Bromopyruvate Inactivation of 2-Keto-3-deoxy-6-phosphogalactonate Aldolase of *Pseudomonas saccharophila*. Kinetics and Stereochemistry†

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ABSTRACT: The enzyme 2-keto-3-deoxy-6-phosphogalactonate aldolase of *Pseudomonas saccharophila* is inactivated by the substrate analog β-bromopyruvate, which satisfies several criteria of being an active site directed reagent. The inactivation exhibits saturation kinetics, and both bromopyruvate and pyruvate (substrate) compete for free enzyme. Upon prolonged incubation, inactivation is virtually complete. The K_{inact} for bromopyruvate is 12 m*M* and the minimum inactivation half-time is 16 min with a *k* of 0.0433 min^{-1}. Bromopyruvate is also a substrate for the enzyme in that 3(*R,S*)-[3-$^3H_2$]bromopyruvate is asymmetrically detritiated by the enzyme yielding 3(*S*)-[3-^{3}H,H]bromopyruvate concomitant with inactivation. At various concentrations of bromopyruvate which affect the inactivation rate, the ratio of nanomoles of bromopyruvate turned over/unit of enzyme inactivated remains constant averaging 12:1, consistent with both inactivation and catalysis occurring at a single protein site, the catalytic site. The above value does not take into account a possible hydrogen isotope effect and is not thus an absolute value. The stereochemistry of bromopyruvate turnover catalyzed by this enzyme is the same as that for 2-keto-3-deoxy-6-phosphogluconate aldolase of *P. putida*. This fact provides the first evidence that the pyruvate-specific portions of the two active sites may have evolved from a common precursor.

The enzyme 2-keto-3-deoxy-6-phosphogalactonate aldolase catalyzes the condensation of pyruvate with D-glyceraldehyde-3-P (Shuster and Doudoroff, 1967) and the exchange of pyruvate's methyl hydrogens with protons of water (Meloche and Monti, 1975). The enzyme is found in extracts of *Pseudomonas saccharophila* grown on galactose or its derivatives. The catalytic reaction, mediated by this enzyme, is identical with that mediated by 2-keto-3-deoxy-6-phosphogluconate aldolase with the exception that the former enzyme must orient the *re* face while the latter the *si* face of D-glyceraldehyde-3-P's aldehydic carbon for attack at C-3 of bound enolpyruvate to generate the proper configuration at C-4 of the respective condensation products. It is of interest to compare the microenvironments of these two enzymic active sites. Initial studies could be carried out with the alkylating agent and substrate analog β-bromopyruvic acid.

Extensive work has been done on the interaction of bromopyruvate with the active site of 2-keto-3-deoxy-6-phosphogluconate aldolase of *Pseudomonas putida* (Meloche, 1965, 1967, 1970a, 1973; Meloche and Glusker, 1973; Meloche et al., 1972). It was shown that the reagent was a true substrate analog in that it formed a catalytically competent EI complex prior to covalent bond formation at a protein-bound carboxylate. The K_m for bromopyruvate as a substrate for hydrogen exchange as well as the reagent's K_{inact} as an alkylating agent had the same numerical value which was cited as evidence that both reactions occurred at the same (catalytic) site. The catalytic turnover of 3(*R,S*)-[3-$^3H_2$]bromopyruvate in H_2O formed 3(*S*)-[3-^{3}H,H]bromopyruvate (Meloche et al., 1972). The ability of the covalently fixed reagent to bridge the active site carboxylate and Schiff's base-forming lysine provided evidence that in native enzyme, the carboxylate occurred adjacent to the methyl of pyruvate bound in a catalytic step (Meloche, 1973), and led to a proposed mechanism for carboxylate participation in proton activations during catalysis (Meloche and Glusker, 1973). Thus, with 2-keto-3-deoxy-6-phosphogluconate aldolase of *P. putida*, bromopyruvate served as a useful tool for probing both the chirality and geometry of the "pyruvate" microenvironment of the active site.

Accordingly, if bromopyruvate also interacted with the active site of 2-keto-3-deoxy-6-phosphogalactonate aldolase by the above mechanism, details of the geometry and chirality of the pyruvate portions of the active sites of the two aldolases could be compared. Initial studies showing the reagent to be an active site directed alkylating agent as well as a substrate, and the stereochemistry of bromopyruvate turnover are reported in this paper.

