase activity, but neither of these compounds, nor pyruvate, protects against the inactivation.

Using differentially labeled enzyme, it was shown that modification of one sulfhydryl is sufficient to cause loss of both catalytic activities. Protection by inosine nucleotides was found to be similar in each instance. It would appear that a common sulfhydryl is critical to both carboxykinase and oxalacetate decarboxylase activities, and that each utilizes the same nucleotide binding site, despite the known different roles of the nucleotide in each reaction.

Introduction

Phospho*enol*pyruvate carboxykinase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32), isolated and purified from several

* Present address: Pathology Department, Downstate Medical Centre, Brooklyn, NY 11203, U.S.A.

species, has been shown to be sensitive to sulfhydryl reagents. Thus, the carboxykinase activity is known to be inhibited by *p*-hydroxymercuribenzoate [1], *N*-ethylmaleimide [2], and 5,5′-dithiobis(2-nitrobenzoate) [3]. The oxalacetate decarboxylase activity of this enzyme has been much less studied in this regard, e.g. as to whether it too is inactivated by reagents that inactivate carboxykinase activity. Indeed, 2-mercaptoethanol has been shown to inhibit the oxalacetate decarboxylase activity of the chicken liver mitochondrial enzyme at concentrations that activate corresponding carboxykinase activity [4].

Detailed studies with 5,5′-dithiobis(2-nitrobenzoate) indicate a single critical sulfhydryl group for carboxykinase activity, that is vicinal to a second sulfhydryl [3]. Thus, it is quite conceivable that the sulfhydryl requirements for the two enzyme activities are quite different. Further, it is not clear to what extent the two active-sites overlap. Both activities have a similar nucleotide base specificity, but during the decarboxylase reaction nucleotide functions catalytically, whereas during the carboxykinase reaction the nucleotide acts stoichiometrically in phosphate transfer [4]. Bromopyruvate offers several advantages when examining these questions, it is a highly reactive sulfhydryl alkylating agent [5] and an analog of pyruvate, the product of the decarboxylase reaction. It is also readily prepared in isotopically labeled form to allow both studies of modification stoichiometry and identification of the amino acid residue(s) modified [6]. A preliminary report of this work has appeared previously [7].

Methods

Phosphoenolpyruvate carboxykinase. The hog liver mitochondrial and cytosol enzymes were each purified to homogeneity on sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and the mitochondrial enzyme to a specific activity of 20—25 units · mg^{-1}, as previously described [8]. The specific activity of the pure, fully active mitochondrial enzyme was taken to be 25 units · mg^{-1} and the molecular weight to be 65 000 [8] for calculations of mol of isotope incorporated per mol of enzyme inactivated. Units are expressed as μmol · ml^{-1} · min^{-1} under conditions of the phospho*enol*pyruvate carboxylation assay. Analysis of radioisotope incorporation into the enzyme and comparisons between the rates of bromopyruvate inactivation of carboxykinase and decarboxylase activities were performed with purified enzyme of high specific activity. Determinations of the kinetic order of the inactivation (Fig. 1) and determinations at higher bromopyruvate concentrations, with 0.05 mM IDP present, were performed using a 50—55% crude $(NH_4)_2SO_4$ extract that was shown to be free of interfering activities. For studies involving the oxalacetate decarboxylase activity, it was necessary to remove EDTA from purified enzyme preparations which was accomplished by gel filtration. It should be noted that the oxalacetate decarboxylase activity of the hog liver mitochondrial enzyme, like the chicken liver mitochondrial enzyme [4] is inhibited by EDTA and by Mn^{2+}. EDTA at $5 \cdot 10^{-6}$ M and $5 \cdot 10^{-5}$ M gives 58 and 100% inhibition, respectively. Mn^{2+} at $1.4 \cdot 10^{-4}$ M and $7 \cdot 10^{-4}$ M gives 50 and 100% inhibition, respectively. It should also be noted that the decarboxylase activity was also

found to be similar to that of the chicken liver enzyme in that Mn^{2+}, in low concentration, stimulates when EDTA is not previously removed. In such preparations, no activity is observed in the absence of Mn^{2+}, attributable to inhibition by EDTA.

