PYRUVATE CARBOXYLASE: AFFINITY LABELLING OF THE PYRUVATE BINDING SITE.[†]

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Abstract

The active-site-directed reagent, bromopyruvate has been used to covalently label the pyruvate binding site of pyruvate carboxylase (E.C.6.4.1.1.) isolated from sheep liver. Oxaloacetate proved to be the most effective reaction component in protecting the enzyme against inactivation; pyruvate was less effective although its efficiency was enhanced by the presence of acetyl CoA. The other reaction components, $MgATP^{2-}$ and HCO_3^- failed to protect the enzyme against inactivation. Using bromo-[2^{14}C]pyruvate, it was shown that at 100% inactivation, 1.5 pyruvyl residues were bound per mole of biotin and when the reaction was carried out in the presence of acetyl CoA, this ratio was reduced to 1.0. Analysis of pronase digests of the enzyme revealed that more than 90% of the radioactivity was present as carboxy-hydroxy-ethyl cysteine.

It is generally accepted that the tetrameric enzyme pyruvate carboxylase (pyruvate:CO_2 ligase (ADP), E.C.6.4.1.1.) catalyses the following sequence of events;

$$\text{E-biotin} + MgATP^{2-} + HCO_3^- \rightleftharpoons \text{E-biotin-}CO_2^- + MgADP^- + Pi^- \quad \ldots\ldots\text{(i)}$$

$$\text{E-biotin-}CO_2 + \text{pyruvate} \rightleftharpoons \text{E-biotin} + \text{oxaloacetate} \quad \ldots\ldots\text{(ii)}$$

by a non-classical Ping Pong Bi Bi Uni Uni mechanism (1). The initial step involves the formation of carboxy-biotinyl enzyme while in the second step, carboxyl transfer from E-biotin-CO_2^- to the acceptor substrate occurs. This basic scheme is characteristic of all biotin-dependent carboxylases, which differ primarily in the nature of the acceptor molecule. Its validity in the case of pyruvate carboxylase is supported by the isolation of the E-biotin-CO_2^- complex

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and the ability of this enzyme to catalyse three independent exchange reactions (2).

The implication to be drawn from this mechanism is that each active site on pyruvate carboxylase is composed of at least two functionally distinct catalytic sub-sites linked by the biotinyl residue functioning as a mobile carboxyl carrier. The spatial separation of the two sub-sites suggests the possibility of investigating the properties of each sub-site independently and in this communication we report on the use of bromopyruvate as an affinity label for the pyruvate binding site.

METHODS AND MATERIALS

Pyruvate carboxylase was purified from freeze-dried sheep liver mitochondria by a procedure similar to that described by Scrutton and Fung (3). Enzymic activity was assayed by the methods described previously (4,5). Kinetic studies of the inactivation of the enzyme by bromopyruvate were conducted at 30° in 25mM phosphate buffer, containing 1mM EDTA and 50mM $(NH_4)_2SO_4$, pH 7.2. The bromopyruvate solution (47mM) adjusted to pH 7.0 with 1M KOH was prepared within one h of use. Samples of the reaction mixture were removed at various time intervals and assayed immediately by the spectrophotometric assay procedure or by the radioisotopic method after quenching the reaction in 50mM dithioerythritol to prevent further inactivation. Carboxy-hydroxy-ethyl cysteine (CHE-cysteine) was prepared from N-acetyl-cysteine by the method of Barnett *et al.*, (6) while CHE-lysine was prepared from poly-L-lysine using the method of Okamoto and Morino (7). Bromo-[$2^{14}C$]-pyruvate was prepared by direct bromination of pyruvic acid (50μCi, 5mg) using the conditions described by Meloche (8).

RESULTS AND DISCUSSION

The inactivation of pyruvate carboxylase by bromopyruvate

exhibited pseudo first order kinetics and the rate of inactivation remained constant until at least 80% of the enzymic activity had been lost. Linear regression of the pseudo first order inactivation plots was used to determine the rates of inactivation produced by a range of bromopyruvate concentrations in the presence and absence of acetyl CoA (Fig. 1). When the log of the inactivation rate is plotted as a function of the log of irreversible inhibitor concentration, the slope of the line obtained is equal to the number of molecules of inhibitor responsible for the loss

