Piras, R., and Vallee, B. L. (1966a), *Biochemistry 5*, 849.
Piras, R., and Vallee, B. L. (1966b), *Biochemistry 5*, 855.
Siebert, W., Fiore, L., and Dose, K. (1965), *Z. Naturforsch. 206*, 957.
Vallee, B. L. (1964), *Federation Proc. 23*, 8.

Bromopyruvate Inactivation of 2-Keto-3-deoxy-6-phosphogluconic Aldolase. I. Kinetic Evidence for Active Site Specificity*

H. Paul Meloche

ABSTRACT: 2-Keto-3-deoxy-6-phosphogluconic aldolase of *Pseudomonas putida* strain A 3.12 catalyzes the reversible condensation of pyruvate and D-glyceraldehyde 3-phosphate as well as the exchange of the methyl hydrogens of pyruvate with protons of water. Studies were undertaken to demonstrate an active site amino acid residue involved in pyruvate α-hydrogen activation by use of the pyruvate analog monobromopyruvic acid. Bromopyruvate was found to inactivate the aldolase and the inactivation was first order with respect to the remaining active enzyme. Steady-state conditions were assumed to derive kinetic expressions for enzyme–inactivator complex formation and for substrate–inactivator competition. The rate of inactivation of the aldolase was proportional to bromopyruvate at low concentration and constant at high bromopyruvate concentration. The concentration of bromopyruvate giving the half-maximum rate of inactivation was 1 mM and the rate observed at infinite bromopyruvate concentration was equivalent to an apparent first-order rate constant of 0.0115 sec^{-1} at pH 6.0 and 24.5°. Pyruvate and 2-keto-3-deoxy-6-phosphogluconate protected competitively, but D-glyceraldehyde 3-phosphate had little or no effect on the bromopyruvate inactivation rate.

The apparent enzyme–pyruvate dissociation constant at pH 6.0 in the range 0.126–0.148 mM was obtained from protection experiments. Studies with radioactive bromopyruvate showed that two carboxyketomethyl residues were stably incorporated per mole of enzyme inactivated. This value agrees with the number of pyruvates bound when this enzyme is treated with $NaBH_4$. The results are consistent with the occurrence of a basic amino acid residue adjacent to the methyl carbon of pyruvate that is bound to the active site of the enzyme.

The enzyme KDP-gluconic[1] aldolase catalyzes the reversible condensation of pyruvate and D-glyceraldehyde 3-phosphate (Meloche and Wood, 1964a). The aldolase purified from *Pseudomonas putida* (formerly *fluorescens*) A.3.12 extracts was crystallized and found to have a molecular weight of approximately 90,000 (Meloche and Wood, 1964b). There is no evidence that the enzyme requires metal ions for activity. In the presence of borohydride and labeled pyruvate, 2 moles of pyruvate is stably fixed per mole of enzyme with virtually complete loss of activity (Meloche and Wood, 1964b), suggesting that the enzyme molecule contains two active sites. The data of Ingram and Wood (1965) are consistent with an azomethine occurring between the carbonyl carbon atom of pyruvate and the ϵ amino of active site lysine being reduced by borohydride.

One of the early steps in enolate–anion reactions is carbanion formation at the carbon atom adjacent to the carbonyl. This anion generation results from the loss of a proton. Thus, among enzymes catalyzing reactions that involve carbanion generation (*e.g.*, aldolases, isomerases, and β-decarboxylases), one might expect to find an active site basic amino acid residue involved in substrate deprotonation. This in an aldolase would contribute to the over-all reaction by helping tautomerize the azomethine to the enamine form as shown in Figure 1. A basic amino acid residue having this function in the active site of KDP-gluconic aldolase could be alkylated by an analog of pyruvate in which carbon atom three is electrophilic, such as monobromopyruvic acid. Alkylation and identification of such an amino acid would contribute to the over-all knowledge of proton-transfer reactions, as well as provide a possible means of bridging active site pep-

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[1] Abbreviations used: KDP-gluconic(ate), 2-keto-3-deoxy-6-phosphogluconic(ate); CKM, carboxyketomethyl; NADH, reduced nicotinamide–adenine dinucleotide.