Reagents. Bromopyruvate was first synthesized as previously described [9,10] and also obtained from Sigma Chemical Co. with equivalent results. [3-^{14}C]bromopyruvate was prepared and analyzed for specific radioactivity as previously described [10]. The starting material for this preparation sodium [3-^{14}C]pyruvate was obtained from New England Nuclear Corp. and was of the highest specific radioactivity available. [^{3}H]Bromopyruvate was a generous gift from H.P. Meloche. Phospho*enol*pyruvate, monocyclohexylammonium salt, was prepared as previously described [11], and used for the assay of enzyme activity. The monopotassium salt obtained from Sigma was shown to give equivalent results. Nucleotides, imidazole, acetopyruvate, and oxalate were Sigma products. Malate dehydrogenase, lactate dehydrogenase and NADH were obtained from Boehringer-Mannheim. Urea and sodium dodecyl sulfate were obtained from Bio-Rad Labs. Inorganic reagents were reagent-grade commercial products. Tris-HCl was obtained from Sigma, and recrystallized in the presence of EDTA [4].

Enzyme assays. Assay components of phospho*enol*pyruvate carboxylation were in 1 ml: 110 μmol imidazole-Cl, pH 5.8/5 μmol $MnCl_2$/5 μmol phospho*enol*pyruvate/0.5 μmol IDP/0.1—0.15 μmol NADH/1.0 unit, malate dehydrogenase. After a 3 min preincubation at 37°C, the assay reaction was initiated by addition of 75 μmol $NaHCO_3$. Components for the oxalacetate decarboxylation to phospho*enol*pyruvate and to pyruvate assays were as previously described [4]. These reactions were initiated after 3-min preincubation period at 37°C, upon addition of ITP and IDP, respectively.

Application of these assays to monitoring inactivation of the enzyme by bromopyruvate required immediate quenching of the inactivation process upon dilution of aliquots from the inactivation mixtures into the assay solutions. Quenching was confirmed by showing that direct injection of bromopyruvate into assay solutions containing phospho*enol*pyruvate carboxykinase results in no inactivation. Factors that contribute to effective quenching are dilution, protecting substrates, and the presence of 2-mercaptoethanol in the assay solutions. Accordingly, in studies of bromopyruvate inactivation of carboxykinase activity, in either direction, 140 μmol 2-mercaptoethanol were added to the 1 ml solutions. For monitoring oxalacetate decarboxylase inactivation by bromopyruvate, the requirements for effective quenching and reliable assay of residual activity were complicated by the inhibitory effect of 2-mercaptoethanol in high concentration and by the need for IDP protection during the preincubation period, as well as during the assay. Accordingly, aliquots of the inactivation mixture were assayed for residual levels of this activity by dilution first into a solution containing 0.04 M phosphate, pH 7.0/$2.5 \cdot 10^{-3}$ M GDP and $5 \cdot 10^{-3}$ M 2-mercaptoethanol at 0°C. Activity was fully stable for 1 h in these solutions. Subsequent assay of aliquots from this dilution for oxalacetate decarboxylase activity was initiated by the addition of 0.16 μmol of oxalacetate to the assay solutions after the usual preincubation period, final volume, 1 ml. Correction was made for nonenzymatic oxalacetate decarboxylation.

Modification of phosphoenolpyruvate carboxykinase by [3-^{14}C]bromopyruvate. The purified enzyme was first differentially labeled with $5 \cdot 10^{-4}$ M bromopyruvate in 0.04 M phosphate in the presence of $1 \cdot 10^{-3}$ M GDP, final pH 6.2, at 25°C. After a 1 h incubation, GDP and bromopyruvate were immediately removed by gel filtration. Overall recovery of activity was typically 80% or greater (Table I). The thus pretreated enzyme was then incubated with bromo[3-^{14}C]pyruvate of known specific radioactivity. After a given period of time, an aliquot was then removed for assay of residual activity (see above). Simultaneously, 2-mercaptoethanol was added to the remainder of the inactivation mixture to a final concentration of 0.14 M, sufficient to quench effectively inactivation of each activity. After 5 min at room temperature, 0.06 ml of 1.8% sodium dodecyl sulfate and 0.06 ml of 0.2 M sodium borohydride were added to chemically reduce the bromopyruvate α-keto group, and thus stabilize the incorporated radioactive label [6]. This mixture was then allowed to stand an additional 60 min at room temperature, after which time 480 mg of urea were added to a final volume of 1.1 ml, and the resulting solution chromatographed immediately on a Biogel P-10 column, 1.5 X 40.5 cm, equilibrated and eluted with 8 M urea at ambient temperature. Protein concentration of individual fractions was determined from the absorbance at 280 nm [12] and colorimetrically [13] with equivalent results. An 8 M urea blank was used in each instance. Aliquots of the corresponding column fractions were analyzed for radioactivity, correcting for quenching with [^{14}C]-toluene as internal standard. The residual content of the protein column fractions were then pooled, frozen, and utilized as described below. The control experiment confirmed that when 5 mM GDP was added to the initial incubation mixture, subsequent addition of bromo[3-^{14}C]pyruvate did not inactivate the enzyme nor was the radioactive label incorporated.