Methods

Enzyme Purification. Enzyme was purified from sonic extracts of *Pseudomonas saccharophila* grown in the mineral media described by Shuster and Doudoroff (1967) containing 0.2% galactose. The organism was grown to the stationary phase in 20-l. carboys using forced aeration through a sparger (0.17 volume of air per volume of medium per min). Cells were harvested by centrifugation in a continuous flow system.

The enzyme was assayed at 24.5° by measuring ΔA_{340} in a 150-μl volume using DPNH and excess lactic dehydrogenase in 50 m*M* imidazole (pH 7.5). The concentration of sodium 2-keto-3-deoxy-6-phosphogalactonate was 1.3 m*M*. A unit of activity is defined as the cleavage of 1 μmol of substrate/min. The 2-keto-3-deoxy-6-phosphogalactonate

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used early in the study was a generous gift of Dr. L. Szabó, Universite de Paris, Orsay, France. Subsequent work was done using substrate produced by a net condensation between pyruvate and D-glyceraldehyde-3-P, as modified from the procedure of Meloche and Wood (1966), catalyzed by the aldolase which had been resolved from 2-keto-3-deoxy-6-phosphogluconate aldolase.

Crude extracts were prepared by sonication of cell pastes suspended in twice their weight of 50 m*M* phosphate buffer (pH 6.5). The ratio of 2-keto-3-deoxy-6-phosphogalactonate aldolase to 2-keto-3-deoxy-6-phosphogluconate aldolase in crude extracts ranged 1.8:1. Details of the resolution of aldolase activities as well as the purification procedure outlined below will be published elsewhere. Initial separation of the two aldolases was achieved as follows. The crude extract was heated to 60° and then centrifuged. The supernatant was dialyzed against 50 m*M* phosphate (pH 6.5) and then passed through a DEAE-cellulose column equilibrated against the same buffer, and containing a bed resin volume equivalent to five times the volume of the extract to be resolved. The 2-keto-3-deoxy-6-phosphogalactonate aldolase was found to wash through the column eluted with 50 m*M* pH 6.5 phosphate buffer. The recovery of activity ranged 60%, and the ratio of aldolase activities increased to 20:1 favoring the 2-keto-3-deoxy-6-phosphogalactonate enzyme. The pooled tubes were treated with ammonium sulfate (2.5 *M*) to precipitate the protein which was then dissolved in a small volume of, and dialyzed against, 100 m*M* NH_4HCO_3.

Further separation of the two aldolases was achieved by chromatography on DEAE-Sephadex A-50 equilibrated against 100 m*M* NH_4HCO_3. The sample from above was applied to the column which was then washed with the equilibrating buffer until A_{280} returned to base line. Additional proteins were then eluted as a single peak with 100 m*M* NH_4HCO_3–250 m*M* NaCl. 2-Keto-3-deoxy-6-phosphogalactonate aldolase activity is associated with the backside of the peak. Yield is 50% of activity applied and the ratio of aldolase activities increased to 40:1. Protein was then concentrated by ultrafiltration.

Further purification was achieved by two sequential gel filtrations on a 2.5 × 60 cm Sephadex G-150 column equilibrated against 50 m*M* phosphate (pH 6.5). The resulting protein is not homogeneous but does represent a 250-fold purification in 20% overall yield at a specific activity of 30 units/mg of protein.

Where indicated in the text, enzyme was pretreated with 1 m*M* sodium bromopyruvate in the presence of 20 m*M* sodium pyruvate in 50 m*M* citrate buffer (pH 6.5) prior to use. This procedure derivatizes, preferentially, bromopyruvate-sensitive amino acid residues not associated with the catalytic site. Incubation was carried out for 30 min at room temperature, and then the derivatized protein was resolved from reagents by chromatography on a Sephadex G-25 column (2.5 × 30 cm) eluted with 10 m*M* sodium chloride. The protein was concentrated by ultrafiltration. Activity recovery ranged 85%.

