SUBSTRATE ANALOGUE INACTIVATION OF 2-KETO-3-DEOXY-6-PHOSPHOGLUCONIC ALDOLASE*

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Physical organic chemistry points out that a necessary early step, in aldolase reactions, is the activation of hydrogen (or its equivalent) on the carbon alpha to the carbonyl of the substrate. Thus, a basic group(s) functioning by abstracting this hydrogen of the substrate might be found in the active site of an aldolase. There is indirect evidence for the existence of such a group; viz. in enzymatic "enolate anion" reactions (the type reaction for aldolases) studied thus far, hydrogen exchange occurring at an asymmetric carbon is always stereospecific (Rose and Rieder (1955), Topper (1957), Rose and Rieder (1958), Rieder and Rose (1959), Rose and O'Connell (1960) and Lienhard and Rose (1964)). One possible explanation of this fact would be the existence of a sterically fixed basic group which both can mobilize the exchanging hydrogen of the substrate and can equilibrate with protons of water.

If such a basic group did exist in the active site of an aldolase, it might be demonstrated by modifying the substrate in such a way that the alpha carbon became electrophilic. This electrophilic carbon atom could then react with the basic group forming a covalent bond and result in complete inactivation of the enzyme as well as labeling of the active site.

The enzyme chosen for study was 2-keto-3-deoxy-6-phosphogluconic (KDPG) aldolase (from Pseudomonas fluorescens, A 3.12 (Meloche and Wood (1964 a and

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b)) which catalyzes the reversible condensation of pyruvate and D-glyceraldehyde-3-phosphate. It will be shown in this communication that bromopyruvate (an analogue of pyruvate) causes complete inactivation of KDPG aldolase and satisfies several criteria for reaction at the substrate specific site.

Inhibition of KDPG Aldolase by Bromopyruvate. - As shown in Fig. 1, one mM bromopyruvate inactivates KDPG aldolase and the inactivation is virtually complete. Upon incubating for a longer period of time, less than 1 per cent of the original aldolase activity is detectable. At higher levels of bromopyruvate (5 mM), the enzyme was rapidly and completely inactivated. No effect of the quantities of bromopyruvate added with the enzyme sample was detected on the assay used for KDPG aldolase (Meloche and Wood (1964a)). It is also seen (Fig. 1) that in the absence of bromopyruvate KDPG aldolase activity is stable for the duration of the experiment.

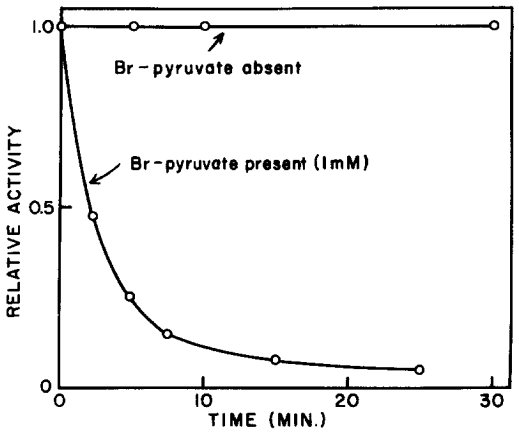

Figure 1. The inactivation of KDPG aldolase by bromopyruvate.

For inactivation, a 0.1 ml reaction mixture consisting of 50% pure KDPG aldolase (20 units, Meloche and Wood (1964a, 1964b)) and cacodylate buffer, pH 6.0 (10 μmoles) was preincubated 10-15 minutes, after which the initial rate was determined (1 μl samples) using the assay described by Meloche and Wood (1964a). The inactivation was then started by adding 1 μl of bromopyruvate (100 mM in acetone) and 1 μl samples were assayed at indicated times (the inactivation was stopped by dilution when adding the sample to the cuvette). All incubations and assays were at 25°. Bromopyruvate (m.p. 58-59) was prepared by the method of Dickens (1962).

In Fig. 2 is plotted the half-time of inactivation (reciprocally related to inactivation rate) vs, the reciprocal of bromopyruvate concentration. A straight line which extrapolates to a half-time of inactivation of 0.75 minutes is observed. Thus a zero-order region is seen in the bromopyruvate dependence of inactivation as well as a first-order region at lower concentration. This resemblance to Michaelis-Menten kinetic behavior of a substrate is an indication that a simple second-order mechanism is not involved, but rather that a reversible enzyme-bromopyruvate complex is formed prior to inactivation. From Fig. 2 it was determined that 1×10^{-3} M bromopyruvate gave one half maximum velocity of inactivation in the absence of substrate. Evidence for the existence of the enzyme-bromopyruvate complex is very important, since it supports the possibility that the active site (presumed) lysine necessary for Schiff's base formation (Grazi et al. (1963), Meloche and Wood (1964a)) is not being alkylated by bromopyruvate, but rather may be functioning normally by orienting the "substrate" within the active site of the enzyme. Thus, it would seem that a residue other than the above mentioned lysine might be alkylated by the substrate analogue.