Confirmation of cysteine residue modification. The glutathione standard was modified by bromopyruvate, treated with sodium borohydride and acid hydrolyzed, as previously described [6]. The bromopyruvate modified enzyme see above, was likewise acid hydrolyzed in 6 N NCl at 110°C, for 24 h, after

TABLE I

CHEMICAL MODIFICATION OF PHOSPHO*ENOL*PYRUVATE CARBOXYKINASE BY BROMO[3-^{14}C]PYRUVATE

Incubation was in 0.04 M phosphate, (pH 6.2), with a temperature of 30°C. Expt. 1: $2.7 \cdot 10^{-4}$ M bromopyruvate, $9.58 \cdot 10^{6}$ cpm μmol^{-1} Expt. 2: $5.2 \cdot 10^{-4}$ M bromopyruvate, $7.82 \cdot 10^{6}$ cpm μmol^{-1}, incubation times 4 min, 6 min. The specific activity of the pure enzyme was taken to be 25 [8]. Total recovery of the pretreated enzyme was after incubation with GDP, bromopyruvate, and subsequent gel filtration. In the case of the 8% inactivation of carboxykinase activity, loss of activity in the absence of bromopyruvate, 8% or less, was corrected for individually. The stoichiometry is expressed in mol of isotopic label incorporated per mol of enzyme inactivated by bromopyruvate.

Expt. No.	Spec. Act.	Recovery of pretreated enzyme (%)	% Inactivation	Stoichiometry
1	24.4	95	43	0.73
2	22.0	79	61	0.57
	22.0	79	86	0.80

first removing the urea and concentrating the enzyme protein in an Amicon microultrafiltration apparatus. Descending paper chromatography was performed in three solvent systems; ethyl acetate/pyridine/H_2O (120 : 50 : 40, v/v), 2-propanol/pyridine/formic acid/H_2O (30 : 20 : 6 : 24, v/v), and 2-butanol/88% formic acid/H_2O (15 : 3 : 2, v/v) [14]. Samples and standards were run adjacent to one another on Whatman No. 1 paper. Solvent fronts were determined individually, the paper was cut into strips and the radioactivity counted for each half-inch segment.

Incubation of phosphoenolpyruvate carboxykinase with [^{3}H]bromopyruvate, analysis for detritiation. Conditions were as previously described [15].

Results

Kinetics of inactivation

Phospho*enol*pyruvate carboxykinase is rapidly and completely inactivated by bromopyruvate. Kinetics are first-order in enzyme, with extrapolation to 100% activity at initial time (Fig. 1). The kinetic order with respect to bromopyruvate is also first-order, calculated as the slope of log $(t_{1/2})^{-1}$ vs. log bromopyruvate concentration 1.01, determined for a bromopyruvate concentration range of $2.5 \cdot 10^{-5}$ M to $1.0 \cdot 10^{-4}$ M. The concentration range was extended to $1 \cdot 10^{-3}$ M bromopyruvate, by adding $5 \cdot 10^{-5}$ M IDP, a protecting substrate, to each incubation mixture. The corresponding slope, between $1 \cdot 10^{-4}$ M and $1 \cdot 10^{-3}$ M bromopyruvate was 1.03, confirming the absence of saturating kinetics of inactivation. The pseudo-first-order rate-constants, for loss of carboxykinase activity, were determined in the forward and reverse directions to be $0.91 \cdot 10^4$ min^{-1} (carboxylation) and $0.89 \cdot 10^4$ min^{-1} (decarboxylation).