Randomly tritiated bromopyruvate was synthesized by bromination of randomly tritiated sodium pyruvate–nonlabeled pyruvic acid using HCl as catalyst (Meloche, 1970a,b). Tritiated bromopyruvate was recovered as the crystalline sodium salt as follows. The tritiated free acid was diluted fourfold with nonlabeled sodium bromopyruvate and the pH was *carefully* adjusted to 3 with dilute $NaHCO_3$ as required, then dried in vacuo. The residue dissolved in water (1500 μmol/ml) was treated with 5 volumes of ethanol. Crystallization initiated upon scratching with a glass rod and was complete upon overnight storage in a refrigerator. Crystals were recovered by centrifugation and dried in vacuo. Yield was 50%. Upon enzymatic analysis (see below) the preparation was determined to be 102% pure by weight. The radiochemical purity of the tritiated sodium bromopyruvate was visualized as follows. A sample was treated with H_2O_2 in large excess which converts tritiated bromopyruvate to tritiated bromoacetate, and any tritiated pyruvate to tritiated acetate. The reaction mixture was then chromatographed on a 1 × 15 cm column of Dowex-1-chloride using a linear 0–0.1 *N* HCl gradient and collecting fractions. Tritium was monitored by liquid scintillation counting either in an ethanol–toluene or Triton–toluene cocktail and correcting for quench. All of the applied tritium was recovered. Only 0.2% was found at the elution position of acetate (or glycolate) while the remaining 99.8% chromatographed as bromoacetate, consistent with the tritiated bromopyruvate being virtually free of tritiated pyruvate.

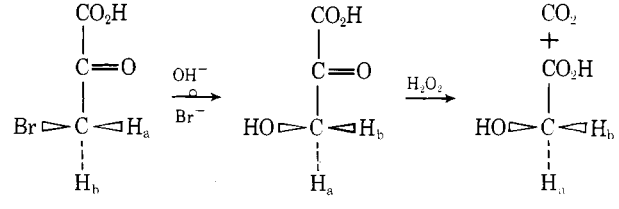
Bromopyruvate was obtained as the free acid from Sigma Chemical Co. The free acid was converted to the sodium salt by *carefully* adjusting the pH to 3 with $NaHCO_3$ solution. This was converted to the crystalline sodium salt as described above.

Pyruvate and bromopyruvate were assayed by monitoring ΔA_{340} in a system containing DPNH and excess lactic dehydrogenase in 50 m*M* imidazole (pH 7.5). The total volume was 150 μl. For bromopyruvate assay it is assumed that upon dilution bromopyruvate is hydrolyzed to hydroxypyruvate which is reduced by DPNH.

Aldolase inactivation studies were carried out at 24.5° in 50 m*M* citrate (pH 6.5). Residual aldolase activity was determined by adding 0.2–1.0 μl samples to cuvets containing DPNH, lactic dehydrogenase, and imidazole buffer. After 15-min incubation to destroy bromopyruvate, the residual aldolase was assayed upon addition of 2-keto-3-deoxy-6-phosphogalactonate. It was assumed that inactivation was terminated upon addition of the sample to the cuvet, predominantly, by dilution (0.2–1.0 μl → 130 μl).

The stereochemistry of 3(*R,S*)-[3-$^3H_2$]bromopyruvate turnover was determined as follows. Asymmetrically labeled [3-^{3}H,H]bromopyruvate was adjusted to pH 9 with $NaHCO_3$ solution to hydrolyze the bromide, thereby generating hydroxypyruvate. In this process inversion of configuration at C-3 occurs (Hughes et al., 1950). After 15-min incubation, excess H_2O_2 is added decarboxylating hydroxypyruvate with the formation of glycolate. The sequence of events is shown in Scheme I, where bromopyruvate of fixed

Scheme I



configuration at C-3 is converted to glycolate of opposite configuration at C-2. Glycolate was recovered by chromatography on Dowex-1-Cl as described above, and located by scintillation counting and assay with 2,7-naphthalenediol (Calkins, 1943). The pooled peak was adjusted to pH 6 with NaOH and dried in vacuo. The residue, dissolved in <5 ml

