Reaction of the Substrate Analog Bromopyruvate with Two Active-Site Conformers of 2-Keto-3-deoxy-6 phosphogluconic Aldolase*

H. Paul Meloche

ABSTRACT: The enzyme 2-keto-3-deoxy-6-phosphogluconic aldolase is inactivated by the substrate analog bromopyruvate. Inactivation is accompanied by the esterification of a protein-bound carboxyl group or the alkylation of the sulfur of protein-bound cysteine. When inactivation is carried out at low salt concentration, most of the bromopyruvate is incorporated by esterification. When inactivation is carried out at elevated salt levels, bromopyruvate is bound both by esterification and mercaptide formation. The data show a direct relationship between salt concentration and sulfur alkylation, and suggests that inactivation at very high salt concentrations would yield bromopyruvate bound primarily by mercaptide formation. Throughout these experiments the ratio of bromopyruvate equivalents covalently incorporated per equivalent of enzyme inactivated remained constant. When enzyme was inactivated in 1 M NaCl, the distribution of bromopyruvate incorporated by esterification and mercaptide formation was the same whether the activation was carried out in the presence or absence of substrate, indicating that protection by substrate was conferred equally for each reaction. This result is consistent with both the bromopyruvate-sensitive carboxyl and cysteine being in the active site. It is concluded that a single bromopyruvate on the enzyme has two alternative ways of reacting, possibly with two functionally distinct protein conformers, one conformer being specific for activation in the pyruvate proton-exchange reaction and the other being specific for activation in the cleavage or condensation reaction.

The enzyme 2-keto-3-deoxy-6-phosphogluconic aldolase 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 (Meloche and Wood, 1964a). Studies on the inactivation of KDP-gluconic aldolase with the substrate analog monobromopyruvate were undertaken in this laboratory. These experiments were directed at locating and identifying a group in the active site which, in conjunction with the azomethine-forming lysine, would function in the proton activation and condensation steps of the catalyzed reaction (Meloche, 1965; Rose, 1966). Bromopyruvate inactivates the enzyme (Meloche, 1965), and a detailed kinetic study provided data consistent with the substrate analog being an active-site-specific alkylating agent (Meloche, 1967). The aldolase, inactivated either by alkylation with bromopyruvate or by the reductive binding of pyruvate to the enzyme's active site (Meloche and Wood, 1964b), covalently incorporated 1 μmole of reagent/4.7 $\times$ 10^5 units of catalytic activity lost. These data can be cited as evidence that 1 μmole of aldolase is a multiple of 4.7 $\times$ 10^5 units (11,374 IU) of catalytic activity. The experiments reported in this paper are directed at identifying the bromopyruvate-sensitive amino acid(s) in the enzyme's active site.

Methods

KDP-gluconic[1] aldolase was purified and assayed by the procedure of Meloche *et al.* (1966) with the following modification in the purification procedure. After the calcium phosphate gel step, the protein was chromatographed on Sephadex G-100 *vs.* 100 mM sodium phosphate (pH 7) and 5 mM EDTA. The aldolase peak had a specific activity range of 9000–10,000 units/mg of protein and was used without further purification. One unit of activity is defined as an A_{340} change of 1.0/min using a coupled assay in a total volume of 150 μl at 24.5°.

Radioactive bromopyruvate was synthesized by the direct bromination of a mixture of [^{14}C]sodium pyruvate and carrier pyruvic acid using glacial acetic acid as solvent (Meloche, 1967). This solvent is not acidic enough to allow bromination to proceed. Consequently, in a synthesis as described in the cited article, acidification was accomplished by the addition of a drop (about 20 μl) of concentrated H_2SO_4 (procedure A). Acidification can also be achieved by the addition of one-tenth volume of 30% formic acid (B), or (C) the addition of a drop of concentrated HCl. Yields of [^{14}C]-bromopyruvate by procedures A and C are good. However, in procedure B, yields range 25–30% of both radioactivity and bromopyruvate. This may be due to the fact that the addition of 30% formic acid introduces substantial amounts of water to the reaction. Solvents were removed from the [^{14}C]bromopyruvate *in vacuo*, and the residue was taken up in a small amount of fresh glacial acetic acid and stored in a freezer. Bromopyruvate was assayed as previously described (Meloche, 1967).

