IRREVERSIBLE INACTIVATION OF UROCANASE BY 4-BROMOCROTONATE

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SUMMARY

Urocanase from *Pseudomonas putida* is irreversibly inactivated by 4-bromocrotonate. At pH 6.7 and 25°, the rate of inactivation is first-order in remaining active enzyme and follows saturation kinetics with a K_I of 180 mM and a maximum inactivation rate of 0.889 min^{-1}. The rate constant of inactivation decreases with pH in the pH range 5.8 to 8.5. 4-Bromocrotonate methyl ester inactivates urocanase at only 3% the rate observed with bromocrotonate while other alkylating reagents are ineffective in promoting a time-dependent loss of activity. Dihydrourocanate protects competitively against bromocrotonate inactivation; an average value of 3.3 mM at pH 6.7 is obtained for the enzyme-dihydrourocanate dissociation constant. Protection against inactivation is also offered by fumarate and crotonate, but not by maleate. The results are consistent with bromocrotonate reacting within the active site region of the enzyme.

The addition of water to urocanic acid yielding imidazolone propionic acid represents the second step in the catabolic pathway for histidine. Urocanase, the enzyme which mediates this conversion, has been obtained in homogeneous form from *Pseudomonas putida* and has been partially characterized (1,2). An unusual structural feature of the protein is the presence of an α-ketobutyryl residue covalently attached to each of two identical or very similar subunits (1,2). Although a role for the α-ketobutyryl moiety in the catalytic process has not been definitively established, its participation as a prosthetic group in the hydration reaction seems likely since borohydride reduction of the enzyme destroys catalytic activity (1). In an attempt to provide insight into the nature of other residues which may be involved at the active site, we have tested 4-bromocrotonate as a possible affinity label

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for urocanase. Since both fumarate and crotonate have been found to competitively inhibit the enzyme (1,3), the reactive bromomethylene group of BCA[2] might be expected to be favorably positioned for covalent modification of a catalytically crucial side chain. In this communication, we show that bromocrotonate rapidly inactivates urocanase from *P. putida*. Kinetic evidence also is presented to suggest that the inactivation reaction is active site-directed.

MATERIALS AND METHODS

4-Bromocrotonic acid was synthesized by bromination of crotonic acid with N-bromosuccinimide in the presence of benzoyl peroxide (4,5). The product obtained after repeated recrystallization from ligroin melted at 72-74° (lit. 74° (6)). The NMR spectrum (CCl_4) of this material was in accord with the expected structure (4). Bromocrotonate methyl ester (b.p. 83-86°/15 mm) was prepared by reaction of the haloacid with methanol-HCl-acetyl chloride (7). 4-Bromobutyric acid (60%, Sigma) was vacuum distilled before use. All other chemicals were of the highest purity commercially available.

Urocanase was isolated from sonic extracts of histidine-adapted *Pseudomonas putida* (ATCC 12633) as described by George and Phillips (1). Enzyme activity was assayed spectrophotometrically at 25° by following the decrease in absorbance at 277 nm in 0.1 M potassium phosphate buffer (pH 7.5) containing 0.1 mM urocanate. The assay was modified from that of Tabor and Mehler (8).

Unless otherwise indicated, kinetic studies of bromocrotonate inactivation of the enzyme were carried out at 25° in 0.2 M potassium phosphate (pH 6.7). Samples were withdrawn periodically from the reaction mixture and transferred directly into cuvets containing standard assay solution supplemented with 5-10 mM dithiothreitol, which rapidly decomposed excess bromocrotonate and effectively terminated the inactivation reaction. Rate constants were calculated from least-squares lines fitted to semilogarithmic plots. Control incubations with enzyme alone established that urocanase activity was stable during the reaction period under all conditions employed.

RESULTS AND DISCUSSION

Exposure of *P. putida* urocanase to 20 mM 4-bromocrotonate for 60 minutes at pH 6.7 and 25° results in the loss of greater than 99 percent of the original enzymatic activity. Incubation for longer periods of time or with greater concentrations of the reagent results in complete inactivation, which can not be reversed by extensive dialysis at neutral pH. Loss of urocanase

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[2]The abbreviation used is: BCA, 4-bromocrotonate

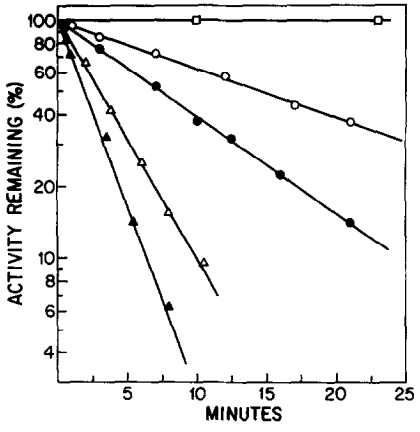

FIGURE 1: Pseudo first-order plots of the inactivation of urocanase (0.09-0.11 enzyme units/ml) by 4-bromocrotonate in 0.2 M potassium phosphate (pH 6.7) at 25°. A unit of enzyme activity is equivalent to the consumption of 1 μmole of urocanate/minute under the assay conditions specified in the text. (○) 10 mM, (●) 20 mM, (△) 60 mM, (▲) 120 mM bromocrotonate. (□), no inhibitor.

