ACTIVE SITE MODIFICATION OF ISOCITRATE LYASE[1]

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SUMMARY. β-Bromopyruvate (BrP) alkylates isocitrate lyase at the active site as indicated by the following: (1) the irreversible alkylation shows saturation kinetics, (2) substrate, isocitrate, is a competitive inhibitor of inactivation by BrP, (3) the combination of products, glyoxylate and succinate, or a competitive inhibitor of the enzyme, oxalate, or glyoxylate plus itaconate (a succinate analogue) all protect against inactivation by BrP, (4) the rate of inactivation can be increased by EDTA, an initiator of the catalytic reaction in the presence of activated (reduced) enzyme and substrate. About four carboxyketomethyl residues are incorporated per mole of inactivated enzyme which implies four catalytic sites in the tetrameric enzyme. Amino acid analysis after H_2O_2 treatment of the alkylated enzyme demonstrates that one cys/monomer is being alkylated.

The glyoxylate cycle is an important anaplerotic pathway in some microorganisms and fatty seedlings (1). It affords the net synthesis of organic acids of the TCA cycle from 2-carbon metabolites. In our laboratory we are investigating the mechanism of catalysis and mode of regulation of the first enzyme unique to the glyoxylate cycle, isocitrate lyase. The enzyme from Pseudomonas indigofera is a tetramer of closely similar or identical subunits (2). The results presented in this communication suggest that β-bromopyruvate inactivates isocitrate lyase through the akylation of a cys at each of four active sites.

Isocitrate lyase was purified to homogeneity from P. indigofera by a

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slight modification of the procedure of Shiio et al. (3). Protein was measured by the procedure of Lowry et al. (4) and enzyme assayed essentially as described by McFadden (5). For studies of ^{14}C-incorporation, labeled protein was precipitated with trichloroacetic acid, collected and washed on a Millipore membrane (0.22 μ) and assayed (6).

The time course of inactivation of isocitrate lyase by β-bromopyruvate (BrP) was studied at pH 7.7 with reduced enzyme, i.e., enzyme that had been incubated at least 10 minutes at 30° with 10 mM dithiothreitol in 0.1 M tris containing 5 mM Mg^{+2} and then separated from the sulfhydryl compound by gel filtration on a Sephadex G-50 column. Reduced enzyme is known to be only slightly catalytically active in the presence of substrate (Dr. J. A. Williams, unpublished) unless EDTA or a sulfhydryl reagent is added (7). When the effects of other compounds on inactivation by BrP were measured, these compounds were

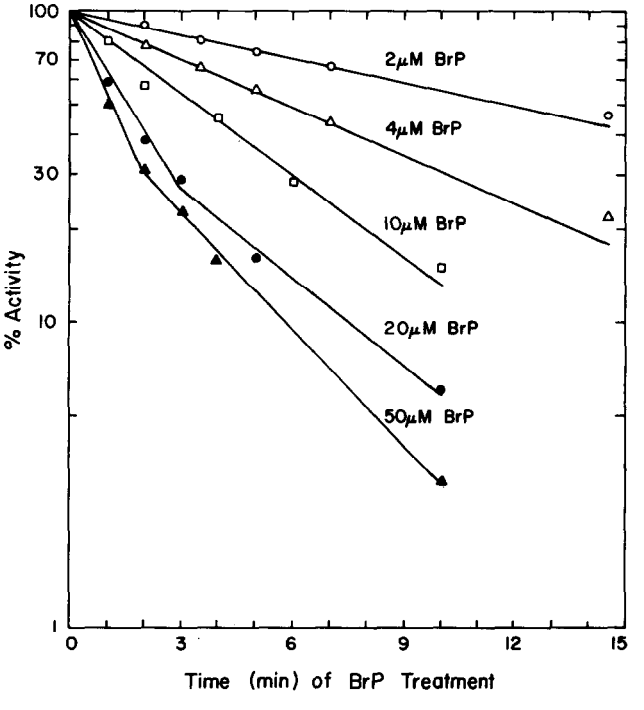

Figure 1. Semi-log plot of time course of inactivation of isocitrate lyase by BrP at 30°. The enzyme concentration was 6 x 10^{-9} M for the upper four curves and 9 x 10^{-9} M for the bottom curve.