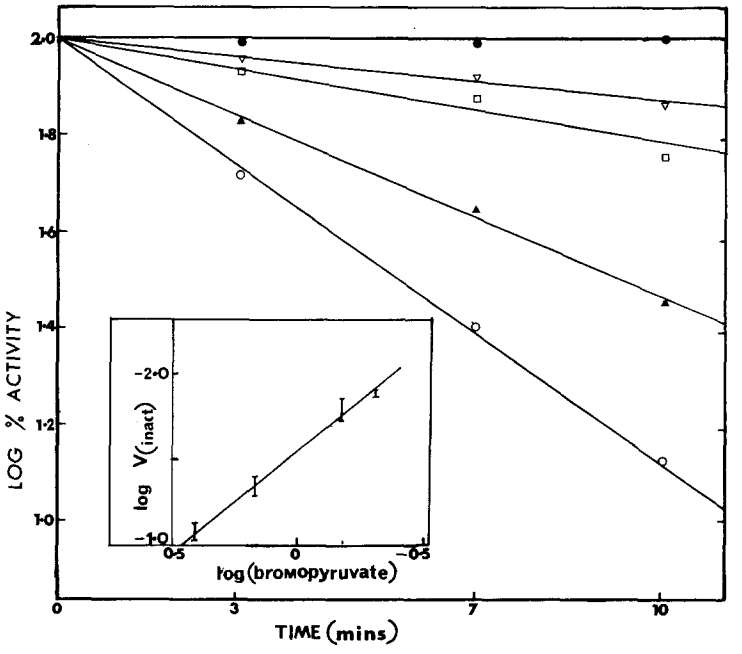

Fig. 1. Effect of varying concentrations of bromopyruvate on pyruvate carboxylase activity. To 100µl of enzyme (20 units/ml; spec. act. 15.2) in phosphate buffer, pH 7.2, was added 5µl of varying concentrations of bromopyruvate to give the following concentrations; (●—●), 0; (▽—▽), 0.4mM; (□—□), 0.6mM; (▲—▲), 1.2mM; (○—○), 2.0mM. Samples were assayed for enzymic activity remaining at various time intervals and expressed as log % of the initial velocity.
Inset: Replot of primary data where the log of the inactivation rate is plotted as a function of the log of bromopyruvate concentration.

of activity per active site (9). The slope of such a plot for the inactivation of pyruvate carboxylase by bromopyruvate (Fig. 1-inset) had an average value of 1.0 $\pm$ 0.24 and was only slightly reduced in the presence of acetyl CoA. This result suggested that bromopyruvate was selectively modifying one amino acid which was essential for enzymic activity.

In order to demonstrate that a compound is acting as an affinity label, Singer (10) has suggested that certain criteria need to be satisfied. One of these is that the substrate affords specific protection of the enzyme against inactivation by the affinity label. Therefore, the effect of pyruvate on the rate of inactivation of pyruvate carboxylase by bromopyruvate was investigated and the results are presented in Fig. 2. From these data it can be seen that 50mM pyruvate resulted in less than 15% reduction in the rate. However, in the presence of acetyl CoA, there was a pronounced reduction in the rate of inactivation. The degree of protection in the presence of acetyl CoA was dependent on the pyruvate concentration. The weak protection obtained by pyruvate alone agrees with the observation made by Mildvan et al., (11) who reported that [^{3}H]pyruvate bound poorly to pyruvate carboxylase isolated from chicken liver. Furthermore, the enhanced protection observed in the presence of acetyl CoA is supported by the findings of Ashman et al., (12) who showed that the apparent K_m value for pyruvate decreased from 6.0mM to 0.8mM in the presence of acetyl CoA.

In contrast, oxaloacetate proved to be a most effective protecting reagent against bromopyruvate inactivation and its effectiveness was also enhanced by the presence of acetyl CoA (Fig. 2). The explanation for this observation is that, unlike pyruvate, oxaloacetate binds readily to the free enzyme. On the other hand

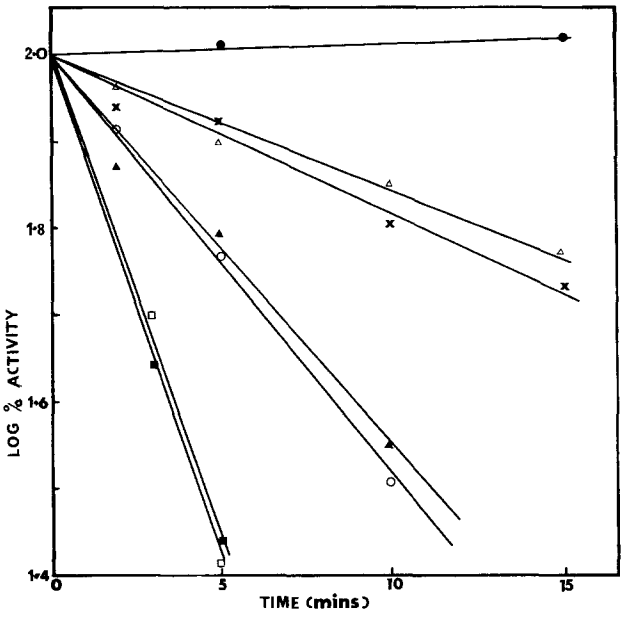

Fig. 2. Effect of pyruvate, oxaloacetate and acetyl CoA on the inactivation of pyruvate carboxylase by bromopyruvate. To 100µl of enzyme (11 units/ml; spec. act. 6.2) containing the additions as shown, was added 2µl (final concentration, 4.2mM) of bromopyruvate. Enzymic activity was determined at the time intervals indicated; (●—●), control; (■—■), no additions; (□—□), 20mM pyruvate; (○—○), 12.5mM oxaloacetate; (▲—▲), 0.5mM acetyl CoA; (△—△), 0.5mM acetyl CoA and 20mM pyruvate; (X—X), 0.5mM acetyl CoA and 12.5mM oxaloacetate.