Glutathione was alkylated by bromopyruvate to provide an authentic *S*-CKM-cysteinyl derivative. The synthesis was carried out at room temperature in aqueous solution by

* From The Institute for Cancer Research, Philadelphia, Pennsylvania 19111. *Received April 2, 1970.* This work was supported by the National Science Foundation Grants GB-5921 and GB-8403, by the National Institutes of Health Grants CA-06927 and FR-05539, and by an appropriation from the Commonwealth of Pennsylvania.

[1] Abbreviations used are: KDP-gluconic, 2-keto-3-deoxy-6-phosphogluconic; CKM, carboxyketomethyl; CHE, 1-carboxy-1-(D,L)-hydroxyethyl; CM, carboxymethyl.

adding 1 equiv of bromopyruvate in four portions to the glutathione and maintaining the pH between the limits 5.5 and 6.0 with dilute alkali. The product was isolated as the pH 3.5, ethanol-insoluble barium salt in 50–60% yield. Peroxide treatment of *S*-CKM-glutathione yielded *S*-CM-glutathione sulfoxide,[2] which was reduced to its sulfide with concentrated HI.[3] Acid hydrolysates of the latter product showed equivalent amounts of *S*-CM-cysteine, glutamic acid, and glycine on the amino acid analyzer.

S-CKM-glutathione was also chemically reduced using a 20-fold molar excess of $NaBH_4$. Acid hydrolysates of this product showed a new amino acid chromatographing just ahead of the elution position of *S*-CM-cysteine on the analyzer. In hydrolysates of reduced *S*-[^{14}C]CKM-glutathione, the new amino acid contained all of the radioactivity; thus it is presumed to be *S*-CHE-cysteine. This material has the same extinction in the ninhydrin reaction as that of *S*-CM-cysteine, which is significantly lower than that of the other α-amino acids.

Amino acid analyses were performed using a Spinco Model 120C. For most of the experiments to be described, effluent from the 60-cm column was taken directly to a fraction collector, bypassing the analyzer's ninhydrin supply and spectrophotometer. Samples of ^{14}C hydrolysate were chromatographed with either nonradioactive or tritiated carrier. The known compound was located with ninhydrin using the procedure of Yem and Cocking (1955), or by simultaneous ^{3}H/^{14}C counting. Radioactivity was measured by liquid scintillation counting in a toluene–ethanol system. All hydrolyses were carried out using 6 N HCl as described by Moore and Stein (1964).

Nonradioactive bromopyruvate was synthesized by the method of Dickens (1962). Crystalline bromopyruvate was converted into hydroxypyruvate by maintaining the pH at 8 with base. D,L-[2-^{3}H]Glyceric acid was synthesized by the reduction of hydroxypyruvate with a 20-fold excess of sodium borotritide. The tritiated glycerate was purified by chromatography on Dowex 1-acetate using a linear gradient established with equal volumes of water and 2 M acetic acid.

[^{14}C]Sodium pyruvate and sodium borotritide were obtained from New England Nuclear Corp. Pronase was obtained from Calbiochem. Derivatized aldolase in 50 mM imidazole (pH 8) was digested with pronase (1 mg/ml) for 16 hr at 35°.

In preparation for alkylation with radioactive bromopyruvate, KDP-gluconic aldolase was treated with 1 mM unlabeled bromopyruvate and 40 mM pyruvate. This step was undertaken to preferentially alkylate nonactive-site amino acid residues. The incubation was carried out at pH 6 in 20 mM sodium cacodylate or 20 mM sodium citrate buffer for 30 min at 20°. The enzymatic activity surviving this step ranged 70–80%. The protein was recovered by precipitation with ammonium sulfate (0.4 g/ml). The pellet was dissolved in a small amount of water and passed through a 2.5 × 30 cm column of coarse Sephadex G-25 equilibrated with 10 mM NaCl. All of the activity was associated with the void volume. The protein was concentrated by precipitation with ammonium sulfate and the pellet was dissolved in 20 mM sodium cacodylate or sodium citrate buffer (pH 6) to a protein concentration of 6–7 mg/ml. This preparation of KDP-gluconic aldolase will be referred to as *pretreated enzyme*.

Pretreated enzyme was inactivated by 1–2 mM [^{14}C]-bromopyruvate essentially as previously described (Meloche, 1967). In all cases, inactivation was allowed to proceed at least 90%, and the stoichiometric relationship of moles of CKM-residue incorporated to loss of catalytic activity was 1 μmole/4.7 × 10^5 units. Inactivation was terminated with two to three ammonium sulfate precipitations, and the radioactive, inactive enzyme dissolved in water was used below.

