The Substrate Analog, Bromopyruvate, as a Bridging Agent for the Active Site of 2-Keto-3-deoxy-6-phosphogluconic Aldolase

CHEMICAL EVIDENCE FOR A CARBOXYLATE ADJACENT TO THE SCHIFF'S BASE-FORMING LYSINE

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SUMMARY

The enzyme, 2-keto-3-deoxy-6-phosphogluconic aldolase is inactivated by the substrate analog, bromopyruvate. Inactivation results from either an active site carboxyl group or a cysteine being alkylated by bromopyruvate. Treatment of enzyme inactivated by carboxylate ester formation by borohydride produces the secondary amine resulting from reduction of a Schiff's base between the covalently fixed carboxyketomethyl group and the ε-amino group of lysine. This is evidenced by the presence of $N\gamma$-(1'-carboxy-2'-hydroxy)ethyl lysine in hydrolysates of inactivated, reduced enzyme. However, reduction of enzyme inactivated by thioether formation does not produce a secondary amine. These results then constitute chemical evidence that the carboxylate occurs adjacent to an active site lysine residue, which is the azomethine-forming lysine, while the bromopyruvate-sensitive cysteine is elsewhere in the active site environment.

The substrate analogue bromopyruvate inactivates 2-keto-3-deoxy-6-phosphogluconic aldolase (1, 2). Kinetic studies of inactivation rates were consistent with bromopyruvate-active site interaction (2), where loss of catalytic activity resulted from alkylation of either a carboxylate anion or cysteine dependent upon salt concentration (3), suggesting the existence of more than one active site conformer. Bromopyruvate is also a substrate for the enzyme. Kinetic experiments revealed that both catalytic turnover and alkylation occurred at the same protein site, and that carboxylate attack on the β-carbon of bound

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(3,5)-[3H,3H]bromopyruvate occurred with inversion (4). It is thus evident that the reagent is bound to the enzyme's active site by a catalytic mechanism in which the β-carbon of bound bromopyruvate may well be adjacent to either the carboxylate or the cysteine or both allowing a specific alkylation reaction to occur. It is possible to resolve whether the active site carboxylate or cysteine residue is adjacent to the Schiff's base-forming lysine, since borohydride reduction would convert the lysine ε-amino into a secondary amino group as shown below

$$\begin{align*}
\text{CO} & \rightarrow \text{CO} \\
\text{C} & \rightarrow \text{C} \\
\text{Br} & \rightarrow \text{Br} \\
\text{H} & \rightarrow \text{H} \\
\text{H} & \rightarrow \text{H} \\
\end{align*}$$

where $-\text{NH}_2$ represents the protein-bound ε-amino of lysine, and $-\text{B}$ represents either the carboxylate or cysteine residues sensitive to bromopyruvate in the low or high salt conformers of the enzyme's active site, respectively. Thus, II upon hydrolysis would yield as unique amino acids either $N\gamma$-(1'-carboxy-2'-hydroxy)ethyl lysine or $N$-(5'-amino-5'-carboxy)pentyl lanthionine or both. Identification of products of reduced-inactivated enzyme hydrolysates would then serve to demonstrate whether bound bromopyruvate could interact with either carboxylate or cysteine or both, respectively. The results of this study are reported in this paper.

METHODS

2-Keto-3-deoxy-6-P-gluconic aldolase was purified to the specific activity of homogenous enzyme and assayed, radioactive bromopyruvate was synthesized in the presence of HCl, and the aldolase was inactivated at low salt concentration (20 mM sodium citrate) as well as in 1 M NaCl as previously described (3). Crystalline sodium-[3-3H]pyruvate was prepared by an aldolase catalyzed exchange reaction (5). Prior to inactivation, the enzyme was pretreated with nonradioactive sodium bromopyruvate in the presence of sodium pyruvate as previously described (2). One unit of activity represents the
The crystalline sodium salt of bromopyruvate was prepared as follows: bromopyruvic acid (1.5 g) was dissolved in 20 ml of H2O, and the pH of the solution was very carefully adjusted to 3.5 with 0.1 N NaOH (a titration curve showed bromopyruvate's carboxyl to have a pK of 2.5). Five volumes of ethanol were added to the sodium bromopyruvate solution forming an oil. The mixture was stored for 5 days at -10°C during which time crystallization occurred on the walls of the storage vessel. The crystals were collected, washed with absolute ethanol, and dried in vacuo. The yield was 0.9 g or 53%.