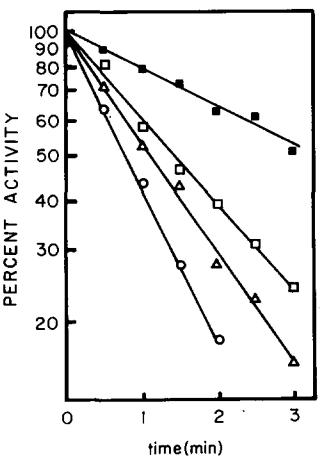


Fig. 1. Inactivation of phospho*enol*pyruvate carboxykinase by bromopyruvate. Bromopyruvate concentrations: ■———■, 0.025 mM; □———□, 0.05 mM; △———△, 0.07 mM; ○———○, 0.10 mM. Each incubation mixture also contained 0.04 M phosphate/phospho*enol*pyruvate carboxykinase. Final pH 6.2. Temperature was 37° C.

The equivalence of these rate constants and the inability to reactivate the enzyme after gel filtration are further indications that the inactivation is, in fact, irreversible. The pseudo-first-order rate-constants increase with pH as follows: pH 5.7, 6.2, 6.5, 7.0, 7.5, 8.0; corresponding rate constants: 0.62, 1.13, 2.23, 3.18, 5.64, 11.4 min^{-1}. Conditions: 0.1 mM phosphate (pH 6.2) at 37°C.

The inactivation depends significantly on the presence of the bromopyruvate α-keto group. Iodoacetate, bromoacetate, and chloroacetate do not inactivate and 3-bromopropionate inactivates very slowly. The pseudo-first-order rate-constant with 5 mM bromopropionate is 0.44 min^{-1}, as compared with 1.50 min^{-1} obtained with 0.05 mM bromopyruvate.

Inactivation of mitochondrial and cytosolic phosphoenolpyruvate carboxykinase

Mitochondrial and cytosolic phospho*enol*pyruvate carboxykinase were earlier purified to homogeneity by a common hydrophobic and affinity chromatography procedure and found to behave differently upon gel electrophoresis in the absence of sodium dodecyl sulfate [8]. We therefore compared their rates of inactivation with bromopyruvate but found the rate constants to be experimentally equivalent. With 0.05 mM bromopyruvate: 1.67 min^{-1} (mitochondrial), 1.88 min^{-1} (cytosolic), pH 6.2.

Modification with bromopyruvate

With any modification reagent, there is always the potential that the reagent may react not only at the active site but with chemically reactive groups elsewhere in the enzyme molecule. This is of particular concern with a reagent as reactive as bromopyruvate. Indeed, preliminary results with ^{14}C-labeled bromopyruvate showed 2—3 mol of label incorporated per mol of enzyme inactivated.

This problem may generally be circumvented by first modifying the enzyme with unlabeled reagent, with the active-site protected by substrate, and then later modifying the now exposed active-site with labeled reagent [6,16]. To use this method it is first necessary to find appropriate protection conditions.

Nucleotide substrates protect against carboxykinase inactivation with a base specificity that parallels that of the catalytic reaction. Thus, $2 \cdot 10^{-4}$ M GDP, GTP, or IDP protect fully, whereas $1 \cdot 10^{-3}$ M of the corresponding cytidine and uridine nucleotides protect only 9—12%, and $1 \cdot 10^{-3}$ M ADP or ATP by 24 and 17%, respectively. Conditions were using $1 \cdot 10^{-4}$ M bromopyruvate, 0.04 M phosphate (pH 6.2) with a temperature of 37°C. Percentage of protection = $100 \times (k_{\text{no nucleotide}} - k_{\text{nucleotide}})/(k_{\text{no nucleotide}})$. Phospho*enol*pyruvate and Mn^{2+} also protect, but not completely.

While the above results refer to loss of carboxykinase activity, it was found that the oxalacetate decarboxylase activity of the pure enzyme was also lost upon incubation with bromopyruvate, with a similar degree of protection conferred by inosine and guanine nucleotides (Table II).

Thus, it was decided to differentially label the enzyme first with non-radioactive bromopyruvate in the presence of the nucleotide protecting agent, GDP, and then subsequently with ^{14}C-labeled bromopyruvate. The labeled enzyme was separated from excess reagent by gel filtration and it was shown that 0.6—0.8 mol of label were incorporated per mol of enzyme inactivated (Table I).