In preparation for hydrolysis, [^{14}C]CKM-enzyme was chemically reduced to [^{14}C]CHE-enzyme. This was done since extensive radioactivity of [1-^{14}C]CKM-aldolase is lost during either acid or enzymatic hydrolysis, probably due to decarboxylation. Similarly, [1-^{14}C]CKM-glutathione lost substantial amounts of label during acid hydrolysis and underwent loss of radioactivity during storage in solution.[2] However, chemical reduction of the CKM-carbonyl stabilized carbon-1 of both the enzyme and glutathione derivatives.

Consequently, in all experiments, inactivated radioactive enzyme was denatured by the addition of sodium lauryl sulfate to a final concentration of 0.18%, and the solution was then made 20 mM by the addition of 0.04 volume of 500 mM $NaBH_4$ and incubated 30–60 min at room temperature. It should be noted that 0.1% sodium lauryl sulfate will rapidly and completely inactivate the pretreated aldolase. Consequently, it is reasonable to assume that the reagent destroys the conformation of the enzyme's active sites. The reaction mixture was then passed through the Sephadex G-25 column described above. The breakthrough containing the protein and all of the radioactivity was concentrated by lyophilization. Where indicated in the text, prior to hydrolysis, the dried residue in an ampoule was treated with 2 ml of 57% HI for 1 hr at room temperature to reduce any sulfoxides (a few drops of hypophosphorous acid were added to the stock HI reagent to retard air oxidation during storage). The HI was removed *in vacuo* prior to hydrolysis. The HI treatment will only work if enough hypophosphorous acid is present to completely destroy residual I_2, which would otherwise oxidize sulfides during hydrolysis. Samples were also alternately evacuated and then flushed with prepurified N_2 prior to sealing and heating. Under these conditions, decomposition of sulfide during hydrolysis was minimal.

Results

Early work showed that two major radioactive peaks were obtained in hydrolysates of [1-^{14}C]CHE-aldolase chromatographed on the 60-cm column of the amino acid analyzer as shown in Figure 1. One peak is shown to cochromatograph with *S*-CHE-cysteine in tubes 17–19; the other peak was found in tubes 10–12, which is beyond column breakthrough (tube 6 ±0.5). The small peak seen in tube 8 in this and other experiments appears to be a decomposition product of the cysteine derivative in these protein hydrolysates. The ninhydrin-positive peak adjacent to *S*-CHE-cysteine represents aspartic acid in the protein hydrolysate. Of 5000 cpm applied to the column, 4200 cpm (84%) was recovered; 2000 behaved as *S*-CHE-cysteine; and 1900 were found in the second major peak. The results in Figure 1 show that of the CKM residues incorporated into KDP-gluconic aldolase during inactivation in this experiment about half result from the alkylation of the

[2] H. P. Meloche, unpublished results.

[3] We are indebted to Dr. T. F. Lavine for suggesting the use of HI to reduce sulfoxides to sulfides.

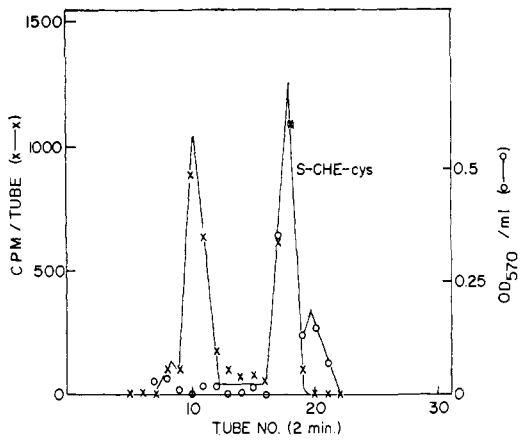

FIGURE 1: Chromatography of radioactivity of [1-^{14}C]CHE-aldolase hydrolysate on the 60-cm column of the amino acid analyzer. Pretreated enzyme was alkylated using [1-^{14}C]bromopyruvate synthesized by procedure A (Methods), in 100 mM cacodylate buffer (pH 6). Additional NaOH was added to the enzyme–bromopyruvate mixture to make the final pH 6. A total of 5000 cpm was applied to the analyzer along with 1 μmole of carrier *S*-CHE-cysteine obtained from an *S*-CHE-glutathione hydrolysate. Radioactivity (×—×) was located by scintillation counting; carrier *S*-CHE-cysteine was located using ninhydrin (O–O). A total of 4200 cpm was recovered. Elution was carried out with 0.20 M sodium (citrate) (pH 3.28) at a flow rate of 1.167 ml/min.