Amino acids were studied using a Spinco model 130 C amino acid analyzer, operated using the principles of Moore and Stein (6). For the experiments reported in this paper the analyzer's 60-cm column was packed with type AA 15 resin supplied by Spinco. The column output was taken directly to a fraction collector. Ninhydrin-positive material was detected by mixing 1 volume of sample with 0.5 volume of ninhydrin solution (from the analyzer's reservoir) and heating at 100°C for 15 min. The mixture was then cooled, 5 volumes of 50% ethanol were added and the A570 was determined. Radioactivity was determined using either scintillation counting in an ethanol-toluene system or flow through counting using a cerium activated lithium glass solid scintillator supplied by Packard Instrument Co. All isotopes used were purchased from New England Nuclear Corp.

Proteins were hydrolyzed either in 6 N HCl or enzymatically using pronase (3). Pronase was used at 1 mg per ml in 0.05 M imidazole, pH 7, at 30°C for 16 to 24 hours. Enzyme containing L-[3,4-3H]lysine (generally labeled) was produced by growing Pseudomonas putida A 3.12 in a mineral salts medium, containing 1% potassium gluconate as the sole source of carbon (7). Prior to inoculation tritiated lysine, 10 mCi, was added to 1 liter of medium. Fifty to 70% of the radioactivity was taken up by the cells during growth. L-[3H]-Lysine-containing enzyme was partially purified and then mixed with 2,400 units (10.7 mg) of purified nonradioactive 2-keto-3-deoxy-2-gluconic aldolase. This mixture was purified to the specific activity of homogenous protein, namely 225 units per mg of protein, by chromatography on Sephadex G-100 versus 10 mM sodium phosphate, pH 6, containing 1 mM EDTA. The specific radioactivity of the [3H]lysine in the enzyme was determined by inactivating the enzyme with [1-14C]pyruvate (7.0 × 10^4 cpm per pmole) and borohydride. The inactive enzyme was dialyzed against H2O acid hydrolyzed, and the Nε-[1-14C]carboxyethyl-[3H]lysine was isolated by chromatography on the amino acid analyzer and determined by simultaneous 14C:3H counting as shown in Fig. 1. The ratio of 14C:3H was 2.0. Therefore, the specific activity of lysine in the enzyme was calculated to be 7.0 × 10^4/2.0 or 3.5 × 10^4 cpm per mole.

Enzyme containing 35S was obtained by growing the organism in mineral media containing 1% gluconate and 10 mCi of Na2 [35S]SO4. Radioactive enzyme was purified to homogeneity using the methodology described for preparing tritiated lysine-containing enzyme, and the specific activity of [35S]methionine in hydrolysates was determined on the amino acid analyzer. It was assumed that the specific activity of methionine (counts per min per pmole) represented that of cystine.

RESULTS

Estimation of Secondary Amine Produced by Reduction of CKM1 Aldolase Generated at Low Salt Concentration

Preliminary Studies—Inactivation at low ionic strength results from formation of a carboxylate ester. Reduction of such [35S]CKM aldolase would produce a secondary amine if carbon-2 of the CKM-residue was bound to the protein as an azomethine. The unique amino acid produced would be Nε-[1-carboxy-2-hydroxy]ethyl lysine. However, if no azomethine linkage were formed, the radioactivity would appear in hydrolysates as diazoglycerate (3). Preliminary experiments were directed at showing whether nooxylglycerate material could be detected in acid hydrolysates. Aldolase-[35S]CKM was reduced with 20 mCi sodium borohydride at room temperature. After dialysis, the protein was acid hydrolyzed. Acid was removed in vacuo and the residue was dissolved in water and adjusted to pH 5 with NaOH and passed through a Dowex 1 (acetate) column (0.9 × 10 cm). It was found that about 25% of the

1 The abbreviations used are: CKM, carboxyketomethyl; CHE, diazoglyceratehydroxyethyl.
total radioactivity would wash through the column. Samples of this radioactive material were spotted and subjected to electrophoresis at pH 4. At 1000 volts the radioactivity migrated 1 cm from the origin towards the cathode in 60 min, indicating an isoelectric point slightly higher than 4.0. The electrophoretogram showed no evidence whatsoever for radioactive glyceraldehyde which should have been absorbed by the Dowex 1, and which under the same conditions would migrate 5 cm in the opposite direction. Thus, the conversion of CKM-aldolase to a neutral compound on reduction suggested secondary amine formation.