TABLE II

PROTECTION BY IMP, IDP, ITP

Nucleotide concentration, $2 \cdot 10^{-4}$ M. Inactivation conditions were $1 \cdot 10^{-4}$ M bromopyruvate, 0.04 M phosphate, final pH, 6.2. Temperature 37°C.

Nucleotide	% Protection		
	Phospho*enol*-pyruvate carboxylation	Oxalacetate decarboxylation to phospho*enol*pyruvate	Oxalacetate decarboxylation to pyruvate
IMP	18	32	25
IDP	97	99	100
ITP	54	66	41

Further, the loss of carboxykinase activity parallels that of oxalacetate decarboxylase inactivation (Table III), indicating that one covalent modification per enzyme molecule results in loss of both activities. Descending paper chromatography of the amino acid hydrolysate, in three solvent systems, confirms that the modification is to a cysteine residue. The solvent systems, and mobilities for the modified enzyme, and the modified glutathione standard given in parentheses are as follows: 2-butanol/formic acid/H_2O: 0.152 (0.154); ethyl acetate/pyridine/H_2O: 0.044 (0.048); 2-propanol/pyridine/formic acid/H_2O: 0.695 (0.693). The profiles obtained with this last solvent system are considered to be the most significant, because of the relatively high mobilities.

The kinetic order of the reaction with respect to bromopyruvate, suggests that the enzyme has little affinity for pyruvate though not necessarily for *enol*-pyruvate. Therefore, we compared the effects of pyruvate and two analogs of *enol*pyruvate, firstly as to potential inhibition of carboxykinase activity and secondly as potential protecting agents against carboxykinase inactivation by bromopyruvate. Oxalate has, in several instances, been employed as an analog of *enol*pyruvate in transition-state analog inhibitor studies [17] and aceto-

TABLE III

LOSS OF CARBOXYKINASE AND OXALACETATE DECARBOXYLASE ACTIVITIES UPON INCUBATION OF PHOSPHO*ENOL*PYRUVATE CARBOXYKINASE WITH BROMOPYRUVATE

Conditions: 0.04 M phosphate, $5 \cdot 10^{-4}$ M bromopyruvate, phospho*enol*pyruvate carboxykinase, specific activity, 24.4 at a temperature of 30°C.

Incubation time (min)	% Inactivation	
	Carboxykinase	Oxalacetate decarboxylase *
1.5	43	31
2.5	49	43
5	65	58
8	71;68	64;62

* Each measurement of residual activity was made at three enzyme concentrations, and confirmed to be proportional to enzyme concentration. Incubation for 8 min in the absence of bromopyruvate showed no loss of either catalytic activity.

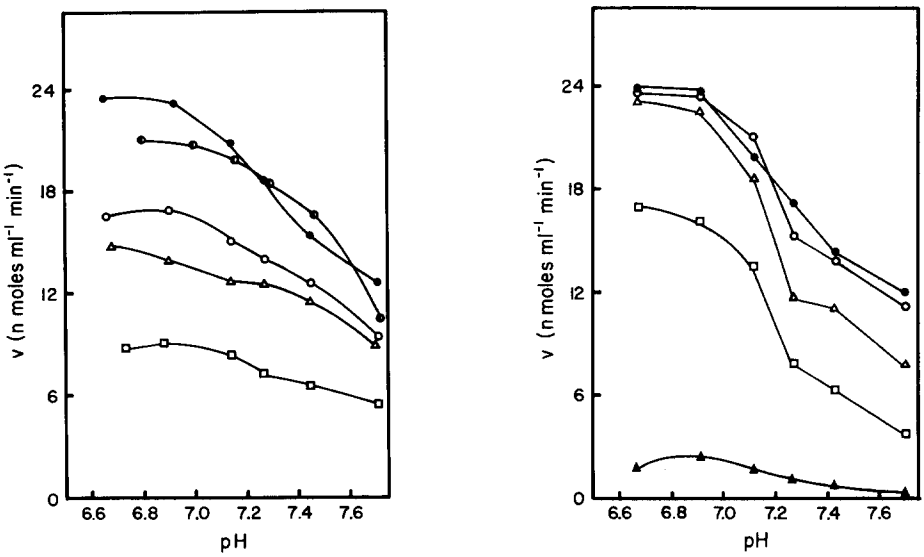


Fig. 2. Oxalate inhibition of phospho*enol*pyruvate carboxylation activity as a function of pH. The pH is that of the final assay solution. ●———●, no added components; ○———○, 1 mM oxalate; △———△, 2 mM oxalate: □———□, 5 mM oxalate; ◐———◐, 10 mM pyruvate.