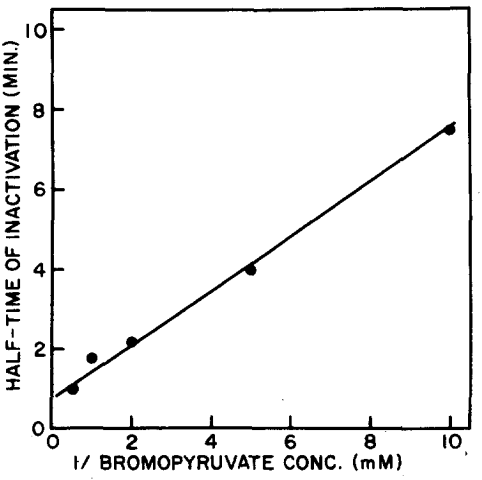

Figure 2. The effect of bromopyruvate concentration on the half-time of inactivation of KDPG aldolase.

All conditions were as described in Fig. 1, except that the concentration of the stock bromopyruvate solution (in acetone) was adjusted so that the addition of 1-2 µl would result in the final concentration indicated.

Further evidence for the analogue's attack within the active site is shown in Fig. 3 in which is seen the effect of pyruvate on the inactivation of KDPG aldolase by bromopyruvate. In the presence of three levels of the inhibitor (1, 2 and 4 mM), pyruvate protects the enzyme from inactivation; the half-time of inactivation increasing as the substrate concentration increases. In addition, it has been found that with 1 mM bromopyruvate, the protective effect of KDPG concentrations is similar to those of pyruvate. Interestingly, 8 mM D-glyceraldehyde-3-phosphate has little or no effect on the half-time of inactivation resultin from 1 mM bromopyruvate. Thus substrate protects the enzyme from inactivation by the analogue, and the protection seems to occur at a pyruvate specific site.

The complete inactivation of the enzyme by bromopyruvate, the conformity of the inactivation to Michaelis-Menten kinetics and the protective effect of substrate suggest that the analogue reacts within the active site of KDPG aldolase. This conclusion is further supported by the following additional observations: 1) the ethyl ester of bromopyruvate also completely inactivates

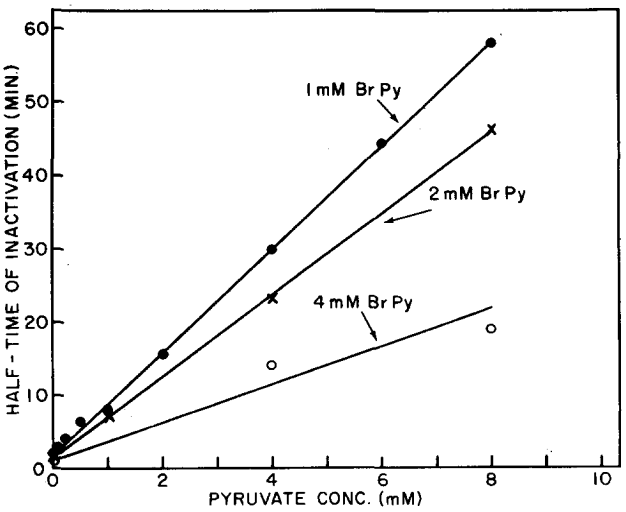

Figure 3. The protective effect of pyruvate on the inactivation of KDPG aldolase by bromopyruvate (Br py).

All conditions were as described in Figs. 1 and 2, except that aliquots of a Na pyruvate solution(s) (pH 6) were added prior to preincubation to give the indicated concentrations. The pyruvate stock solution(s) concentration was determined using lactic dehydrogenase and DPNH.

the enzyme but at only 2 per cent the rate of bromopyruvate; 2) bromoacetate, which lacks substrate similarity, on exhaustive treatment causes only partial (35 per cent) inactivation.

Currently, kinetic analyses of substrate protection data is underway to further confirm bromopyruvate's attack within the action site of the enzyme and to study the stoichiometry of inactivation. In addition, experiments are being conducted to identify the amino acid residue(s) alkylated by the analogue. If carbon three of bromopyruvate indeed does form a covalent bond with a basic group involved in alpha-hydrogen activation, a most intriguing possibility presents itself, viz. borohydride reduction of the Schiff's base existing between the carbonyl carbon of the covalently bound pyruvate and the lysine thought to be involved in catalysis. This presents the possibility of ultimately isolating and determining the amino acid sequence of a bridged peptide representing a significant portion of the active site of KDPG aldolase.

Other experiments are currently under way in this laboratory to study the effect of a similar substrate analogue (acetaldehyde-X) on another aldolase - deoxyribose-5-phosphate aldolase.

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