Further work was then undertaken to find conditions affecting the yield of total radioactivity of reduced $[^{14}C]$CKM-aldolase hydrolysates behaving as nonglyceraldehyde material. In these experiments freshly inactivated $[^{14}C]$CKM-enzyme was dialyzed against 1000 volumes of 10 mM sodium citrate, pH 6. Reduction was carried out for 30 min with 20 mM NaBH$_4$ at different temperatures. The borohydride reduced $^{14}$C-enzyme was adjusted to pH 4 to destroy residual BH$_4^-$ and dialyzed overnight against 1000 volumes of water. The sample was lyophilized, acid hydrolyzed, and the acid was removed in vacuo. The neutralized hydrolysate was then passed through a Dowex 1-antimonate column. The results are shown in Table 1. As seen, the temperature at which reduction was carried out effected the yield of labeled nonglyceraldehyde material. Reduction at 0° yielded 18% of the total label breaking through the column; 22° yielded 30%, 35° yielded 55%, and reduction at 50° resulted in 70% of the label breaking through the column.

The data extrapolate to all of the radioactivity being neutral if reduction were carried out at 70°. It should be pointed out that the native enzyme's activity is stable to 70°. In separate experiments we have found that 80% of the activity of purified enzyme survived 30 min at 72°. Thus, the active site conformation of native enzyme is not denatured at the elevated temperatures used in the reduction studies, which implies that the active site conformation of derived enzyme might be equally stable. Consequently, the temperature-dependence of appearance of nonglyceraldehyde radioactivity resulting from the reduction of an active site complex is consistent with stabilization of a Schiff's base between the carbonyl of a covalently bound CKM-residue and the ε-amino of lysine which is then reduced to a secondary amine.

Chromatographic Behavior of Presumed Secondary Amine on the Amino Acid Analyzer—$[^{14}C]$CKM-aldolase was prepared, dialyzed against water, and chemically reduced at 50° in the presence and absence of a denaturant agent. As shown in Fig. 2B, a portion of the $[^{14}C]$CKM-aldolase was reduced in the presence of 0.1% sodium lauryl sulfate. The 6 x HCl hydrolysate was examined on the 60-cm column of the amino acid analyzer using pH 4.25 buffer as the eluting agent and radioactivity was monitored by flow through counting. A single, radioactive peak accounting for all of the applied label appeared at column breakthrough. This is the chromatographic behavior of glyceraldehyde (or S-CHE-cysteine) on this column at pH 4.25. However, when chemical reduction of $[^{14}C]$CKM-aldolase at 50° was carried out in the absence of a protein denaturant, two radioactive peaks are evident as shown in Fig. 2A. One peak representing 40% of the label once again appears at column breakthrough. However, 60% of the label was retained by and chromatographed on the column, and was found in a region where glycine, alanine, and cysteine would co-elute under the conditions employed. This latter neutral fraction was pooled, adjusted to pH 2, and applied to the 60-cm column for rechromatography at pH 3.28. As seen in Fig. 3, the bulk of the applied radioactivity (76%) elutes in tubes 50 to 54, occupying an elution position close to that of valine. A minor component is found in tubes 20 to 23 representing 24% of the label, and eluting in the vicinity where the threonine-serine pair would be expected (55 min). Separate experiments have shown that at both pH 4.25 and 3.28, the radioactive major component elutes separate from, and just ahead of a 50%-fold molar excess of carrier valine. Note that the radioactive material also elutes ahead of N$_\alpha$-(1-carboxy)ethyl lysine which results from the reduction of a pyruvate-lysine azomethine (cf. Fig. 1).