Fig. 3. Same as Fig. 2, except with acetopyruvate as the added component. ●———●, no added component; ○———○, 0.1 mM acetopyruvate; △———△, 0.25 mM acetopyruvate; □———□, 0.4 mM acetopyruvate; ▲———▲, 0.7 mM acetopyruvate.

pyruvate, which is an effective inhibitor of acetoacetate decarboxylase [18], ionizes in solution to the enolate form, with a pK_a of 7.6 [19]. Pyruvate itself exists in solution primarily in the keto form. Inhibition by oxalate is essentially independent of pH, and reasonably effective, considering the concentrations of the assay reagents. For example, with 2 mM oxalate inhibition is 37% at pH 5.8, and 29% at pH 7.2 (Fig. 2). Acetopyruvate is a more effective inhibitor, with inhibition much greater at a slightly alkaline pH. Thus, at 0.25 mM acetopyruvate, for example, inhibition is only 4% at pH 5.8, but 35% at pH 7.2. With 0.4 mM acetopyruvate, inhibition increases to 30% at pH 5.8, and to 69% at pH 7.2 (Fig. 3). This concentration of acetopyruvate is still lower than that of any of the components in the carboxylation assay. The pH profiles do not follow that of a single titratable group but this could simply reflect the complexity of the assay solutions. Pyruvate has little effect on enzyme activity (Fig. 2). Acetopyruvate, pyruvate, and oxalate do not protect against inactivation by bromopyruvate, at either pH 6.2 or pH 7.5, when tested at the highest concentrations used in the inhibition studies (Figs. 2 and 3). Further indication that inactivation by bromopyruvate does not occur at an enolate binding site arises from the inability of this enzyme to catalyze the detritiation of bromo-[^{3}H]pyruvate.

Discussion

The decarboxylation of oxalacetate to phospho*enol*pyruvate catalyzed by phospho*enol*pyruvate carboxykinase may be conceived as proceeding via an *enol*pyruvate or pyruvate intermediate, enzyme bound, which is then phosphorylated by nucleotide triphosphate to phospho*enol*pyruvate. Steady-state kinetic analysis supports this general formulation [20,21], but from such an analysis alone it is not possible to distinguish between the two possibilities, or as to whether *enol*pyruvate and pyruvate are both bound to the enzyme as part of the catalytic mechanism. The same considerations apply also to the oxalacetate decarboxylase activity of the enzyme where nucleotide diphosphate acts catalytically and wherein other differences from carboxykinase activity have been found, e.g. effects of 2-mercaptoethanol, EDTA and Mn^{2+}, which inhibits rather than activates (see above).

Bromopyruvate has proved to be a most useful active-site probe of enzymes that utilize pyruvate as substrate or product during catalysis. These enzymes include 2-keto-3-deoxy-6-phosphogluconic aldolase [22], pyruvate kinase [23], pyruvate, phosphate dikinase [24] and malic enzyme [14]. In each instance, bromopyruvate inactivates with binding preceding inactivation, as evidenced by saturating kinetics of inactivation. The interpretation, in each instance, is that bromopyruvate binds to a pyruvate binding site. With the aldolase and malic enzymes, bromopyruvate has also been found to be a pseudo-substrate [14,15].