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the components of the first partial reaction (equation (i)), i.e., $MgATP^{2-}$ and HCO_3^-, failed to protect the enzyme either singly or together in the presence or absence of acetyl CoA.

The above evidence suggested that brompyruvate was reacting specifically at the pyruvate binding site. Also, the possibility that it was acting as a non-specific alkylating reagent of residues essential for activity was eliminated when it was found that treatment of the enzyme with bromo- and iodo-acetate under identical conditions resulted in an inactivation rate of less than 5% of the bromopyruvate rate. The stoichiometry of the reaction was

determined using bromo-[2^{14}C]pyruvate and at 100% inactivation there were six pyruvyl residues bound for each four moles of biotin. However, when the reaction was carried out in the presence of acetyl CoA, a one-to-one ratio was observed. Although this could be interpreted to mean that bromopyruvate was also reacting at the acetyl CoA binding site, it was negated by the observation that bromopyruvate inactivated both the acetyl CoA-dependent and -independent activities to the same extent. These findings imply that acetyl CoA, in addition to protecting four residues which are essential for enzymic activity, also completely masks two residues

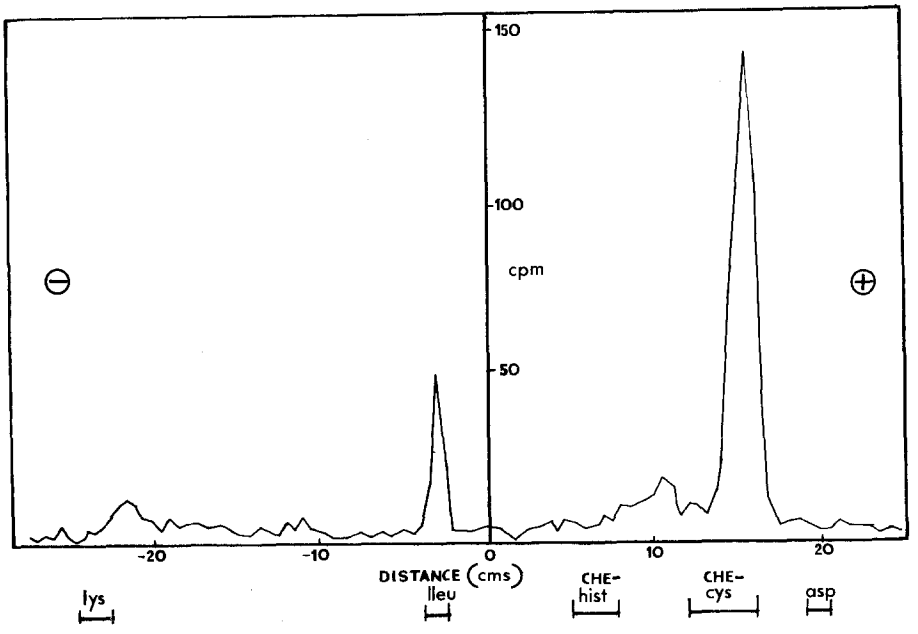

Fig. 3. Electrophoretogram of an enzymic digest of pyruvate carboxylase modified with radio-active bromopyruvate. The enzyme (3.3mg; spec. act. 8.2) was inactivated with bromo-[2^{14}C]-pyruvate (7.5 x 10^{5}cpm per μmole; final concentration, 1mM) until the enzyme was 50% inactivated. The protein was enzymically digested using a combination of trypsin, chymotrypsin, pronase and α-amino peptidase. The digest was applied to Whatman 3mM paper and the electrophoretogram was developed using 3,000 V, 60mA for one h in 0.04M acetate buffer, pH 5.5. Markers were detected by spraying with ninhydrin.

which are not involved in the catalytic process.

In order to identify the residue modified by bromopyruvate, an analysis was carried out on the enzyme after modifying with bromo-[2^{14}C]pyruvate. After 50% of the enzyme had been inactivated, the reaction was quenched with 50mM dithioerythritol and then incubated for one h with 40mM sodium borohydride. The enzyme was precipitated with 10% trichloroacetic acid at 0° and washed with trichloroacetic acid until there was no detectable radioactivity in the supernatant. The denatured protein was digested with pronase and subjected to high voltage paper electrophoresis. After development, analysis of the electrophoretogram (Fig. 3) showed a major band of radioactivity which coincided with the CHE-cysteine marker. The smaller band of radioactivity moving toward the cathode has not been identified but does not correspond to any CHE-derivative that we were able to examine.

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