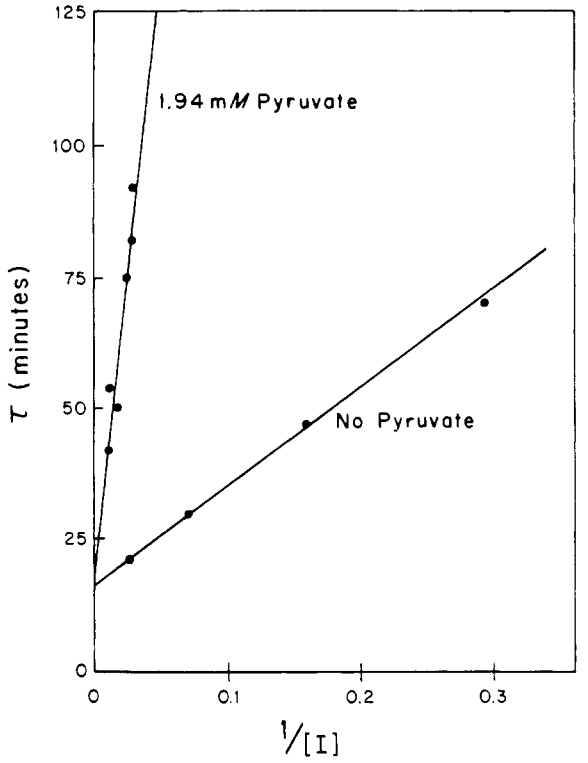


FIGURE 1: Saturation kinetics and substrate competition in bromopyruvate inactivation of 2-keto-3-deoxy-6-phosphogalactonate aldolase.

of H_2O was then chromatographed on Sephadex G-10 and eluted with water to remove the bulk of NaCl. Authentic 2(*R,S*)-[2-$^3H_2$]glycolate was synthesized by degradation of 3(*R,S*)-[3-$^3H_2$]bromopyruvate. The sodium glycolate, located by scintillation counting of fractions, was pooled and dried in vacuo. The configuration at C-2 of asymmetrically tritiated glycolate was determined using glycolic acid oxidase which is specific for L-lactate (Zelitch, 1955) and thus is stereospecific for the *pro-R*[1] hydrogen at C-2 of glycolate (Rose, 1958) as confirmed by Johnson et al., (1965). Noncovalently bound tritium was determined by passing an aliquot of the reaction mixture through a 4 × 0.75 column of Dowex-1-Cl, washing with 3 ml of H_2O, and counting a portion of the wash.

Results

Bromopyruvate Inactivation of 2-Keto-3-deoxy-6-phosphogalactonate Aldolase. Preliminary experiments showed that the enzyme was inactivated by bromopyruvate, and that the reaction was first order through the first 70% of activity loss, after which the reaction slowed. In this respect, the inactivation was analogous to the bromopyruvate–2-keto-3-deoxy-6-phosphogluconate aldolase interaction previously studied where inactivation was first order through the first 70% of activity loss and then slowed (Meloche, 1967). This could well result from bromopyruvate's reacting slowly at an amino acid residue(s) not critical to catalysis, but whose derivatization perturbs kinetic parameters for inactivation. However, it was clear that the first 50% activity loss was within the linear portion of the rapid reaction, so that determination of inactivation half-time would be a valid measure of the inactivation rate.

[1] The prochirality of ligands was determined using the priority of mass number rule of Cahn et al. (1966) assuming that the ligand being determined had priority over its enantiomeric position.

In previous work, using the steady-state assumption for the interaction

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

where E is free enzyme, I inactivator, C_{inact} the EI complex (catalytically active), and E_{dead} inactivated enzyme, a rate equation was derived, which in linear form is

$$\tau = (1/[I])(TK_{inact}) + T \qquad (2)$$

where τ is inactivation half-time at [I], T is the inactivation half-time when [I] is infinite, and K_{inact} is $[(k_2 + k_{-1})/k_1]$ and represents [I] giving T/2 (Meloche, 1967). The equation predicts that in a plot of τ vs. 1/[I] a straight line is generated which extrapolates to a finite inactivation half-time (T) as 1/[I] approaches zero. From the slope of the line one calculates K_{inact}. Data are presented in Figure 1. In the absence of the added substrate pyruvate, the inactivation of 2-keto-3-deoxy-6-phosphogalactonate aldolase obeys eq 2, giving a T of 16 min and a K_{inact} of 12 m*M*. From T, *k* for inactivation calculates to 0.0433 min^{-1}.