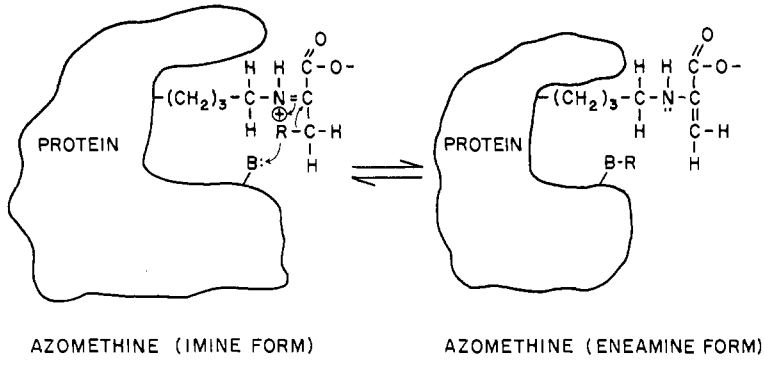

FIGURE 1: Tautomerization of the azomethine in an aldolase reaction. R represents hydrogen.

tide(s) by chemical reduction of the azomethine which should occur between the active site lysine and the CKM residue resulting from alkylation.

It will be shown in this paper that bromopyruvate does inactivate KDP-gluconic aldolase and that the reaction satisfies several criteria of active site specificity: (1) the inactivation is complete, (2) the inactivation obeys saturation kinetics, (3) pyruvate acts as a competitive inhibitor of inactivation, and (4) two CKM-residues are stably incorporated per mole of enzyme inactivated, corresponding to the assumed number of catalytic sites in the enzyme molecule.

Materials and Methods

KDP-gluconic aldolase was purified by either the method of Meloche and Wood (1964b) or Meloche *et al.* (1966) to a minimum specific activity of 7000 units/mg of protein (53% purity). The KDP-gluconate was enzymatically synthesized by the method of Meloche and Wood (1966).

Radioactive bromopyruvate was synthesized, using glacial acetic acid as solvent, by treating a mixture of sodium [^{14}C]pyruvate and freshly redistilled pyruvic acid with bromine previously dried over H_2SO_4 (Dickens, 1962). Commercial radioactive sodium pyruvate of the highest available specific activity was used. Preliminary experiments indicated that at molar ratios of pyruvic acid to sodium pyruvate less than 5, yields of bromopyruvate were poor. For synthesis, 10 mg of pyruvic acid was dissolved in 1 ml of glacial acetic acid, and this solution was used to dissolve 2 mg of radioactive sodium pyruvate. Bromine (0.1 ml) was diluted to 10 ml with acetic acid, and 0.73 ml of this solution was added to the pyruvate–acetic acid solution above. Concentrated H_2SO_4 (1 drop) was added to the reaction mixture as a catalyst (Dickens, 1962). The reaction was carried out at 65° for 1 hr in micro-reflux glassware fitted with a $CaCl_2$ drying tube. The specific radioactivity of the bromopyruvate was taken to be that of the pyruvic acid–sodium pyruvate mixture employed for synthesis, which was determined after chromatography on Dowex 1-Cl (Meloche and Wood, 1964a).

The [^{14}C]bromopyruvate reaction mixture stored in a freezer was stable for at least 5 months. Before use a sample (about 35 μmoles of [^{14}C]bromopyruvate/ml) was taken to dryness *in vacuo*. The residue was twice dissolved in a small amount of acetone and dried and finally so dissolved in 0.2 M potassium cacodylate (pH 6) as to contain 2–3.5 μmoles of bromopyruvate/ml as determined by enzymatic assay (see below). The pH of the solution was adjusted to 6 with KOH as necessary.

Nonradioactive bromopyruvic acid was synthesized by the method of Dickens (1962). The material used in this study melted at 58–59° and assayed 90–95% pure, both on titration with alkali and enzymatic assay. All other reagents used were obtained commercially.

KDP-gluconic aldolase was assayed spectrophotometrically as described by Meloche *et al.* (1966). One unit of activity was equivalent to the cleavage of 0.0242 μmole of KDP-gluconate/min. It has been determined that 9.4×10^5 units of enzymatic activity are equivalent to 1 μmole of enzyme (mol wt 90,000) containing two catalytic sites.

Pyruvate and KDP-gluconate were assayed either enzymatically or by measuring semicarbazone formation (Meloche and Wood, 1964a). Bromopyruvate upon incubation in pH 8 potassium phosphate or imidazole-HCl buffer at room temperature was rapidly hydrolyzed to hydroxypyruvate, which could be assayed either enzymatically or with semicarbizide as described for pyruvate.

Radioactivity was determined by liquid scintillation counting in an ethanol–toluene system.