incubated with reduced enzyme for 5 minutes prior to the addition of BrP. The BrP reaction with enzyme was instantaneously stopped by the addition of dithiothreitol to a final concentration of 5 mM. Control experiments established that BrP was stable throughout the course of all experiments described. For example the half-time of hydrolysis of 4 mM BrP at pH 7.7 was 162 minutes at 30°. The pH was maintained either with 0.1 M tris-acetate or a pH stat, and the hydrolysis measured both titrimetrically and by bromide release (8).

Figure 1 shows a semi-log plot of the percent enzyme activity remaining versus the time of BrP treatment at several different BrP concentrations. There is a marked change in slope (Fig. 1) above 70% inactivation. It should be emphasized that this change in slope has been observed reproducibly over a wide enzyme concentration range including 400-fold higher concentrations and that the lower linear limb always extrapolates to zero time at ca. 50% activity.

Assuming the "pre-equilibrium" model, $E+BrP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EBrP \xrightarrow{k_2} EP$ where $k_2 << k_{-1}$ and $\frac{dEP}{dt} = k_2[EBrP] = k_2(E_T - EP) \Big/ 1+\frac{K_{BrP}}{[BrP]}$, integration of the rate equation yields: $\ln \frac{E_T}{E_T - EP} = \frac{k_2 t}{1+\frac{K_{BrP}}{[BrP]}}$ where E_T equals the total enzyme concentration and K_{BrP} is the dissociation constant, $\frac{k_{-1}}{k_1}$, for the EBrP complex. The model predicts that $\tau = \frac{\ln 2}{k_2} + \frac{\ln 2}{k_2}\frac{K_{BrP}}{[BrP]}$, where τ is the inactivation half-time. Thus a plot of τ versus $\frac{1}{[BrP]}$ should give a straight line with intercept $\frac{\ln 2}{k_2}$ which would be the half-time at saturating BrP or the minimum inactivation half-time. The open circles of Figure 2 show that such a plot yields a straight line which extrapolates to 0.7 minutes for the minimum inactivation half-time. Thus the inactivation shows saturation kinetics as would be expected if BrP were reversibly binding the active site prior to alkylation, presumably at or near the same site. From the slope of the line, K_{BrP} is obtained and equals 3×10^{-5} M. In this connection pyruvate, a competitive inhibitor, has a K_i of 5.2×10^{-5} M.

If BrP is a competitive inhibitor of the binding of substrate, S, i.e., both cannot be bound to the enzyme simultaneously, the expressions for the

binding of S (assuming no cooperative effects) are $E+S \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ES$ and $K_s = \frac{k_{-3}}{k_3}$ and the integrated rate equation becomes $\ln \frac{E_T}{E_T - EP} = \frac{k_2 t}{1+\frac{K_{BrP}}{[BrP]}\left(1+\frac{[S]}{K_s}\right)}$. From this expression, it can be shown that $\tau = \frac{\ln 2}{k_2} + \frac{K_{BrP}}{[BrP]} \frac{\ln 2}{k_2}\left(1+\frac{[S]}{K_s}\right)$; thus a plot of τ versus $\frac{1}{[BrP]}$ should give a straight line with the intercept also at $\frac{\ln 2}{k_2}$, the minimum inactivation half-time. In Figure 2, a τ versus $\frac{1}{[BrP]}$ plot is shown for 0.5 and 2 mM D_s-isocitrate. Both give straight lines that extrapolate to the same minimum inactivation half-time, 0.7 minutes, as found in the absence of isocitrate. From the slope of the two lines, K_s can be calculated and 0.088 and 0.090 mM are obtained for 0.5 and 2 mM D_s-isocitrate, respectively. These values are in good agreement with the K_m of 0.11 mM obtained recently by Dr. J. A. Williams in our laboratory. The competitive mode of the protection by isocitrate against BrP inactivation indicates that isocitrate protects by excluding BrP from the active site.