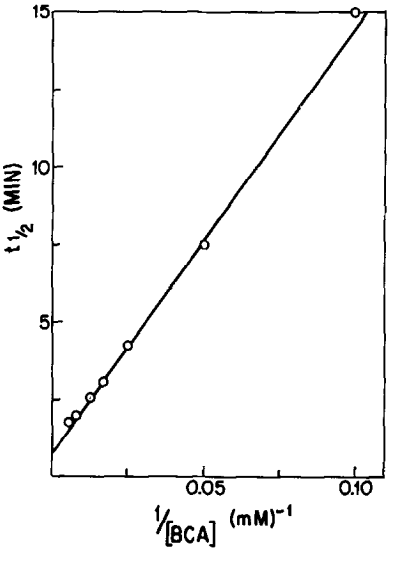

FIGURE 2: Inactivation half-life ($t_{\frac{1}{2}}$) for inactivation of urocanase as a function of the reciprocal of bromocrotonate concentration. Values of $t_{\frac{1}{2}}$ were calculated from plots as shown in Figure 1.

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activity in the presence of excess bromocrotonate is a pseudo first-order process to at least 95% inactivation and increasing concentrations of the inhibitor accelerate the rate of inactivation (Figure 1). The calculated

apparent first-order rate constants of inactivation show a nonlinear dependence upon BCA concentration, implying that inactive enzyme is formed *via* an intermediate complex of bromocrotonate with urocanase. For such reactions, the half-life of inactivation ($t_{\frac{1}{2}}$) should be a linear function of the reciprocal of BCA concentration intercepting the ordinate at a finite value, T, the minimum inactivation half-life at infinite inhibitor concentration (9-11). A straight line is obtained (Figure 2) which extrapolates to a half-life of inactivation of 0.78 minute. The concentration of bromocrotonate giving the half-maximal inactivation rate (K_I) is 180 mM, and a first-order rate constant of 0.889 min^{-1} at pH 6.7 and 25° is computed from T.

The structural requirements for time-dependent inhibition of urocanase appear to be quite stringent (Table I). Only 14% inactivation is achieved

TABLE I: Time-dependent Inhibition of *P. putida* Urocanase

Additions	Activity Remaining (%)	
	30 min.	60 min.
None	100	100
4-Bromocrotonate	6	< 1
4-Bromocrotonate methyl ester	93	86
Iodoacetate	100	100
Bromoacetate	100	100
3-Bromopropionate	102	99
4-Bromobutyrate	100	98

Each inhibitor at 20 mM concentration was incubated with urocanase under the conditions described in the legend of Figure 1. Aliquots were withdrawn after 30 and 60 minutes and assayed for enzyme activity.

with the methyl ester of 4-bromocrotonate (20 mM) in 1 hour; the rate of inactivation (0.003 min^{-1}) is approximately 30 times slower than that observed (0.093 min^{-1}) with 20 mM bromocrotonate. In addition, iodoacetate, bromoacetate, 3-bromopropionate and 4-bromobutyrate have no measurable effect on enzyme activity under the same conditions.

The rate of BCA-inactivation is substantially diminished in the presence of dihydrourocanate, a competitive inhibitor of bacterial urocanase (1,12,13). In order to provide evidence that this effect is mediated by competition between dihydrourocanate and bromocrotonate for binding to the same site (or sites) on the protein molecule, protection studies were carried out using the approach described by Meloche (11). Plots of $t_{\frac{1}{2}}$ *vs.* increasing concentrations of dihydrourocanate for inactivation of urocanase by 10 and 20 mM bromocrotonate are depicted in Figure 3. In both cases, linear relationships are observed with the respective slopes equal to 5.25 and 1.8, from which an average value of 3.3 ± 0.6 mM is calculated for the apparent dissociation constant of the enzyme-dihydrourocanate complex. The intercepts obtained