The elution of hydroxy amino acid derivatives earlier than their

![Fig. 2. Chromatographic behavior of hydrolysates of $[^{14}C]$-carboxyketomethylaldolase on the amino acid analyzer. Radioactivity was detected using flow through counting. Elution was carried out using pH 4.25 0.2 M sodium citrate at a flow rate of 1.16 ml per min. Total column effluent was directed through the flow cell to a fraction collector. A, reduction of labeled CKM-protein was carried out in the absence of a protein denaturant. B, reduction of labeled CKM-protein was carried out in the presence of a protein denaturant as described in the text. For both A and B, approximately 20,000 of 25,000 applied counts (liquid scintillation counting) were recovered.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Reduction temperature</th>
<th>Initial radioactivity</th>
<th>Radioactivity breaking through</th>
<th>Neutral radioactivity</th>
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<tr>
<td></td>
<td>cpm</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>2000</td>
<td>300</td>
<td>18</td>
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<td>3500</td>
<td>1050</td>
<td>30</td>
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<td>35</td>
<td>1500</td>
<td>750</td>
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</tr>
<tr>
<td>50</td>
<td>2000</td>
<td>1400</td>
<td>70</td>
</tr>
</tbody>
</table>
hydrogen containing analogues is a common feature of amino acid analysis. Thus, the result of Fig. 3 is consistent with the major component being \( \text{N}-(1'\text{-carboxy-2'-hydroxy})\text{ethyl lysine} \). The minor component, which will be discussed below, was seen in all experiments unless the protein had been passed through a Sephadex G-25 column prior to labeling and is thus considered an artifact resulting from an impurity. The data in Figs. 2 and 3 are consistent with chemical reduction of \([^{14}\text{C}]\text{CM aldolase}\) producing a secondary amine which appears as a unique amino acid upon hydrolysis.

**Evidence for 1'-Carboxy, and 2'-Hydroxy Groups Adjacent to 1'-Amino Group in Presumed Secondary Amine**—Experiments were undertaken to determine whether this new component found in hydrolysates of borohydride-reduced radioactive CM aldolase produced at low salt concentration possesses both carboxyl and hydroxymethyl groups adjacent to what was carbon atom 2 of bromopyruvate, and whether nitrogen was covalently attached to that carbon.

The first experiments to be discussed are those designed to show that what was the carbonyl carbon of bromopyruvate (equivalent to the covalently bound CMK-residue) has been converted, by chemical reduction, to a secondary amine carbon. This was done by investigating the peroxide stability of the 1'-carboxyl in contrast to its sensitivity towards ninhydrin. Studies were carried out using \([^{14}\text{C}]\text{bromopyruvate}\) inactivated enzyme which was reduced to the presumed secondary amine. The acid hydrolysate was partially purified by chromatography at pH 4.25 using the 60-cm column of the amino acid analyzer. The labeled peak appearing at about tube 15 was pooled (cf. Fig. 2A). A series of \( [^{14}\text{C}] \) standards as well as the \( [^{14}\text{C}] \) experimental sample were placed in Warburg flasks containing a NaOH-saturated wick in its center well which served as a CO\(_2\) trap. For ninhydrin lability studies, a side arm contained one-half volume of ninhydrin reagent, the other side arm contained 0.1 ml of 4 M H\(_2\)PO\(_4\). The vessel was shaken and then opened and the wick was examined for \( ^{14}\text{CO}_2 \) by liquid scintillation counting. As controls, \( [^{14}\text{C}]\)alanine and \( [^{14}\text{C}]\)glycerate (from sodium lauryl sulfate denatured reduced \([^{14}\text{C}]\text{CM aldolase hydrolysate}\)) were reacted with ninhydrin as described above. As additional controls, the \( [^{14}\text{C}] \) labeled experimental sample and \( [^{14}\text{C}] \) bromopyruvate in 20 mM sodium citrate pH 6 were reacted with 200 mM H\(_2\)O\(_2\) at room temperature in a stoppered Warburg vessel containing a NaOH wick. After a 15-min incubation, 0.1 ml of 4 M H\(_2\)PO\(_4\) was tipped in as above, the vessel was opened, and the \( ^{14}\text{CO}_2 \) was counted. Results are shown in Table II. \( [^{14}\text{C}] \)Bromopyruvate solution when reacted with H\(_2\)O\(_2\) lost 95% of its radioactivity by oxidative decarboxylation. Of this, 49.6% could be accounted for by the radioactivity trapped on the NaOH saturated wick, indicating that the method decreases the counting efficiency by a factor of 3. The second experiment with H\(_2\)O\(_2\) showed that the 1'-\( ^{14}\text{C} \) experimental