Kinetic studies of bromopyruvate inactivation of the aldolase were conducted at 24.5° and pH 6. Titrametric studies of the spontaneous hydrolysis of bromopyruvate indicated that the compound was stable below pH 6.9. In a typical kinetic experiment, 1–2 μl of an appropriate dilution of bromopyruvic acid in water or acetone was added to 100 μl of 0.1 M potassium cacodylate (pH 6) containing 15–20 units of the aldolase. At various time intervals, 1-μl samples of the reaction mixture were transferred to microcuvets containing NADH, imidazole buffer, pH 8, and lactic

dehydrogenase as described for KDP-gluconic aldolase assay. Thus inactivation was terminated by dilution (1:140) and by the spontaneous conversion of bromopyruvate to hydroxypyruvate (which was then rapidly converted to glycerate by the NADH and lactic dehydrogenase). Two minutes after the last sample had been taken, 10 μl of KDP-gluconate solution was added to each cuvet and aldolase activity was assayed.

Inactivation studies with radioactive bromopyruvate were conducted using aldolase which had been pretreated with unlabeled bromopyruvate in the presence of pyruvate. This was accomplished by incubating the enzyme (>2 mg of protein/ml) in 10 mM cacodylate or phosphate buffer (pH 6) with 1 mM bromopyruvate and 40 mM sodium pyruvate for 30 min at 22°. The protein was precipitated by the addition of 0.5 g of ammonium sulfate/ml of solution and centrifugation. The pellet was dissolved in water and reprecipitated with ammonium sulfate twice more. Under these conditions of pretreatment only 10–20% enzyme activity was lost. If the pretreated enzyme was to be stored, it was dissolved in 0.02 M potassium phosphate buffer (pH 6) and frozen. If the enzyme was to be used immediately, it was dissolved in water to a concentration of about 4 mg of protein/ml. This solution was then added to an equal volume of [^{14}C]bromopyruvate in cacodylate buffer (above) and incubated at room temperature for the desired interval of time. At the end of the incubation period the protein was precipitated with ammonium sulfate three times. The final pellet was dissolved in water and assayed for residual aldolase activity. The protein was dialyzed twice against 2000 volumes of water which was sufficient to eliminate all the dialyzable radioactivity. After dialysis, the radioactivity of the protein solution was measured.

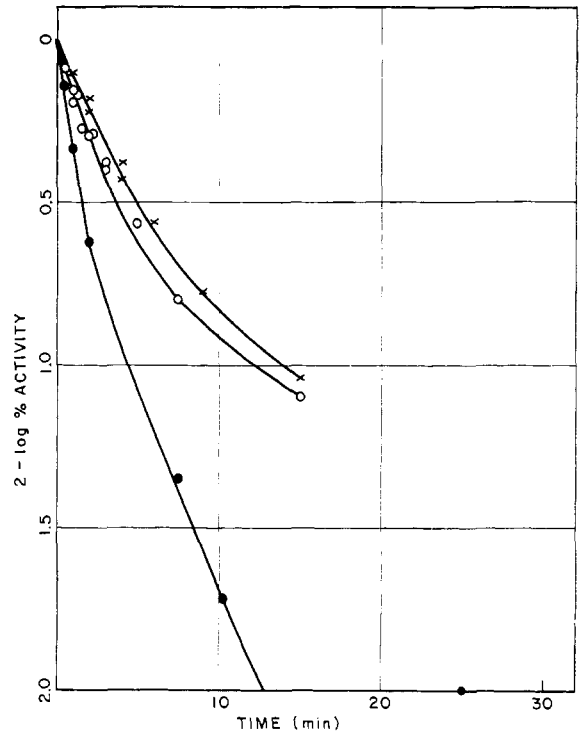

FIGURE 2: Bromopyruvate inactivation of KDP-gluconic aldolase as a function of time. Activity values on the ordinate are in exponential form where 0.0 represents 100% activity and 2.0 represents 99% inactivation. The inactivation experiment was conducted as described in Materials and Methods except that incubation was at room temperature: (●—●) 20 mM, (○—○) 1 mM, and (×—×) 0.5 mM bromopyruvate.