Equation 2 can be expanded, assuming both I and S (substrate) compete for free enzyme, to give the linear relationship

$$\tau = \frac{1}{[I]} T\left(K_{inact} + \frac{K_{inact}[S]}{K_S}\right) + T \qquad (3)$$

where K_S is the enzyme–pyruvate dissociation constant (Meloche, 1967). Thus a plot of τ vs. 1/[I] would show increased slope in the presence of pyruvate, but the generated line would extrapolate to T as [I] approached infinity. From the slope of the line one could calculate K_S. Data showing that the aldolase inactivation obeys eq 3 in the presence of 1.94 m*M* pyruvate are also shown in Figure 1. The K_S of pyruvate is calculated to be 0.18 m*M*. From the data of Figure 1 it is concluded that the aldolase–bromopyruvate interaction exhibits two characteristics of active site involvement viz. saturation kinetics and competition with substrate.

A third characteristic for an active site directed alkylation was observed. Upon incubation of enzyme in the presence of 48.4 m*M* bromopyruvate for 40 hr, loss of catalytic activity was 99.4%, i.e., virtually complete. In the absence of reagent, the catalytic activity was stable over the course of the experiment.

Bromopyruvate as a Substrate for the Aldolase. If C_{inact} is catalytically competent and analogous to the aldolase–pyruvate, i.e., ES complex, catalytic detritiation of 3(*R,S*)-[3-$^3H_2$]bromopyruvate during its incubation with the enzyme would be anticipated. One, then, can attempt a sensitive test of whether inactivation is active site specific, based upon the kinetic model shown in

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C_{inact} \begin{matrix} \overset{k_2}{\nearrow} E_{dead} \\ \underset{k_3}{\searrow} E + P \end{matrix} \qquad (4)$$

where P is detritiated reagent (Meloche et al., 1972). This model assumes that steps 2 and 3 are alternative pathways for turnover of a single complex, C_{inact}, and that k_2 and k_3 values are invariant. Under such conditions, one would see a constant P/E_{dead} concentration ratio independent of [I], which would show K_m and K_{inact} to have the same numerical value (Meloche et al., 1972). Such a result would demonstrate that both inactivation and catalysis are occurring at the *same* protein site. Results are shown in Table I. For

Table I: The 2-Keto-3-deoxy-6-phosphogalactonate Aldolase Catalytic Turnover of 3(*R,S*)-[3-$^3H_2$]Bromopyruvate.

Expt	[BrPy] (m*M*)	Volume (μl)	Incubation Time (hr)	^{3}HOH Formed (cpm/ml)	Total ^{3}HOH Formed (cpm)	Total BrPy Turned-over[a] (nmol)	Total Enzyme Present: Initial (units)	Total Enzyme Present: Final (units)	Inactivated (units)	BrPy/E (nmol/unit)
1	9.5	240	5.0	183,200	43,970	397	39.5	3.8	35.7	11.1
2	22.3	102	3.5	540,000	55,080	513	39.5	3.4	36.1	14.2
3	47.0	110	1.0	371,400	40,855	352	39.5	6.7	32.8	10.7
										Av 12.0 ± 1.9

[a] Assuming a specific activity half that of the initial bromopyruvate (BrPy).

this experiment, the stoichiometry of enzyme inactivation is expressed as loss of units of activity which assumes a linear relationship between fraction of catalytic activity lost and catalytic sites alkylated. Experiments were conducted at three levels of [I] affecting the inactivation rate (column A), 9.5, 22.3, and 47.0 m*M*. Columns D and E show that catalytic detritiation occurred. From the total ^{3}HOH formed (column E) is calculated nanomoles of bromopyruvate turned over using the first-order correction

$$\text{nanomoles} = (-[I] \ln (1 - f_{eq}))\text{volume} \quad (5)$$

where f_{eq} = (total cpm released as ^{3}HOH)/(total cpm available × 0.5) (Meloche et al., 1972) and I concentration is expressed as μ*M*. This correction takes into account the first-order loss of the tritiated species during incubation and assumes that only one of the two prochiral positions at C-3 of the reagent is catalytically exchanged with H_2O. It should be noted that the tritiated reagent is reasonably stable under the experimental conditions since in the absence of enzyme the half-time of spontaneous detritiation was found to be about 100 hr. The units of enzyme inactivated are shown in column G. In column H it is seen that at the three levels of reagent employed, the ratio of nanomoles of bromopyruvate turned over per unit of activity lost, 11.1, 14.2, and 10.7, remained constant averaging 12.0 ± 1.9. This does not take into account a hydrogen isotope effect and is not, thus, an absolute value. However, the data of Table I are consistent with bromopyruvate's being a *substrate* as well as an *alkylating agent* for 2-keto-3-deoxy-6-phosphogalactonate aldolase, with both catalysis and inactivation occurring at the same protein site, the catalytic site. Further, both K_{inact} and K_m for bromopyruvate are 12 m*M*.