### Table II

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial Radioactivity</th>
<th>Radioactivity in solution after reaction</th>
<th>Radioactivity trapped by NaOH</th>
</tr>
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<tbody>
<tr>
<td>( [^{14}\text{C}] )Bromopyruvate</td>
<td>227,000</td>
<td>17,000</td>
<td>151,000</td>
</tr>
<tr>
<td>( [^{14}\text{C}] )Experimental</td>
<td>500</td>
<td>520</td>
<td>110</td>
</tr>
<tr>
<td>Ninhydrin treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( [^{14}\text{C}] )Glycerate</td>
<td>1,000</td>
<td>1,050</td>
<td>100</td>
</tr>
<tr>
<td>( [^{14}\text{C}] )Alanine</td>
<td>69,000</td>
<td>3,000</td>
<td>400</td>
</tr>
<tr>
<td>( [^{14}\text{C}] )Experimental</td>
<td>800</td>
<td>20</td>
<td>2.5</td>
</tr>
<tr>
<td>sample</td>
<td>300</td>
<td>0</td>
<td>125</td>
</tr>
</tbody>
</table>

was introduced to the reaction mixture to displace dissolved CO\(_2\), the vessel was shaken and then opened and the wick was examined for \( ^{14}\text{CO}_2 \) by liquid scintillation counting. As controls, \( [^{14}\text{C}]\)alanine and \( [^{14}\text{C}]\)glycerate (from sodium lauryl sulfate denatured reduced \([^{14}\text{C}]\text{CM aldolase hydrolysate}\)) were reacted with ninhydrin as described above. As additional controls, the 1'-\( ^{14}\text{C} \) labeled experimental sample and \( [^{14}\text{C}] \) bromopyruvate in 20 mM sodium citrate pH 6 were reacted with 200 mM H\(_2\)O\(_2\) at room temperature in a stoppered Warburg vessel containing a NaOH wick. After a 15-min incubation, 0.1 ml of 4 M H\(_2\)PO\(_4\) was tipped in as above, the vessel was opened, and the \( ^{14}\text{CO}_2 \) was counted. Results are shown in Table II. \( [^{14}\text{C}] \)Bromopyruvate solution when reacted with H\(_2\)O\(_2\) lost 95% of its radioactivity by oxidative decarboxylation. Of this, 49.6% could be accounted for by the radioactivity trapped on the NaOH saturated wick, indicating that the method decreases the counting efficiency by a factor of 3. The second experiment with H\(_2\)O\(_2\) showed that the 1'-\( ^{14}\text{C} \) experimental
sample was not decarboxylated and thus was stable to a general oxidizing agent, confirming that chemical reduction had destroyed the carboxyl of the CKM-residue. Similarly, no radioactivity was released from \( \text{NL-}[^{1-14}\text{C}] \) glycerate upon reaction with ninhydrin. However, \( \text{NL-}[^{1-14}\text{C}] \) alanine released 95.5\% of its radioactivity during the ninhydrin reaction. Of this label, 56.5\% was detected as \( ^{14}\text{CO}_2 \), indicating a quench factor of 1.77. When the \( ^{1-14}\text{C} \)-labeled experimental sample was reacted with ninhydrin in two separate experiments, 38.5 and 41.5\% of the radioactivity was trapped as \( ^{14}\text{CO}_2 \). Applying the quench factors calculated for \( ^{14}\text{C} \) bromopyruvate and \( \text{NL-}[^{1-14}\text{C}] \) alanine in the \( ^{14}\text{O}_2 \) and ninhydrin experiments, respectively, the above result shows that up to 82\% of the carboxyl radioactivity that was in bromopyruvate prior to enzyme inactivation was released as \( ^{14}\text{CO}_2 \) upon treatment of the presumed secondary amine with ninhydrin. Virtually none of the label was found in solution after reaction. The result clearly shows that ninhydrin decarboxylates most, if not all, of the 1'-position of the new amino acid. Ninhydrin is a general reagent for decarboxylating \( \alpha \)-keto and \( \alpha \)-amino acids (primary and secondary).