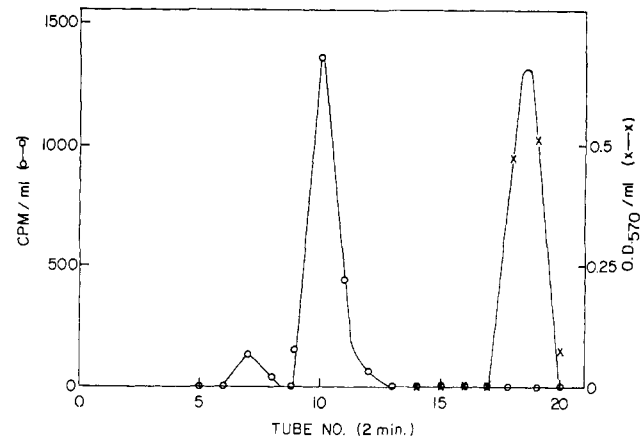

FIGURE 2: Chromatography of radioactivity of [1-^{14}C]CHE-aldolase hydrolysate on the 60-cm column of the amino acid analyzer. Pretreated enzyme was alkylated using [1-^{14}C]bromopyruvate synthesized by procedure B (Methods), in 20 mM sodium cacodylate buffer (pH 6). A total of 2500 cpm was applied to the analyzer with carrier *S*-CHE-cysteine. Radioactivity was located by scintillation counting. A total of 2200 cpm was recovered. Elution was carried out with 0.20 M sodium citrate buffer (pH 3.28) at a flow rate of 1.167 ml/min.

mercaptyl of cysteine and the remainder from an unexplained mechanism to be considered below.

In other experiments it was observed that the distribution of radioactivity between the two forms was quite variable. The two peaks always accounted for virtually all of the counts which comprised 1 equiv/active subunit, and the relationship of the two components was reciprocal. These results implied that two distinct chemical events were occurring during reaction of the protein with bromopyruvate, each of which resulted in activity loss. Further, it appeared that the selectivity of bromopyruvate could be controlled by manipulating some physical or chemical condition(s).

The [^{14}C]bromopyruvate used to inactivate the enzyme was synthesized by procedure A (Methods). After removing the acetic acid *in vacuo*, the bromopyruvate contains a considerable amount of H_2SO_4, which contributes a significant salt concentration to the inactivation system. Thus, the possibility that a salt effect might contribute to the variable results giving either *S*-CHE-cysteine or the unknown in hydrolysates was tested.

Radioactive bromopyruvate was, therefore, synthesized by procedure B. Since both acetic and formic acids are volatile, removal of solvent from the [^{14}C]bromopyruvate would give a product of low salt content. The chromatographic behavior of a hydrolysate of [1-^{14}C]CHE-aldolase inactivated at low salt concentration is shown in Figure 2. In contrast to Figure 1, virtually *all* of the label is the unknown. Identical results were obtained using bromopyruvate synthesized by either procedure B or C.

The elution position of the radioactive unknown suggested that it was a free acid. This could result from bromopyruvate inactivation of the aldolase by reaction with an active-site carboxyl group or phenolic OH to form an ester. Denaturation and reduction of [^{14}C]CKM covalently bound to protein in this way would produce [^{14}C]glyceric acid upon acid hydrolysis. Evidence for an ester linkage in enzyme inactivated at low salt concentration was obtained, *viz.*, (1) virtually all of the label of CHE-aldolase was released from the protein by overnight incubation in 0.05 N NaOH at room temperature, (2) 90% of the label was released from the protein by overnight incubation in 10 M urea containing 1 M hydroxylamine at pH 9. Both of these results rule out secondary amine attachment to the protein as would result from Schiff base reduction. In addition, they rule out reaction with the phenol group of a tyrosine since phenyl ethers are only hydrolyzed by acid (Greenstein and Winitz, 1960). In addition, it was demonstrated that the label of [^{14}C]CHE-aldolase was covalently bound to the protein since, as noted, it remained associated through several ammonium sulfate precipitations. It thus seemed reasonable that a carboxy ester to carbon-3 of D,L-lactic acid existed, which upon acid hydrolysis would lead to D,L-glyceric acid.