Results

The time course of inactivation of KDP-gluconic aldolase by three levels of bromopyruvate is shown in Figure 2. Note that inactivation is depicted on the ordinate in terms of the logarithm of the activity remaining. In the presence of 20 mM bromopyruvate at least 99% inactivation was achieved (as shown) at 25 min (on further incubation inactivation was complete), whereas 90% inactivation occurred with 1.0 and 0.5 mM bromopyruvate in the data shown. It is seen that in all three cases the reaction was pseudo first order for the first 70–75% inactivation. Finally, it is noted that the initial rate of inactivation is directly related to, but not proportional to, bromopyruvate concentration.

Other alkylating agents were not as effective as bromopyruvate. Incubation with bromoacetate at 1 mM for 15 min or 10 mM for 60 min resulted in only 30% inactivation of the aldolase at room temperature. This agrees with the result of Kovachevich and Wood (1955) with iodoacetate. In addition, α-iodoacetamide (10 mM) in 60 min at room temperature had no measurable effect on the aldolase. Thus the effect of bromopyruvate can be attributed to its structural similarity with pyruvate, suggesting that bromopyruvate alkylates within the active site. This is consistent with the complete inactivation attainable. Incubation of the aldolase with nonsaturating levels of bromopyruvate (it will be shown that 1 mM bromopyruvate half-saturates the enzyme) persistently leaves residual activity (<2%). This phenomenon as well as the deviation from pseudo-first-order kinetics will be discussed later.

The specificity of bromopyruvate for the enzyme's active site was tested applying steady-state kinetics. For this it is assumed that enzyme and bromopyruvate form a complex prior to inactivation (alkylation)

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C_{inact} \xrightarrow{k_2} E_{inact} \qquad (1)$$

where E represents free enzyme, I bromopyruvate, C_{inact} the enzyme–inactivator complex, and E_{inact} inactivated enzyme. In addition the complex concentration is in a steady state ($dC_{inact}/dt = 0$). It is further assumed that

$$[E_t] = [E] + [C_{inact}] + [E_{inact}] \qquad (2)$$

where E_t represents the sum of all forms of the enzyme.

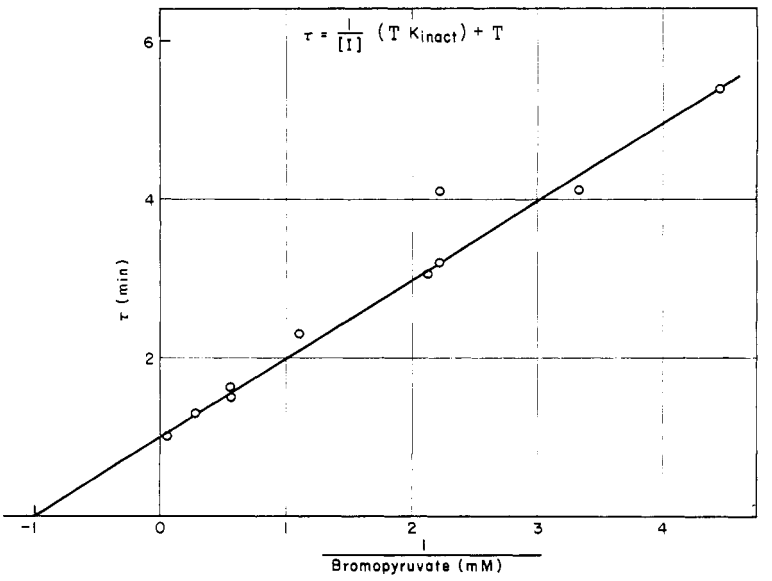

FIGURE 3: Inactivation half-time as a function of the reciprocal of bromopyruvate concentration. Conditions were as described in Materials and Methods.

Note that E and C_{inact} are catalytically active forms. Finally it is assumed that

$$v_{inact} = k_2[C_{inact}] \quad (3)$$

and

$$V_{inact} = k_2([E_t] - [E_{inact}]) \quad (4)$$

where v_{inact} represents the inactivation velocity at finite inactivator concentration [I] and V_{inact} is the inactivation velocity when [I] is infinite. From the above assumptions, the derived rate equation is

$$v_{inact} = \frac{V_{inact}}{\frac{K_{inact}}{[I]} + 1} \quad (5)$$

where K_{inact} is $(k_{-1} + k_2)/k_1$ and represents that concentration of bromopyruvate giving the half-maximum inactivation rate and presumably half-saturating the enzyme. The similarity of this rate equation to that of the simple Michaelis–Menten expression derived from steady-state assumptions should be noted.