Chemical reduction destroys the carboxyl of the \( \text{[1-14C]} \text{CKM residue as shown by the failure to liberate counts with peroxide, thus ninhydrin-dependent decarboxylation results from attack at an amino group. With amino acids, decarboxylation is an intrinsic part of the reaction mechanism (8). In fact, Hollerman and Coffey (9) used ninhydrin decarboxylation of \( N_e (1'-\text{carboxyethyl}-\text{lysine as partial proof of structure of the authentic compound. Thus, the data in Table II are consistent with what was the carboxyl of bromopyruvate being adjacent to an amino group, consistent with the structure of the proposed secondary amine. A subsequent experiment was directed at demonstrating a hydroxymethyl group covalently bound to the secondary amine carbon. The aldolase was inactivated with \( \text{[3-H]} \text{bromopyruvate. After dialysis, the [3-H]CKM-aldolase was reduced with 40 mm borohydride at 50^\circ \text{C}. Residual borohydride was destroyed at pH 4 and the sample was diazolized and then hydrolyzed with pronase. The secondary amine was isolated by passing the sample (10,000 cpm) through a Dowex 1 (acetate) column (1.5 \times 10 \text{ cm}) and collecting the breakthrough. About 5500 cpm recovered (equivalent to 14 nmole of derivative) in a volume of 2 ml was treated with peridote in \text{Na}_{2}\text{CO}_3 buffer as described by Reeves (10). After destruction of residual peridote with \text{NaHSO}_4, 60 \text{ mmole of carboxylic acid were added and the formylidenedon was obtained upon addition of 5,5-dimethylhydroxy-resorcinol. The dimedon was collected by filtration and resuspended five times in 10 ml portions of ice, distilled water and titered. The dimedon was then washed on a filter under vacuum with 250 ml of iced water. The dimedon yield was 12.6 mg or 72\% based on added formaldehyde. The dimedon contained radioactivity equivalent to 3600/0.72 or 5000 cpm of tritium. Consequently, 91\% of the tritium of the 3'-H secondary amine was converted to \( \text{[PH]} \) formaldehyde by peridote cleavage. \text{NL-}[^{3-14}\text{C}] \text{Glyceraldehyde is completely excluded from the hydrolysate by passage through Dowex 1 (acetate). Thus, the results of this experiment are consistent with peridote cleaving a \( \text{[PH]} \) hydroxymethyl group vicinal to a deprotonated amino group to yield \( \text{[PH]} \) formaldehyde, a known peridote reaction (11).}

In the results of this section are consistent with bromopyruvate inactivating the aldolase by carboxylate esterification (low salt (3)). Chemical reduction of the protein-CKM adduct and hydrolysis yield the former CKM-residue converted to the species shown below.

\[
\begin{align*}
\text{CO}_2 & \quad \text{H} \\
\text{H} & \quad \text{CHN} \quad \text{R} \\
\text{H} & \quad \text{C} \quad \text{OH}
\end{align*}
\]

\[\text{(2)}\]

**Protein-bound Lysine and Bromopyruvate in Structure of Presumed Secondary Amine—Tritiated lysine-containing 2-keto-3-deoxy-P-glucose aldolase (3.5 \times 10^4 \text{ cpm per pmole protein bound lysine}) was reacted with \( \text{[1-14C]} \text{bromopyruvate (7.45 \times 10^4 \text{ cpm per pmole}) in 50 \text{ mm citrate buffer, pH 6. After inactivation, the protein was precipitated three times with ammonium sulfate and dissolved in water. Enzyme inactivation amounted to 840 units with the incorporation of 7890 cpm. This is equivalent to the incorporation of 1 \text{ pmole of CKM residue per 8000 units of activity lost and corresponds to the same stoichiometry found previously by inactivation of the enzyme either by pyruvate-borohydride treatment (2, 12), or alkylation with bromopyruvate (2). The reaction mixture was then heated to 50^\circ \text{C}, and reduced by adding NaBH}_4 \text{ to 40 mm. After dialysis and concentration in vacuo the reaction mixture was acid hydrolyzed for 26 hours. The radioactivity chromatographing at pH 4.25 (cf. Fig. 2A) was pooled, and rechromatographed at pH 3.18 on the 60-cm column of the amino acid analyzer. The location of \text{[14C]} label detected by solid scintillation counting is presented in Fig. 4.1. As shown, two peaks are evident, analogous to those seen in Fig. 2. Each peak was re-examined by the liquid scintillation technique, simultaneously determining tritium and \text{[14C]. The results presented in Fig. 4B show that each peak contains \text{[14C} and \text{[H} in a counting ratio of about 2:1. The ratio of specific activities (counts per min per \text{[14C} pmole of the \text{[1-14C]} bromopyruvate (7.45 \times 10^4 \text{ cpm per pmole}) and protein bound lysine (3.5 \times 10^4 \text{ cpm per pmole}) is 2.15. Consequently, the radioactive peaks seen in Fig. 4 result from the coherent reaction of the carbon skeletons of bromopyruvate and aldolase bound lysine in a stoichiometry of 1:1.**