For identification of the unknown as glyceric acid, 100 mμmoles of purified material was prepared by inactivating 100,000 units of enzyme with [1-^{14}C]bromopyruvate. The radioactivity of the hydrolysate of denatured-reduced enzyme was purified by chromatography on Dowex 1-acetate, paper electrophoresis, and elution, followed by a second Dowex 1-acetate chromatography. The purified radioactive material was obtained in 50% overall yield. Chromatography on the 60-cm column of the amino acid analyzer using the machine's ninhydrin supply and spectrophotometer showed the radioactive unknown to be ninhydrin negative, which is consistent with its being glyceric acid.

Identification of the purified radioactive unknown as glyceric acid is shown in Figures 3 and 4, in which the radioactivity of ^{14}C unknown and D,L-[2-^{3}H]glycerate coincide on the amino acid analyzer and Dowex 1-acetate chromatography. In addition the ^{14}C unknown and D,L-[2-^{3}H]glycerate cochromatograph on Sephadex G-10 and have the same

TABLE I: Effect of Salt Concentration on the Alkylation of KDP-gluconic Aldolase.

Inactivation		CKM Residues Fixed/Enzyme Inactivated (moles/equiv)	$^3H:^{14}C$		
Sodium Citrate (mM)	pH		Hydrolysate	Dowex 1-Acetate Glycerate Peak	^{14}C as Glycerate (%)
20	6.0	1.02	7.4	7.47	99.0
100	6.0	0.97	4.0	7.7	52.0
250	5.5	1.05	3.75	13.75	27.3

electrophoretic mobility at pH 4. These results leave little doubt that the unknown radioactive peak seen in Figures 1 and 2 is glyceric acid, which would arise from the bromopyruvate alkylation of a protein-bound carboxyl group, chemical reduction, and hydrolysis.

As already suggested, the selectivity of bromopyruvate in the alkylation of KDP-gluconic aldolase appeared to be controlled by the concentration of salt present during inactivation. A study was undertaken in which pretreated enzyme was inactivated at various concentrations of buffer. Sodium citrate was employed in these experiments since it would provide high ionic strength. The inactivated [^{14}C]CKM-enzyme was denatured and reduced to [^{14}C]CHE-enzyme and chromatographed on Sephadex G-25 (Methods). Samples of the inactivated enzyme were degraded with pronase. Control experiments showed that, although pronase did not release the thioether derivative very efficiently, it did release all of the glyceric acid from the treated aldolase. Whereas acid hydrolysis and subsequent removal of HCl required 2.5 days, pronase hydrolysis (presumably by esterase action) was complete in a few hours and the sample could be used below without further treatment. The radioactive [^{14}C]-hydrolysate was mixed with a known amount of D,L-[2-^{3}H]-glycerate and the ratio of $^3H:^{14}C$ was determined by simultaneous counting. The sample was then chromatographed on a 0.75 × 10 cm column of Dowex 1-acetate employing a linear gradient established using equal volumes of water and 2 M acetic acid. The ratio of $^3H:^{14}C$ in the eluted peak was determined by simultaneous counting. The per cent total ^{14}C in the hydrolysate occurring as glycerate was calculated using the relationship

$$\% \ ^{14}C \text{ as glycerate} = \frac{(^3H:^{14}C)\ \text{final}}{(^3H:^{14}C)\ \text{initial}} \times 100$$

The data of this experiment are shown in Table I. As seen, when 20, 100 (pH 6), or 250 mM sodium citrate (pH 5.5) was