As was seen in Figure 2, the inactivation of the enzyme was pseudo first order for the first 70–75% over a 40-fold concentration of bromopyruvate. This indicates that a kinetic measurement made within the first 75% of inactivation would represent an initial rate. Initial inactivation rates were measured by determining the time required for [I] to cause a 50% loss of enzyme activity, *i.e.*, the inactivation half-time (τ). Kinetically, τ = ln 2/first-order rate constant (Laidler, 1958); thus $\tau \propto 1/v_{inact}$ and $T \propto 1/V_{inact}$. Substituting these two terms in eq 5, the linear form of the rate expression is

$$\tau = \frac{1}{[I]}(TK_{inact}) + T \quad (6)$$

Thus a plot of τ *vs.* 1/[I] should give a straight line with intercept at T, the minimum inactivation half-time at infinite inactivator concentration. Data are shown in Figure 3. It is seen that a straight line is obtained extrapolating to a minimum inactivation half-time of 1 min. Thus the inactivation obeys saturation kinetics providing evidence that bromopyruvate and enzyme do form a dissociable complex prior to inactivation. Since bromopyruvate is structurally similar to substrate, this suggests that the reagent alkylates within the enzyme's active site. The K_{inact} for bromopyruvate is 1 mM, and a first-order rate constant of 0.0115 sec^{-1} at 24.5° (pH 6) can be calculated from T.

The kinetic studies were extended to test the effect of substrate on the rate of enzyme inactivation by bromopyruvate. Pyruvate was studied in detail since it provided a kinetic system in which substrate would bind to the enzyme without undergoing subsequent reactions. Steady-state kinetics were again used to derive an equation for competition between substrate and inactivator. Assume that

$$E + S \underset{k_{-s}}{\overset{k_s}{\rightleftharpoons}} C \quad (7)$$

where S represents pyruvate and C is the enzyme–pyruvate complex. Then the sum of all of the forms

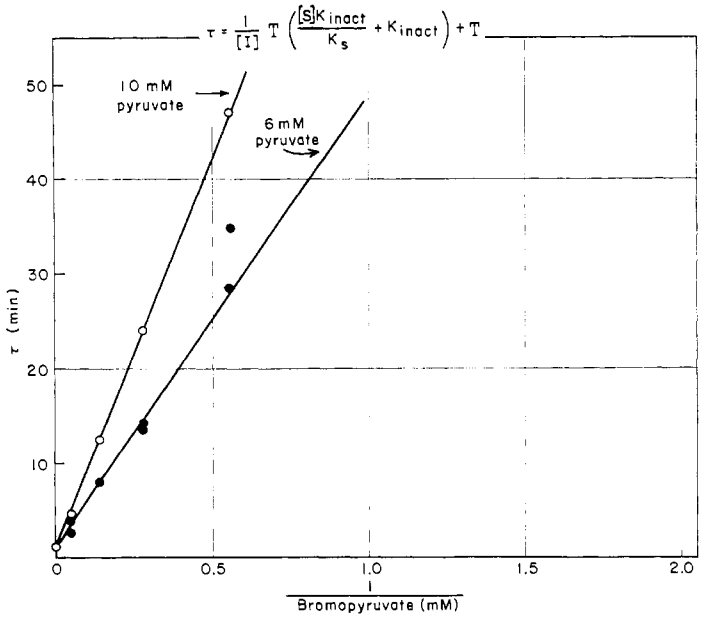

FIGURE 4: Inactivation half-time as a function of the reciprocal of bromopyruvate concentration in the presence of pyruvate. Conditions were as described in Materials and Methods.

of enzyme is

$$[E_t] = [E] + [C_{inact}] + [C] + [E_{inact}] \quad (8)$$

The rate equation derived is

$$v_{inact} = \frac{V_{inact}}{\frac{K_{inact}}{[I]} + \frac{K_{inact}}{[I]}\frac{[S]}{K_s} + 1} \quad (9)$$

where K_s is k_{-3}/k_3 and represents the apparent dissociation constant of the enzyme-pyruvate complex. The linear form of eq 9 expressed as half-time is

$$\tau = \frac{1}{[I]} T \left(K_{inact} + \frac{K_{inact}[S]}{K_s} \right) + T \quad (10)$$

Equation 10 states that in a plot of τ *vs.* 1/[I] at constant pyruvate levels, straight lines should result whose slopes are determined by the pyruvate concentration and that the lines should intercept the ordinate at the minimum inactivation half-time (1 min). In addition, from the slopes of the lines one can calculate the K_s of pyruvate. Figure 4 shows a plot of τ *vs.* 1/[I] in the presence of 10 and 6 mM pyruvate. It is seen that both lines extrapolate through 1 min on the ordinate, the previously determined T value. The slope of the line resulting from 10 mM pyruvate is 81.3 while that from 6 mM pyruvate is 48.3, corresponding to K_s values of 0.125 and 0.127 mM, respectively. These results provide kinetic evidence that both pyruvate and bromopyruvate compete for the same site on the enzyme.