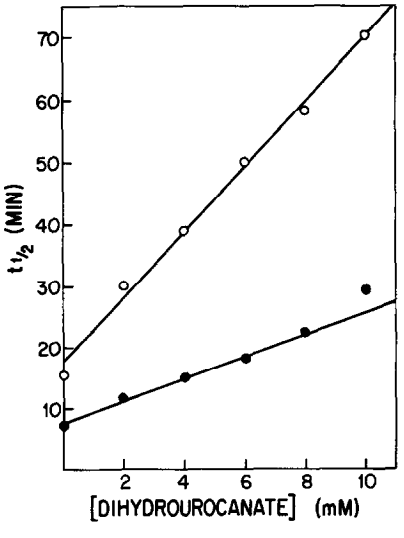

FIGURE 3: Inactivation half-life ($t_{\frac{1}{2}}$) as a function of dihydrourocanate concentration in the presence of 10 mM (○) and 20 mM (●) bromocrotonate.

from the data of Figure 3 are 17 and 7.6 minutes, respectively, values which agree closely with those (15 and 7.8 minutes) derived by calculation (11). These results support the postulate that dihydrourocanate protects urocanase against bromocrotonate inactivation by binding at the active site.

A similar protective influence against inactivation is provided by fumarate and by crotonate; almost no activity is lost in 30 minutes when either compound (50 mM each) is present in a reaction mixture containing bromocrotonate at 10 mM concentration. In contrast, no significant difference in the inactivation rate is observed in the presence of 50 mM maleate. Unlike fumarate and crotonate, maleate also has no effect on urocanase activity (1).

Figure 4 shows the variation of the rate of inactivation as a function of pH. The reason for the pronounced decrease in rate (*ca.* 9-fold) observed between pH 5.8 and 8.5 is not obvious, since the opposite result would be anticipated for the covalent reaction of bromocrotonate with a nucleophilic group on the enzyme and dissociation of the carboxyl proton of the reagent should not be a contributing factor over the pH range examined. The possi-

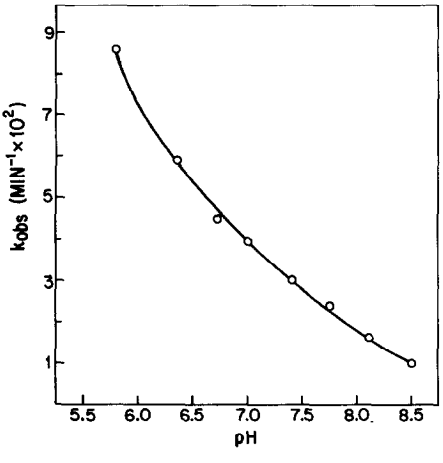

FIGURE 4: Effect of pH on the apparent first-order rate constant (k_{obs}) of bromocrotonate inactivation. Rates of inactivation were determined at 25° in 0.2 M phosphate or 0.2 M Tris-HCl buffers containing 10 mM bromocrotonate.

bility that this result may be due to decomposition of bromocrotonate during these experiments has also been ruled out on the basis of NMR spectroscopy measurements. However, since the rate of the reaction is likely controlled by the concentration of a dissociable enzyme-BCA complex (Figure 2), a decrease in the affinity of urocanase for the reagent with increasing pH could give rise to the pH-rate profile observed. This effect might also be due, of course, to the influence of an ionizable group (or groups) not directly involved in complex formation or to a pH-dependent structural change in the enzyme.

The reaction of bromocrotonate with urocanase exhibits several characteristics expected for an affinity label. The enzyme is completely and irreversibly inactivated by the analog, a reversible enzyme-inactivator complex is formed prior to inactivation (*vide supra*), and the inactivation rate is retarded in the presence of competitive inhibitors. Additional evidence for active-site modification of urocanase by BCA includes the failure of maleate to protect against inactivation and the demonstration that dihydrourocanate and bromocrotonate compete for the same site. The observation that bromocrotonate inactivates urocanase more rapidly than does BCA methyl ester may indicate that bromocrotonate initially binds to the enzyme by ionic interaction of a positively charged group at the urocanate binding site with the carboxylate group of the haloacid, thus orienting its γ carbon atom properly for electrophilic attack on a functional side chain. A similar process may be responsible for the selective modification by BCA of active site structures in swine heart fumarase (5). Further work is needed, however, to determine whether the apparent specificity of BCA in inactivating urocanase (Table I) is due to inherent differences in reactivity of these halogen compounds towards nucleophiles, or to differences in the binding constants or the stereochemistry of enzyme-inhibitor complexes.

Currently, experiments are in progress to identify the residue(s) modified by bromocrotonate and to substantiate the kinetic implications of active

site specificity reported here. Bromocrotonate may also prove useful as an active site probe for urocanase from other organisms.

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