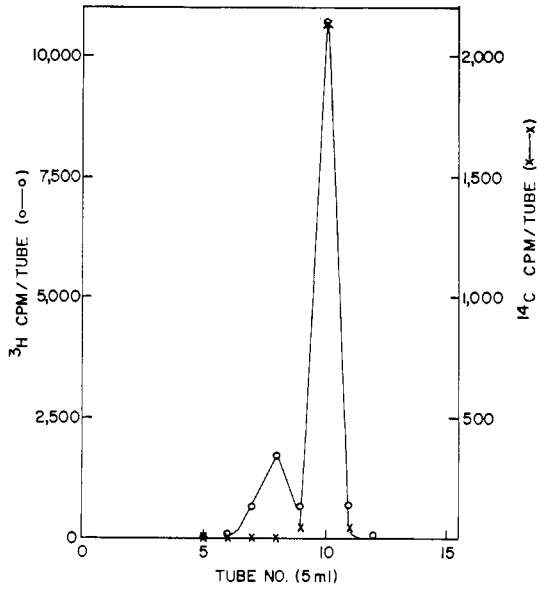

FIGURE 3: Cochromatography of ^{14}C unknown from [1-^{14}C]CHE-aldolase hydrolysate and D,L-[2-^{3}H]glycerate on the 60-cm column of the amino acid analyzer. Purified ^{14}C unknown (2200 cpm) and D,L-[2-^{3}H]glyceric acid (14,600 cpm) were applied to the column and located by simultaneous counting. The recovery of each isotope was quantitative. Elution was carried out with 0.20 M sodium citrate buffer at a flow rate of 1.167 ml/min.

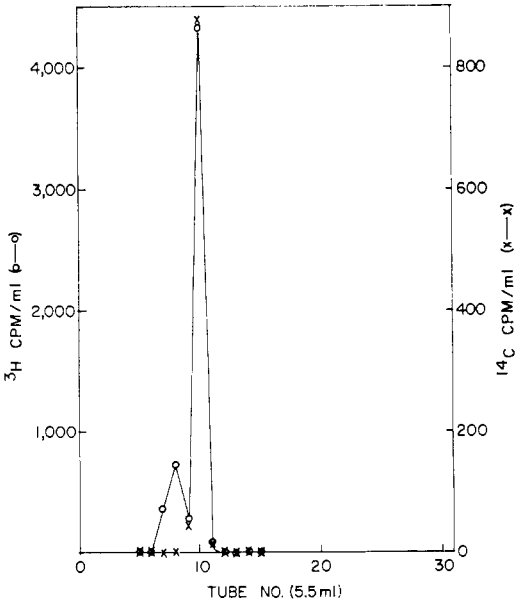

FIGURE 4: Cochromatography of ^{14}C unknown from [1-^{14}C]CHE-aldolase hydrolysate and D,L-[2-^{3}H]glyceric acid on Dowex 1-acetate. Purified ^{14}C unknown (7400 cpm) and D,L-[2-^{3}H]glyceric acid (37,000 cpm) were chromatographed on a 1 × 10 cm column of Dowex 1-acetate. Elution was achieved using a linear gradient resulting from equal volumes of water and 2 M acetic acid. Tritium and radioactive carbon were located by simultaneous counting; 5.5-ml fractions were collected.

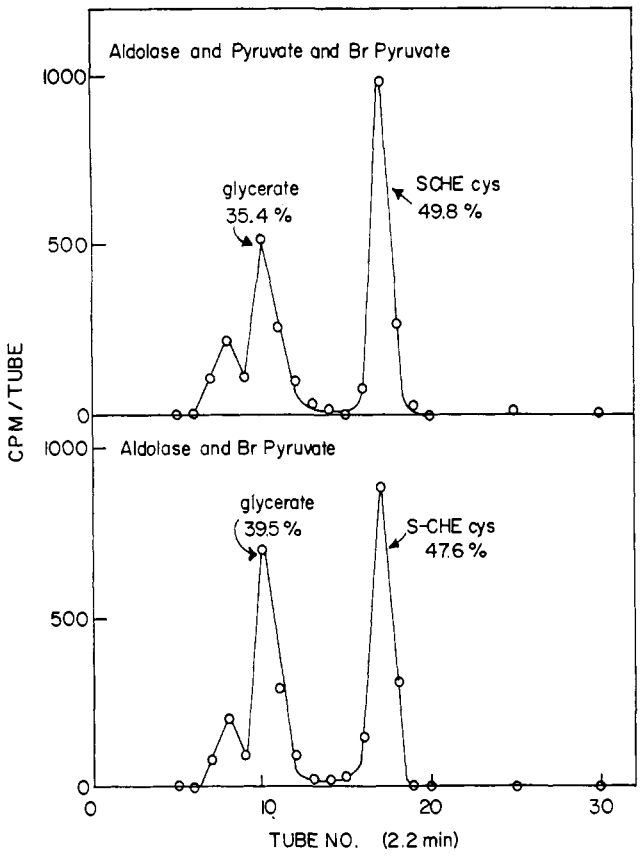

FIGURE 5: Distribution of label of CHE-aldolase formed at high salt concentration in the presence and absence of substrate. The aldolase was inactivated as described in the text in 1 M NaCl containing 20 mM sodium citrate (pH 6). A total of 3000 cpm of an acid hydrolysate was applied to the 60-cm column of the amino acid analyzer in each case. Radioactivity was located using liquid scintillation counting. Elution was carried out with 0.20 M sodium citrate (pH 3.28) at a flow rate of 1.167 ml/min.