Further evidence for competition could be obtained in another experiment by measuring inactivation half-times with constant bromopyruvate concentration and varying pyruvate concentrations, since eq 10 can be rearranged in the following way

$$\tau = [S]T\frac{1}{[I]}\left(\frac{K_{inact}}{K_s}\right) + \left(T + T\frac{K_{inact}}{[I]}\right) \quad (11)$$

From this equation a plot of τ *vs.* [S] should give straight lines whose slopes are proportional to 1/[I] and whose intercepts are influenced by the bromopyruvate concentration ($T + T(K_{inact}/[I])$). In addition, the K_s for pyruvate can be calculated from the slopes. In Figure 5 is shown a plot of half-time *vs.* pyruvate concentrations in the presence of 0.375, 1, 2, and 4 mM bromopyruvate. The calculated intercepts are respectively 3.66, 2.0, 1.5, and 1.25 min, values which agree with those obtained from the data (3.6, 2.0, 1.6, and 1.2). The respective slopes of the lines are 11.1, 7, 5.35, and 2.2, which correspond to K_s values of 0.242, 0.143, 0.093, and 0.114 mM. The last three values agree very well with the average value of K_s obtained from Figure 4 in which the same preparation of bromopyruvate was used. The somewhat higher value obtained with 0.375 mM bromopyruvate seems beyond experimental error but may be the result of using a different preparation of bromopyruvate.

The results shown in Figures 4 and 5 provide convincing kinetic evidence that pyruvate and bromopyruvate compete for the same site of KDP-gluconic aldolase. KDP-Gluconate was also found to retard the bromopyruvate inactivation rate. However, 8 mM

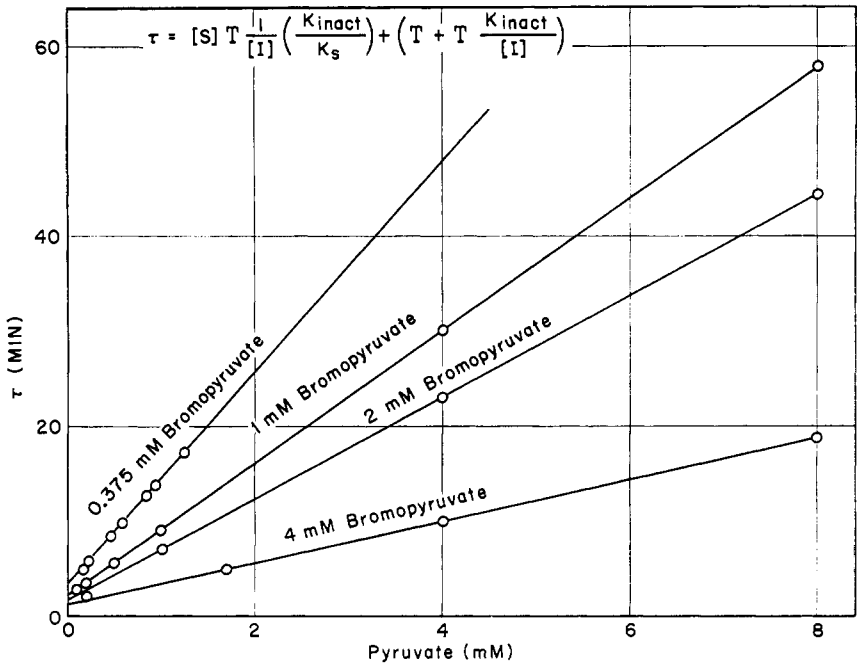

FIGURE 5: Inactivation half-time as a function of pyruvate concentration in the presence of bromopyruvate. Conditions were as described in Materials and Methods.

D-glyceraldehyde 3-phosphate[2] had no measurable effect on the inactivation half-time resulting from 1 mM bromopyruvate, indicating that only the pyruvate-specific portion of the active site is alkylated by bromopyruvate.