**Estimation of Secondary Amine Produced by Reduction of CKM-aldolase Generated at High Salt Concentration**

Studies reported in this section were carried out using enzyme inactivated with bromopyruvate in 1 m \text{NaCl at pH 6. Under these conditions about one-half of the incorporated CKM residues are covalently bound to an active site cysteine residue with the other half covalently bound via ester linkage (3). After inactivation, the enzyme was isolated by ammonium sulfate precipitation and the dissolved pellet was dialyzed against 1 m \text{NaCl for 4 hours. The enzyme preparation was then treated with 20 mm \text{NaBH}_4 at 50^\circ \text{C and concentrated by ultrafiltration. In the first series of experiments, \text{[1-14C]} bromopyruvate inactivated, reduced enzyme was divided into two equal portions. One portion was treated with bromine-water to ensure oxidation of any sulfoether derivatives to the sulfone level. Residual...**
and HCl was removed in vacuo. The hydrolysate was chroma-
tically, bromine was destroyed by treating the preparation with formic acid until CO2 evolution ceased. The preparation was then lyophilized from water four times. Both the oxidized and untreated [1-14C]CKM-reduced aldolase samples were acid hydrolyzed. After removal of HCl in vacuo the preparations were chromatographed at pH 4.25 on the amino acid analyzer and column output was diverted to a fraction collector. For both oxidized and nonoxidized samples, the only ninhydrin-sensitive carboxyl groups found were associated with the peak representing Nε-(1'-carboxy-2'-hydroxy)[1'-14C]ethyl lysine (cf. Fig. 2A). The failure of bromine oxidation to produce a more acidic secondary amine species suggests that the chemical reduction of S-CKM-aldolase does not produce N-(5'-amino-5'-carboxy)pentyl lanthionine which bromine-water would oxidize to the sulfone.

The second series of experiments was carried out using protein resulting from mixing isotopically equivalent amounts of [35S]-cysteine-aldolase and [3H]lysine aldolase which were inactivated by nonradioactive bromopyruvate. After reduction and handling as described above, the protein was acid hydrolyzed and HCl was removed in vacuo. The hydrolysate was chroma-
tographed at pH 4.25 on the amino acid analyzer and the column effluent was diverted to a fraction collector. Fractions were assayed for [35S] and [3H] using the simultaneous counting technique. There was no evidence for material in the hydrolysate containing both [35S] and [3H]. This result is also consistent with reduction of S-CKM-aldolase not producing a secondary amine, but producing S-nitrocarboxyhydroxy-ethyl-cysteine.

DISCUSSION

The results presented in this paper show that when the active site of 2-keto-3-deoxy-6-P-gluconate aldolase interacts with bromopyruvate at low salt concentration so that the bulk of inactivation occurs via protein-bound carboxylate esterification (3), subsequent chemical reduction of this inactivated enzyme produces a secondary amine. Thus, hydrolysates of such an enzyme contain a unique amino acid, namely Nε(1'-carboxy-2'-hydroxy)ethyl lysine whose structure is shown below.

\[
\begin{align*}
\text{CO}_2\text{H} & \quad \text{NH}_2 \\
\text{CH}_2-\text{C}\text{-OH} & \quad \text{C-H} \\
\end{align*}
\]