present during inactivation, 1 CKM-residue was covalently bound per 4.7×10^5 units of enzyme inactivated. However, the fraction of total ^{14}C behaving as glycerate is, in per cent, 99, 52, and 27.3. It is assumed that nonglyceric acid counts are *S*-CHE-cysteine peptide. They were observed to chromatograph early in the elution profile. The data in Table I show that there is an inverse relation between the salt concentration of the medium during inactivation and the esterification of a protein-bound carboxyl group.

Table I also shows the inactivation stoichiometry to remain constant even though the incorporated label partitioned between two functional groups. This implies that both functional groups are at the active site of the enzyme. An experiment was undertaken to demonstrate this point in which the aldolase was partially inactivated at high salt concentration in the presence and absence of pyruvate. In preparation for this experiment the enzyme was pretreated with unlabeled bromopyruvate in the presence of protecting amounts of pyruvate and 1 M NaCl and then extensively dialyzed against 20 mM sodium citrate (pH 6) prior to use. The pretreated aldolase was then inactivated by 0.5 mM [1-^{14}C]bromopyruvate in 1 M NaCl containing 20 mM sodium citrate (pH 6). Inactivation was carried out for 6.5 min at 24°. The extent of activity loss was 40% with the incorporation of 12,500 cpm. A parallel labeling experiment was carried out in the presence of 10 mM sodium pyruvate. In this case 40% inactivation was achieved in 26 min with the incorporation of 12,000 cpm. Both protein samples were denatured, reduced, and acid hydrolyzed. A portion of the hydrolysate was examined on the amino acid analyzer and is shown in Figure 5. As seen, protein, whether inactivated in the presence or absence of pyruvate, has a very similar distribution of label between glyceric acid and *S*-CHE-cysteine. Thus the rate of isotope incorporation into both sites is equally suppressed by pyruvate. This is consistent with both bromopyruvate-sensitive groups being within the enzyme's active site.

Discussion

The data presented in this paper show that the bromopyruvate inactivation of KDP-gluconic aldolase is accompanied by either the esterification of a protein-bound carboxyl group or the alkylation of cysteine. The selectivity of the system for reaction with either the carboxyl or cysteine is controlled by the salt concentration during inactivation. In addition, the data support the assumption that both the reactive carboxyl and cysteine are in the enzyme's active site.

The explanation we wish to offer for the labeling data obtained with this system is that bromopyruvate sees two active site conformers. One possesses a reactive carboxylate anion and the other a cysteine mercaptyl. This hypothesis presupposes that the equilibrium between conformers is affected by salt. A mechanism eventually explaining the two nucleophiles in the enzyme's active site would be contingent upon salt having a specific effect on the catalytic reactions carried out by the enzyme. In separate experiments,[2] we have found that added NaCl has a pronounced effect on the cleavage reaction. The log of per cent remaining maximum velocity was a linear function of salt concentration; the enzyme was only 1% as active in 3.2 M NaCl. In addition, the K_m of KDP-gluconate increased with the square of the NaCl concentration which is similar to the effect of salt on fructose diphosphate aldolase of muscle (Rose and O'Connell, 1969). In contrast, added salt *does not alter the* V_{max} *of the pyruvate–proton-exchange reaction.* Consequently, a variable (salt), which markedly affects the total reaction, has little effect on the partial reaction. This supports the view that the transaldolization (cleavage) and protonation (exchange) steps depend on nonidentical sites, and suggests that the two nucleophiles seen by bromopyruvate are somehow related to the two catalytic steps. Transaldolization may occur in the carbanion possessing conformer while exchange occurs in the cysteine conformer.

Rose *et al.* (1965) were the first to postulate separate sites for protonation and transaldolization steps. This was based upon the kinetic behavior of muscle aldolase after selective modification by carboxypeptidase. The present results with KDP-gluconic aldolase provide the first chemical evidence to support the two-site hypothesis. Consequently, the occurrence of different aldolase conformers for carrying out the transaldolization and protonation steps may be a common phenomenon in nature. The extent of conformational and chemical difference in the active site involved in the two

catalytic roles is a question to which future experiments will be addressed.