Further evidence for the active site specificity of bromopyruvate was obtained by determining the number of CKM residues incorporated per mole of enzyme inactivated. This was accomplished by first pretreating the enzyme with nonradioactive bromopyruvate in the presence of a large excess of pyruvate so that nonactive site amino acid residues would be preferentially alkylated. The isolated enzyme was then treated with radioactive bromopyruvate for the time periods indicated in Table I. A summary of five experiments is given with enzyme varying in specific activity from 7000 to 10,000 units/mg of protein. The counts incorporated into the protein (column 8) per specific activity of bromopyruvate gives the micromoles of CKM residues incorporated. The total units of enzyme lost in the inactivation (column 6)/9.4 $\times$ 10^5 gives the micromoles of enzyme inactivated. From these two values the calculated result (column 9) indicates the number of moles of CKM residues stably incorporated in the inactivation of a mole of enzyme. It is seen that over the last 64–98.5% inactivation of the enzyme, an average of 2.06 moles of CKM residues was stably incorporated/mole of enzyme *inactivated*. It should be noted that the degree of isotope incorporation remained constant even on prolonged exposure of the enzyme to radioactive bromopyruvate. This provides evidence that in pretreated enzyme, only two amino acid residues are capable of being alkylated by the reagent under the conditions used. That the incorporated residues are carboxyketomethyl was supported by the observation that protein resulting from [1-^{14}C]-bromopyruvate treatment lost more than 90% of its radioactivity as carbon dioxide upon treatment with excess peroxide. For comparison, Table I also shows that in the presence of radioactive pyruvate and borohydride, 99.4% of the enzyme was inactivated with the incorporation of 2.08 pyruvyl residues/mole. Thus, the number of CKM residues incorporated into the enzyme during inactivation is identical with the pyruvyl residues stably fixed by reduction of the azomethine occurring between the ϵ amino of active site lysine and pyruvate. The latter has been cited as evidence for two active sites in the enzyme molecule (Meloche and Wood, 1964b).

Discussion

A number of examples of substrate analog directed enzyme inactivations are known. Cohen and Bridger (1964) showed that heavy metal inactivation of adenylosuccinase was promoted by the substrate analog thioinosinic acid. Schoellmann and Shaw (1963) designed the substrate analog L-1-tosylamido-2-phenylethyl chloromethyl ketone as an active site reagent for α-chymotrypsin. The analog inactivated the enzyme and appeared to be specific for histidine. Gold and Fahrney (1964) studied the inactivation of α-chymotrypsin, using another substrate analog, phenylmethane-

[2] A gift from Dr. I. A. Rose.

TABLE I: Isotope Incorporation during the Inactivation of KDP-Gluconic Aldolase.[a]

Experiment	Sp Act. of Bromopyruvate (cpm/μmole)	Incubn Time (hr)	Enzyme Inactivation (total units)				Radioactivity in Protein (total cpm)	CKM Residues/ Enzyme Inactivated (moles/mole)
			Initial	Final	Inactivated	% Inactivated		
Bromopyruvate + KDP-gluconic aldolase								
1	6.17×10^5	0.33	27,875	10,100	17,775	64	23,900	2.05
2	5.9×10^5	3	26,200	5,100	21,100	80.5	27,800	2.1
3	5.9×10^5	4	36,000	2,200	33,780	93.9	43,600	2.06
4	1.1×10^6	17	20,500	500	20,000	97.5	45,700	1.955
5	1.1×10^6	17	14,200	200	14,000	98.5	35,000	2.13
								Av 2.06
	Sp Act. of sodium pyruvate (cpm/ μmole)							Pyruvate Fixed/ Enzyme Inactivated (moles/mole)
Sodium [^{14}C]pyruvate + borohydride								
6	1.1×10^6		11,000	50	10,950	99.4	26,640	2.08

[a] Conditions were as described in Materials and Methods. In expt 6, borohydride reduction was carried out as described by Grazi *et al.* (1963); the sodium pyruvate concentration was 12 mM. After reduction the protein was dialyzed to constant specific radioactivity.

sulfonyl fluoride. Their data were consistent with sulfonation of an active site serine residue which they postulated to be the same residue attacked by diisopropylphosphofluoridate. In this work was shown kinetic evidence for active site specificity, *viz.*, a double-reciprocal plot of the apparent first-order rate constant *vs.* phenylmethanesulfonyl fluoride concentration gave Michaelis–Menten kinetics.