The conclusion made from the data of Table I is further supported by the experiment shown in Table II which reveals the catalytic detritiation to be asymmetric. In this experiment 48.4 units of enzyme were treated with 243.2 nmol of reagent, thereby the ratio of nanomoles of reagent to units of enzyme was 5:1 so that, kinetically, the aldolase was in excess. The data show that through 24-hr incubation, net catalytic detritiation proceed to 64.6%. Also, at 24 hr, an enzyme assay showed that 45.75 units or 94.5% of the original activity was lost. Thus, inactivation had proceeded through 4.25 half-lives. Equation 5 can be rearranged as

$$-\ln (1 - f_{eq}) = \frac{\text{nanomoles of BrPy detritiated}}{\text{nanomoles of BrPy}} \quad (6)$$

so that one can solve for the degree of detritiation possible under the experimental conditions employed. The nanomoles of bromopyruvate detritiated are 45.75 × 12 or 548.88 corresponding to both the units of enzyme inactivated and the ratio of nanomoles of reagent detritiated per unit enzyme inactivated (Table I). The amount of reagent in the experiment was 243.2 nmol. Since, $-\ln (1 - f_{eq})$ is 548.88/243.2 or 2.257, then $(1 - f_{eq})$ is 0.105. Accordingly, under the kinetic conditions employed, 89.5% of the total tritium of labeled bromopyruvate could have been released during enzyme inactivation. The exchange of only 64.6% of the label under experimental conditions where 89.5% could be labilized shows stereopreference by the enzyme for activating one of the two prochiral positions at C-3 of bromopyruvate. It should also be noted that substantial spontaneous detritiation occurred during the experiment. The spontaneous reaction, presumably, proceeds through a planar bromopyruvyl enolate. If a primary hydrogen isotope effect is operating, proton exchange then could occur 20 times faster than detritiation, with protonation of either face at C-3 of the enolate occurring. Consequently, the increase in degree of spontaneous detritiation observed between 2 and 24 hr is sufficient to totally randomize the configuration of tritium at C-3 of an enzyme generated asymmetric [3-^{3}H,H]bromopyruvate. As a result, the enzyme-catalyzed detritiation increase of 49–65% seen between 2 and 24 hr may well reflect rerandomization of an asymmetric product through spontaneous turnover, making the tritium once again susceptible to residual enzymatic activity present. Thus, the stereopreference for a prochiral position at C-3, shown by the data of Table II, may well be absolute.

The stereochemistry of bromopyruvate turnover was de-

Table II: Detritiation of 3(*R,S*)-[3-$^3H_2$]Bromopyruvate by Excess 2-Keto-3-deoxy-6-phosphogalactonate Aldolase.[a]

Time (hr)	Enzyme Absent: ^{3}HOH Formed (cpm/ml)	Enzyme Absent: ^{3}HOH Formed (%)	Enzyme Present: ^{3}HOH Formed (cpm/ml)	Enzyme Present: Net ^{3}HOH Formed[b] (cpm/ml)	Enzyme Present: Covalently Bound Tritium Available[b] (cpm/ml)	Enzyme Present: Detritiation Net (%)
0	10,400	2.0	11,400	−40	560,560	0
2	21,600	4.1	292,500	268,752	548,262	49.0
3	32,400	6.2	345,900	310,264	536,364	57.8
4	47,700	9.2	354,900	302,447	519,548	58.2
24	92,456	17.8	405,490	303,788	470,290	64.6

[a] Enzyme, 48.4 units, was reacted with 1.27 m*M* tritiated sodium bromopyruvate in 100 m*M* citrate (pH 6) in a total volume of 0.1915 ml. A control devoid of enzyme in which the concentration of reagent was 1.15 m*M* was run in parallel. Radioactivity in the sample was 572,000 cpm/ml, and in the control was 520,000 cpm/ml. [b] Corrected for ^{3}HOH formed by spontaneous detritiation.