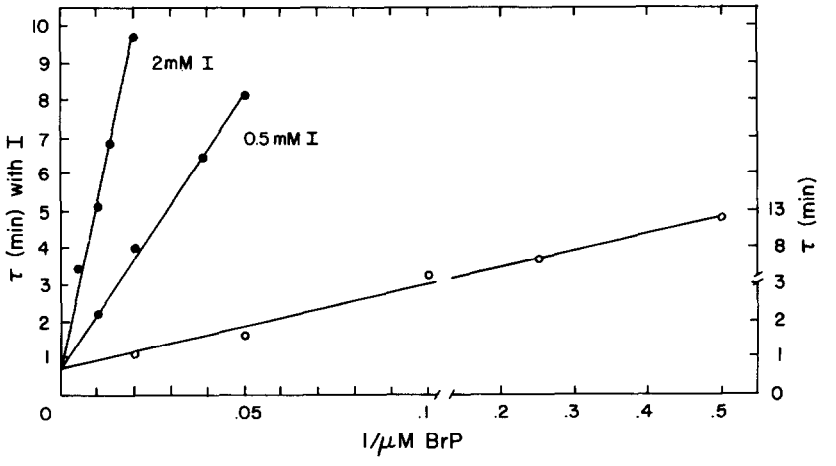

Figure 2. Inactivation half-time as a function of the reciprocal of the BrP concentration.

Table I shows the effects of various compounds on the inactivation by BrP. Preincubation with the combination of products glyoxylate and succinate, both at 10 mM, resulted in 95% retention of activity which was equal to that

obtained in the presence of 4 mM D_s-isocitrate. Oxalate, a competitive inhibitor of the cleavage reaction (9) also reduced the rate of inactivation by BrP. Either succinate or itaconate, a structural analogue of succinate that is known to be an uncompetitive inhibitor (9), fail to give any protection against BrP inactivation. However, each in the presence of glyoxylate gave excellent protection against BrP inactivation. Itaconate has been shown to combine with the enzyme only in the presence of glyoxylate (Dr. J. A. Williams, unpublished). The present results provide added support for this and suggest that the same is also true for succinate.

The results in experiment 4, Table I, show that both glyoxylate and EDTA increase the rate of inactivation by BrP, but, as would be expected if they

TABLE I

Effect of Various Compounds on 3-Minute Treatment of Isocitrate Lyase with 5 μM BrP at 30°

Exp.*	Test Compound**	Concn. (mM)	% Activity Recovered	% Activity Recovered without Test Compound (Control)
1.	D_s-isocitrate	4	95	58
	oxalate	0.5	75	
2.	glyoxylate, succinate	10 (both)	95	58
	succinate	10	54	
3.	glyoxylate, itaconate	10, 0.5	97	44
	itaconate	0.5	43	
4.	glyoxylate	10	29	48
	EDTA	1	25	
	glyoxylate, EDTA	10, 1	28	

*Experiments (Exp.) 1, 2, 3 and 4 were conducted at enzyme concentrations of 6, 6, 12 and 8 x 10^{-8} M, respectively.

**Prior to enzyme assay, compounds except for isocitrate were separated from enzyme by dialysis in Exp. 1 and by filtration on Sephadex G-50 in Exp. 2-4.

were functioning at a common site, fail to have an additive effect. Presumably the binding of glyoxylate at the catalytic site induces a conformational change which sensitizes the enzyme to attack by BrP. The effect of EDTA in increasing the rate of inactivation by BrP is most likely related to the mechanism by which it initiates catalysis by reduced enzyme in the presence of isocitrate.