In the present study kinetic evidence supports the conclusion that the substrate analog monobromopyruvic acid alkylates within the active site of KDP-gluconic aldolase. Kinetic studies were extended to show that both bromopyruvate and substrate (pyruvate) compete for the same site on the enzyme. Studies with radioactive bromopyruvate showed that two carboxyketomethyl residues were incorporated per mole of enzyme inactivated, agreeing with the number of active sites of the enzyme (Meloche and Wood, 1964b). To our knowledge this is the first example of an active site specific alkylating agent among the aldolases.

Two observations in these experiments that could be inconsistent with alkylation of an active site amino acid are: (1) deviation from pseudo-first-order kinetics when 70–75% inactivation has occurred, and (2) the persistence of a trace of residual activity when low levels of inactivator are used (although complete inactivation is observed at high levels of bromopyruvate). There are three possible explanations for these phenomena. First, alkylation of one of the enzyme's active sites may markedly affect the catalytic activity of the second site and its reactivity toward bromopyruvate. However, the close correlation between inactivation and radioactivity bound to the protein argues against this possibility. Secondly, during inactivation a small amount of enzyme may be converted to a form that is alkylated very slowly. The third and most interesting possibility is that the enzyme may catalyze the hydrolysis of bromopyruvate to hydroxypyruvate. This could occur if upon complex formation the reagent found itself in an "alkaline" environment, since bromopyruvate spontaneously hydrolyzes in alkali (Dickens, 1962). The mechanism of this enzyme presumes azomethine formation (Meloche and Wood, 1964b), which assumes the ϵ-amino of lysine to be uncharged (Rose, 1966). Since the pK of this amino group is 10.53 (Haurowitz, 1950) the pyruvate-specific portion of the active site of the aldolase should be equivalent to an alkaline environment. Thus if the enzyme does catalyze bromopyruvate hydrolysis, the enzyme–inactivator complex turnover resulting in 70–75% inactivation could also result in hydrolysis of enough bromopyruvate to cause a change in inactivation rate. At low initial levels of reagent, complex turnover beyond this point could effectively hydrolyze "all" of the reagent so that residual enzyme activity would remain. Then complete inactivation would only be observed at high levels of reagent.

This study was originally undertaken to locate and ultimately identify an active site amino acid involved in the activation and/or transfer of a methyl hydrogen of pyruvate. The ability of the amino acid alkylated by bromopyruvate to act as postulated depends upon its location relative to the azomethine-forming lysine. Thus, to play a catalytic role this new amino acid would have to be adjacent to the carbanion-forming carbon of native enzyme's lysine–pyruvate azomethine. An interesting experiment dependent upon this proper orientation of the CKM residue presents itself, *viz.*, chemical reduction of the azomethine which would occur between the active site lysine and the incorporated residue to yield a new secondary amino acid. Success of this step would provide information concerning the geometry of active site relative to catalytic amino acid residues. It would also provide the first example of bridging two active site amino acids implicated in catalysis through a carbon skeleton having substrate dimensions.

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References

Cohen, L. H., and Bridger, W. A. (1964), *Can. J. Biochem. 42*, 715.

Dickens, F. (1962), *Biochem. Prepn. 9*, 86.

Gold, A. M., and Fahrney, D. (1964), *Biochemistry 3*, 783.

Grazi, E., Meloche, H. P., Martinez, G., Wood, W. A., and Horecker, B. L. (1963), *Biochem. Biophys. Res. Commun. 10*, 4.

Haurowitz, F. (1950), Chemistry and Biology of Proteins, New York, N. Y., Academic, p 65.

Ingram, J. M., and Wood, W. A. (1965), *J. Biol. Chem. 240*, 4146.

Kovachevich, R., and Wood, W. A. (1955), *J. Biol. Chem. 213*, 717.

Laidler, K., Jr. (1958), The Chemical Kinetics of Enzyme Action, London, Oxford, p 23.

Meloche, H. P. (1965), *Biochem. Biophys. Res. Commun. 18*, 277.

Meloche, H. P., Ingram, J. M., and Wood, W. A. (1966), *Methods Enzymol. 9*, 520.

Meloche, H. P., and Wood, W. A. (1964a), *J. Biol. Chem. 239*, 3511.

Meloche, H. P., and Wood, W. A. (1964b), *J. Biol. Chem. 239*, 3515.

Meloche, H. P., and Wood, W. A. (1966), *Methods Enzymol. 9*, 51.

Rose, I. A. (1966), *Ann. Rev. 35*, 23.

Schoellmann, G., and Shaw, E. (1963), *Biochemistry 2*, 252.