This finding provides chemical evidence that the bromopyruvate-sensitive carboxylate of 2-keto-3-deoxy-6-P-gluconate aldolase is adjacent to a lysine ε-amino group in the active site of the en-
zyme. We have reported that bromopyruvate is also a sub-
strate for the enzyme exhibiting kinetics consistent with both catalytic turnover and inactivation occurring at the same pro-
tein site (4). Consequently, our results are consistent with the carboxyl group that is esterified by bromopyruvate being ad-
jacent to the catalytically functional Schiff's base-forming lysine in the catalytic site of the native enzyme. Thus, when bromopyruvate is bound to the active site in a catalytic step, the reagent's β-carbon is positioned adjacent to the carboxylate so that specific alkylation (esterification) can occur. Meloche and Glusker (13) have employed model studies to demonstrate that a γ-carboxylate donated by glutamate (and presumably the β-carboxyl of aspartate) has geometry such that a single residue can participate in proton activation at C-3 of pyruvate and at the C-4 hydroxyl of 2-keto-3-deoxy-6-P-gluconate. These are the two proton activation steps postulated to partici-
pate in aldolase catalysis (14). Consequently, bromopyruvate inactivation of the aldolase via esterification followed by chemi-
ical reduction generates a bridge in the active site between two catalytically functional amino acid residues.

Our initial experiments directed at identifying the amino acid alkylated by bromopyruvate showed that enzyme inactivation was accompanied both by carboxylate-ester formation and cysteine-thioether formation dependent upon salt concentration (3). It was concluded that both bromopyruvate sensitive amino acids were in the active site since, (a) the stoichiometry of labeling remained constant even though the label could be distributed between two amino acid residues; and (b) when inactivation occurred at high salt so that each amino acid was labeled, pyruvate afforded equal protection towards labeling both amino acids (3). However, when the enzyme is inactivated at high salt concentration (1 M NaCl) so that substantial cysteine alklylation occurs, subsequent chemical reduction does not produce detectable secondary amine associated with a thioether. Assuming that the protein does not denature following cysteine alklylation so that the active site environment is destroyed, and that the postulated secondary amine $N(5'\text{-amino-5'\text{-carboxy}})$-pentyl lanthionine or its sulfone would survive the methodology involved, the results suggest that the bromopyruvate sensitive cysteine of the 2-keto-3-deoxy-6-P-gluconate active site is not adjacent to the β-carbon of the bound reagent in the enzyme's high salt conformation (3). Consequently, the cysteine appears to be elsewhere in the active site environment. Whether this amino acid plays a functional role in catalysis cannot be resolved by these experiments.
It was seen throughout this work that associated with radioactive \( N\)-\( (1'\text{-carboxy-2'\text{-hydroxy})ethyl lysine was a more acidic radioactive component appearing early in the elution profile when chromatography was carried out at pH 3.28 (Figs. 3 and 4). It is quite clear that this species is derived in equal proportion from bromopyruvate and lysine (Fig. 3). It is equally clear that the compound is not a sulfur-containing derivative. It was observed later in our experience with this system that the compound was eliminated if protein was passed through G-25 columns prior to derivatization. Under such conditions only labeled \( N\)-\( (1'\text{-carboxy-2'\text{-hydroxy})ethyl lysine was evident. Substantial amounts of phosphate are used in enzyme purification, which would only be removed on specific prior treatment of the protein such as gel filtration. The drying of protein hydrolysates from acid in the presence of traces of phosphate generates phosphoserine (6) which elutes near column breakthrough on the amino acid analyzer. By analogy, the acidic component seen in our experiments may well be the 2'-phosphate ester of \( N\)-\( (1'\text{-carboxy-2'\text{-hydroxy})ethyl lysine.

The results of our series of experiments with bromopyruvate and 2-keto-3-deoxy-6-P-glucosone aldolase can now be summarized as showing a carboxylate adjacent to the Schiff's base-forming lysine in the active site of the enzyme (1-4, 13). The geometry of the active site appears to be such that the single carboxylate can interact with the \( \text{Pro-}1 \) hydrogen on the \( \beta \)-carbon of bound bromopyruvate, a methyl hydrogen of bound pyruvate or the C-4 hydroxyl hydrogen of bound 2-keto-3-deoxy-6-P-glucosone (13). As such, the carboxylate can contribute to electron flow in the protonated azomethine directed catalysis found in the aldolase as shown in Scheme 4. It is of interest to know whether an analogous series of catalytic events can be demonstrated with other aldolases, as well as other enzymes catalyzing C-C synthesis at activated methylene carbons.

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The Substrate Analog, Bromopyruvate, as a Bridging Agent for the Active Site of 2-Keto-3-deoxy-6-phosphogluconic Aldolase: CHEMICAL EVIDENCE FOR A CARBOXYLATE ADJACENT TO THE SCHIFF’S BASE-FORMING LYSINE

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