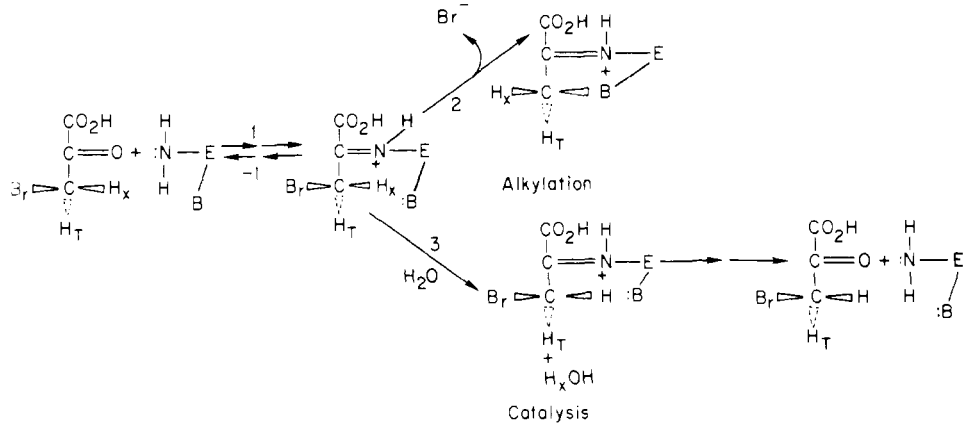


FIGURE 2: Proposed mechanism of 2-keto-3-deoxy-6-phosphogalactonate aldolase active site–bromopyruvate interaction.

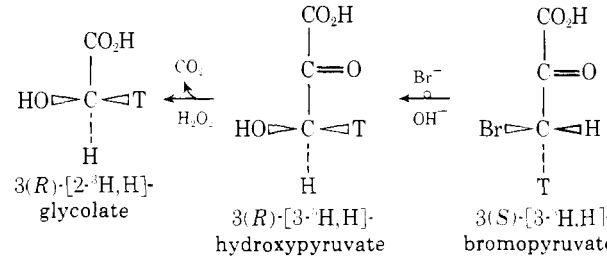
termined in a further study. The experiment described in Table II was scaled up twofold using pretreated enzyme (Methods). At the cessation of aldolase catalyzed detritiation (5 hr), 10 μmol of nonradioactive sodium bromopyruvate was added and the bulk of bromopyruvate was converted to glycolate as described in Methods. The reaction mixture was chromatographed on Dowex-1-Cl and tritiated glycolate was located as described in Methods.

Tritiated glycolate samples were treated with glycolic acid oxidase (Meloche et al., 1972). Authentic 2(*R,S*)-[2-$^3H_2$]glycolate derived from randomly tritiated bromopyruvate equilibrated at 46% release of its label as ^{3}HOH after 22-hr incubation with the oxidase; demonstrating that glycolic acid oxidase is specific for only one of the two prochiral positions at C-2 of glycolate. In contrast, upon 18-hr incubation of glycolic acid oxidase with [2-^{3}H,H]glycolate derived from bromopyruvate asymmetrically detritiated by the aldolase, 81% of the tritium was labilized by the oxidase. The enrichment for tritium at C-2 of this glycolate sample confirms that the bromopyruvate from which it was derived was asymmetrically tritiated at C-3.

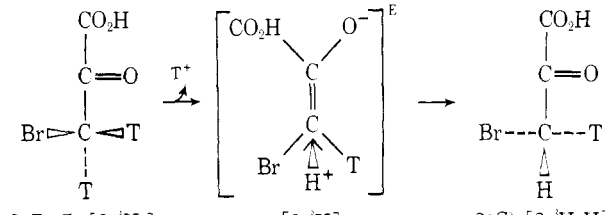
Discussion

The data in this paper show bromopyruvate to be an active site directed alkylating agent for 2-keto-3-deoxy-6-phosphogalactonate aldolase of *Pseudomonas saccharophilia* by the following criteria. The process is pseudo first order in enzyme for a substantial portion of the inactivation, and shows saturation kinetics with both bromopyruvate and pyruvate (substrate) competing for free enzyme. Inactivation is virtually complete at high levels of reagent and upon prolonged incubation. Bromopyruvate randomly tritiated at C-3 is catalytically detritiated by the enzyme during inactivation. The ratio of nanomoles of bromopyruvate catalytically turned over per unit of enzyme inactivated remained constant at 12:1 over a range of bromopyruvate concentrations affecting the inactivation rate. This latter characteristic is consistent with *both* alkylation *and* catalysis occurring at the *same* protein site, the catalytic site. Detritiation of 3(*R,S*)-[3-$^3H_2$]bromopyruvate is asymmetric.