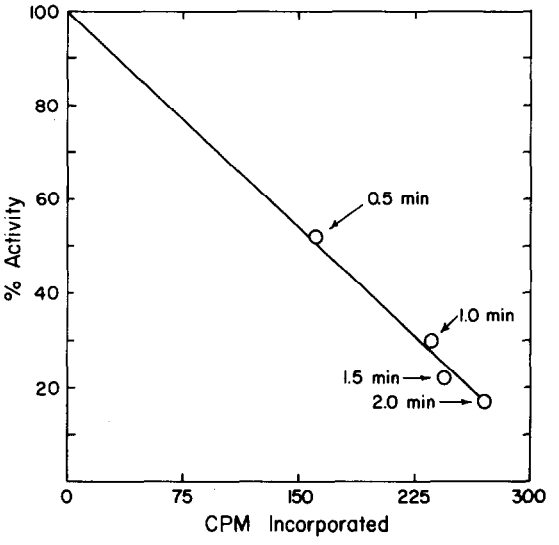

Figure 3. Percent of residual catalytic activity versus incorporation of ^{14}C-BrP into enzyme.

2-^{14}C-bromopyruvate (specific activity 0.0405 μC/μmole), synthesized from 2-^{14}C-pyruvate (10), was used to study the stoichiometry of alkylation of isocitrate lyase. In Figure 3 linearity is shown between incorporation of ^{14}C-BrP and inactivation over a range representing both initial and final slopes on plots like Fig. 1. All investigations of ^{14}C-incorporation into enzyme were carried out with concentrations of enzyme of 1.24 to 4.8 x 10^{-6} M. The stoichiometry for the BrP inactivation process was examined under a variety of experimental conditions and some of the results are tabulated in Table II. As evident, values clustered around four. Other studies have established that virtually complete inactivation (>97%) is associated with saturation of ^{14}C-BrP incorporation providing further evidence for the reagent's site speci-

TABLE II

Incorporation of ^{14}C-Bromopyruvate (^{14}C-BrP) by Isocitrate Lyase

Preincubation with D_s-isocitrate	mM ^{14}C-BrP	Fraction Inhibited	Moles ^{14}C-BrP / Moles Active Enzyme / Fraction Inhibited *
- - -	1.0 (3 min)	0.90	3.98
- - -	0.45 "	0.88	4.01
- - -	0.058 "	0.88	3.90
- - -	0.02 (0.5 min)	0.48	4.02
- - -	" (1.0 min)	0.70	4.02
- - -	" (1.5 min)	0.78	3.77
- - -	" (2.0 min)	0.83	3.90
4 mM (5 min)	" (3.0 min)	0.55	4.15

*These values reflect a correction for a small inactivation of enzyme (7-20%) during gel filtration to remove the thiol as described in a previous section.

ficity. Since all available evidence indicates that the enzyme is a tetramer (2), the above data imply the existence of 1 catalytic site/subunit.

To identify enzymic sites alkylated by BrP, alkylated isocitrate lyase was first dailyzed in 0.01 M tris-Cl, pH 8.0, containing 0.5 mM H_2O_2 for three hours at 25°. This was then dialyzed at 25° for 12 hours against .001 M potassium phosphate, pH 8.0, containing 10^5 units of catalase, lyophilyzed, and hydrolyzed for 21 hours in completely degassed constant boiling HCl. The amino acid analyses were obtained with a Beckman Spinco 120 C amino acid analyzer. Analysis yielded 3.64 nanomoles of carboxymethylcysteine/nanomole of modified enzyme. This is in good agreement with results from the ^{14}C-BrP studies and establishes that BrP is alkylating a cysteine residue. An analogous experiment in which N-acetylcysteine was alkylated with BrP and the product oxidized with H_2O_2 resulted in a quantitative yield of carboxymethylcysteine.

Noting that the loss of activity is directly proportional to BrP incorporation

(as shown in Figure 3) and that four BrP are incorporated into the tetrameric enzyme, the biphasic first-order plots consistently seen and typified by Figure 1 are of particular interest. As noted before, extrapolation to the ordinate of the lower limbs of these curves indicates that approximately 50% of the total enzyme activity is being lost at the slower rate. A closely similar phenomenon was evident in prior work with the much poorer alkylating agent, 2-bromomalonate, which also shows some active site specificity for isocitrate lyase (2). We postulate that the tetrameric enzyme has two nonidentical pairs of subunits with each pair undergoing different rates of inactivation. This hypothesis is under investigation.

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