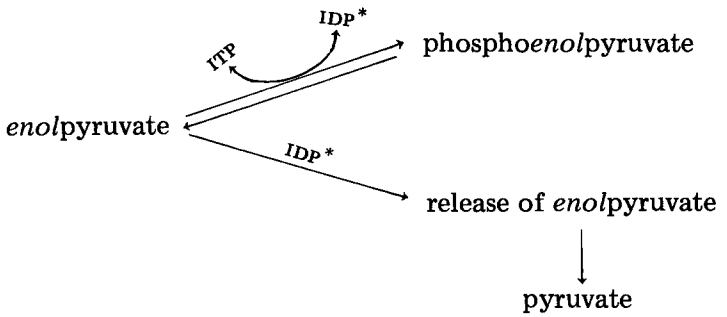
In the present work, the absence of saturation kinetics of inactivation may be taken to indicate that either (a) bromopyruvate is simply excluded from a pyruvate binding site or (b) the carboxykinase active-site accommodates *enol*pyruvate, but not pyruvate, and that the former is then phosphorylated directly. Pyruvate, from the oxalacetate decarboxylase activity, might then be produced at a second site, or nonenzymically, subsequent to release of *enol*pyruvate from the enzyme surface. This latter possibility is suggested by the isotope exchange studies of Willard and Rose with phospho*enol*pyruvate carboxytransphosphorylase [25].

The possibility that bromopyruvate is sterically excluded from the active-site due to the bromo group, comparable in bulk to a methyl group, would seem unlikely. The corresponding methyl analog of phospho*enol*pyruvate, namely phospho*enol* α-ketobutyrate, has been found to be an effective competitive inhibitor of mitochondrial phospho*enol*pyruvate carboxykinase activity, with a K_m/K_i ratio of approx. 15. Phospho*enol* α-ketobutyrate has also been found to be an effective inhibitor of the cytosolic enzyme (Silverstein, R., unpublished results).

More likely, the carboxykinase active-site binds pyruvate poorly if at all. The ability of acetopyruvate to inhibit carboxykinase activity effectively at concentrations lower than that of each substrate, and the pH dependence of that inhibition (see Results) suggests that the active-site is designed to bind the enolate in preference to the keto tautomer and further, that the transition-state of the catalyzed reaction possesses some enolate character. Preferential binding of the enolate is also consistent with the ability of oxalate, but not pyruvate, to inhibit carboxykinase activity (Fig. 2).

It should not, however, be inferred from these considerations that because

oxalacetate decarboxylase activity leads ultimately to the production of pyruvate, a second site must necessarily be invoked. On the contrary, it is more likely that both activities invole a common site, with nucleotide influencing the fate of *enol*pyruvate toward phospho*enol*pyruvate or pyruvate Scheme I). The



*Guanine nucleotides (GDP, GTP) also

Scheme I

observation that modification of one sulfhydryl per enzyme molecule is sufficient to eliminate both activities (Tables I, III) supports this formulation, and the common nucleotide protection patterns (Table II) suggests that binding to a single nucleotide binding site may be sufficient to direct the course of product formation, directly or indirectly. It is not to be inferred from Scheme I that a free flow exists from phospho*enol*pyruvate to pyruvate. Conditions that favor one part of the pathway may inhibit the other. In this regard, a divalent cation is required for carboxykinase activity while inhibiting decarboxylase activity [4]. Further, Mn^{2+} bridging of phospho*enol*pyruvate to the enzyme [26] suggests a divalent cation requirement for the conversion of phospho*enol*-pyruvate, to *enol*pyruvate, and for the reverse, according to microscopic reversibility. Thus, the requirements for *enol*pyruvate production from phospho*enol*pyruvate might be such as to inhibit subsequent pyruvate formation.

The inactivation by bromopyruvate exhibits a pH profile that is similar to that observed previously for *N*-ethylmaleimide inactivation of this enzyme [2], and is consistent with a nucleophilic displacement either by the same sulfhydryl group, or by sulfhydryl groups in similar chemical environments.

The protection effects against inactivation by bromopyruvate, and against other inactivations of phospho*enol*pyruvate carboxykinase [27,28] lend themselves to a two sub-site interpretation of the active-site: one sub-site involving carboxylation and decarboxylation with an affinity for *enol*pyruvate and enolate analogs and a second with a particular nucleotide affinity. In this context, bromopyruvate inactivates phospho*enol*pyruvate carboxykinase because it reacts with an enzyme sulfhydryl at or near the nucleotide binding sub-site, but not at the *enol*pyruvate binding site. 1-Anilinonaphthalene-8-sulfonate appears to overlap both such sites, as nucleotides and *enol*pyruvate analogs protect against inactivation of the enzyme by this reagent, as well as against inactivation by *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid [27,28].

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