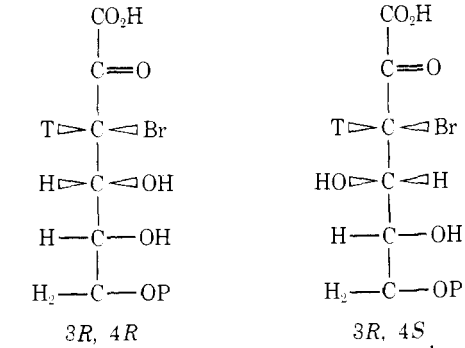
Further experiments showed that the asymmetrically labeled bromopyruvate, when chemically degraded to glycolate through a mechanism involving inversion of configuration, had tritium in that prochiral position at C-2 which is susceptible to glycolic acid oxidase. This is the *pro-R* position (Rose, 1958; Johnson et al., 1965). The stereochemistries are



so that the aldolase must activate the *pro-R* hydrogen isotope at C-3 of 3(*R,S*)-[3-$^3H_2$]bromopyruvate yielding 3(*S*)-[3-^{3}H,H]bromopyruvate. This is the *same* stereochemistry exhibited by 2-keto-3-deoxy-6-phosphogluconate aldolase in its catalytic turnover of bromopyruvate. That is each aldolase protonates the *re*[2] face at C-3 of the bromopyruvyl enolate. This is the face above the plane at C-3 in the diagram shown. (It should be noted that we have no in-



formation concerning stereochemical detail at C-2 of the bromopyruvyl enolate so that in this respect the configuration shown is arbitrary.) Each enzyme catalyzes condensa-



[2] The *re* face at C-3 is that described by the clockwise orientation of ligands substituted with bromine, vinyl carbon, and tritium.

tion with retention of configuration at C-3 (Meloche et al., 1975; Meloche and Monti, 1975). Thus *if* each of these two aldolases *could* condense bromopyruvate and D-glyceraldehyde-3-P, the aldehyde should also attack the *re* face of the enolate shown above. This being the case we would then expect the respective condensation products to have the same configuration at C-3, but opposite configurations at C-4 as shown. The identical stereopreference for ligands at C-3 shown by these two aldolases is the first evidence that the pyruvate portion of the active site of the two enzymes could have evolved from a common precursor.

We can conclude from the data in this paper that bromopyruvate interacts with 2-keto-3-deoxy-6-phosphogalactonate aldolase by an identical mechanism to the 2-keto-3-deoxy-6-phosphogluconate aldolase–reagent interaction. The mechanism is shown in Figure 2 where the active site of the enzyme is depicted as involving both a catalytically competent lysine ϵ-amino group, $-NH_2$,[3] and the bromopyruvate-sensitive amino acid group, -B:. The enzyme–bromopyruvate complex, then, is viewed as a ketimine which can turn over by alternative pathways: (a) abstraction of the *pro-R* hydrogen by the base B: responsible for enamine formation of the (bromo)pyruvate ketamine, with subsequent incorporation of H^+ from solvent followed by dissociation of the EI complex to regenerate free enzyme and asymmetrically labeled bromopyruvate, or (b) alkylation at C-3 of bound bromopyruvate by -B: to form inactive enzyme. It is assumed that the alkylation step is an SN2 process, i.e., occurring with inversion of configuration at C-3 of bromopyruvate as shown to be the case with 2-keto-3-deoxy-6-phosphogluconate aldolase (Meloche et al., 1972). The mechanism of Figure 2 presumes the enzyme bromopyruvate ketimine to be the intermediate in alkylation. Thus the chemically incorporated carboxyketomethyl group resulting from alkylation should have substantial ketimine character (Meloche, 1973), which borohydride reduction would convert into a secondary amine. Accordingly, it is anticipated that bromopyruvate may be a bridging agent for the active site of 2-keto-3-deoxy-6-phosphogalactonate aldolase. Studies to challenge this possibility are underway, as are studies directed at identifying -B:.

[3] The substrate-dependent reductive inactivation of 2-keto-3-deoxy-6-phosphogalactonate aldolase is an unreported observation of Meloche and Monti.

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