Acknowledgment

It is a pleasure to acknowledge the encouragement and advice of Dr. Irwin A. Rose during the course of this work.

References

Dickens, F. (1962), *Biochem. Prepn. 9*, 86.
Greenstein, J. P., and Winitz, M. (1961), Chemistry of the Amino Acids, Vol. 3, New York, N. Y., Wiley, Chapter 40, p 2348.
Meloche, H. P. (1965), *Biochem. Biophys. Res. Commun. 18*, 277.
Meloche, H. P. (1967), *Biochemistry 6*, 2273.
Meloche, H. P., Ingram, J. I., 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.
Moore, S., and Stein, W. H. (1964), *Methods Enzymol. 6*, 19.
Rose, I. A. (1966), *Annu. Rev. Biochem. 35*, 23.
Rose, I. A., and O'Connell, E. L. (1969), *J. Biol. Chem. 244*, 126.
Rose, I. A., O'Connell, E. L., and Mehler, A. H. (1965), *J. Biol. Chem. 240*, 1758.
Yem, E. W., and Cocking, E. C. (1955), *Anal. Biochem. 80*, 209.

Physical Studies on Ribonucleic Acid Polymerase from *Escherichia coli* B*

Darwin Berg and Michael Chamberlin†

ABSTRACT: The sedimentation properties of RNA polymerase from *Escherichia coli* B have been studied. At high ionic strengths core polymerase exists as a single sedimenting species ($s_{20,w}$ = 12.6 S) and has a molecular weight, determined by low-speed sedimentation equilibrium, of $3.8 \times 10^5 \pm 0.2 \times 10^5$. RNA polymerase holoenzyme, containing 1 equiv of σ component, also exists as a single sedimenting species ($s_{20,w}$ = 15.0 S) at high ionic strengths. Studies of the subunit composition of the protein indicate that these species are the protomeric forms of the RNA polymerase molecule.

In solutions which contain high concentrations of ammonium sulfate the apparent values of $s_{20,w}$ and molecular weight decrease for both forms of the enzyme. The effect is not due to dissociation of the polymerase, but appears to be due to multicomponent interactions in the concentrated salt solution. Both core polymerase and RNA polymerase holoenzyme aggregate at low ionic strengths. In the absence of added salt (μ = 0.04) core polymerase forms a mixture of aggregates having a mean sedimentation coefficient of 44–48 S. Thus core polymerase can form aggregates at least as large as a hexamer of the 12.6S species. RNA polymerase holoenzyme sediments as a single species having $s_{20,w}$ = 23 S under these conditions. Thus the presence of 1 equiv of σ component limits the aggregation of RNA polymerase to a dimer of the protomeric form.

Deoxyribonucleic acid directed RNA polymerase (EC 2.7.7.6.) plays a central role in gene expression. The *Escherichia coli* enzyme can be isolated in two different forms: a core polymerase, and a polymerase holoenzyme. The latter form contains an additional subunit which has been designated as σ component (Burgess *et al.*, 1969; Berg *et al.*, 1970). With an intact, bacteriophage DNA as template, RNA polymerase holoenzyme synthesizes asymmetric RNA which is thought to be gene specific (Summers and Siegel, 1969; Travers, 1969). In contrast, core polymerase shows a reduced and variable ability to initiate RNA synthesis with such templates and the RNA produced is not gene specific. σ component appears to confer specificity on holoenzyme prior to the initiation of RNA chains (Travers and Burgess, 1969; Berg *et al.*, 1969), and probably determines the site at which the enzyme binds to DNA (Hinkle and Chamberlin, 1970). After initiation has occurred, σ component is released from the enzyme and can participate in a second round of initiation. The remaining core polymerase continues elongation of the newly formed RNA chain (Travers and Burgess, 1969; Berg *et al.*, 1969). Thus both RNA polymerase holoenzyme and core polym-

* From the Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720. *Received April 23, 1970.* This investigation was supported by research grants from the U. S. Public Health Service, GM 12010 from the Institute of General Medical Sciences, and Training Grant CA 05028 from the National Cancer Institute. Taken in part from a thesis submitted by D. Berg, University of California, Berkeley, Department of Molecular Biology, in partial satisfaction of the requirements for the degree of Doctor of Philosophy, and supported by a National Science Foundation predoctoral fellowship.

† To whom to address correspondence.