Irreversible Inactivation of Bovine Carbonic Anhydrase B by Bromoacetazolamide*

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SUMMARY

Bromoacetazolamide effects rather quickly a partial, and more slowly an almost complete, irreversible inactivation of bovine carbonic anhydrase B (EC 4.2.1.1). Under identical conditions, the enzyme is not inactivated by bromoacetic acid or iodoacetamide, nor does it react significantly with these compounds. The zinc-free enzyme does not undergo irreversible binding with 14C-bromoacetazolamide and can be fully reactivated by zinc after exposure to bromoacetazolamide. At low inhibitor concentrations 1 eq of 14C-bromoacetazolamide reacts with the native enzyme. It is postulated that the enzyme at first forms a complex with the inhibitor. This rapid step is followed by a slower formation of a covalent bond between bromoacetazolamide and an amino acid at or close to the active site of the enzyme resulting in irreversible inactivation. Amino acid analysis of the native and inhibited enzymes revealed that, as a consequence of irreversible inactivation, the 3 nitrogen of 1 eq of histidine had reacted with bromoacetazolamide. At higher inhibitor concentrations, 2 eq of 14C-bromoacetazolamide combined with the enzyme. It is suggested that the initial formation of a complex or covalent bond by 1 molecule of bromoacetazolamide alters the three-dimensional structure of the enzyme so as to make a second combining site available.

The carbonic anhydrases (carbonate hydro-lyase, EC 4.2.1.1) have been found to be strongly inhibited by several sulfonamides (1-3). Studies of the interaction between these inhibitors and different forms of the enzyme should provide information about the active site of the enzyme.

It had been shown that acetazolamide (2 acetaminono-1,3,4-thiadiazole 5-sulfonamide) is a highly potent, reversible (2), noncompetitive (4) inhibitor of carbonic anhydrase. It was postulated that replacement of the acetyl group of acetazolamide by a haloacetyl group might yield an irreversible inhibitor. A nucleophilic side chain of an α-amino acid, at or close to the active site of carbonic anhydrase, should attack the haloacetyl moiety of the inhibitor with the formation of a covalent bond (5). This expectation was fulfilled and we were ready to report our studies on bovine carbonic anhydrase B, with the use of such haloacetyl sulfonamides, when two closely related papers on human carbonic anhydrase B appeared (6, 7). Our results are somewhat different from those reported by Whitney and his coworkers and are presented here.

Carbonic Anhydrase B—The enzyme was separated from a commercial product (Worthington Biochemical Corporation) prepared from bovine erythrocytes. A column of DEAE-cellulose and Tris-chloride buffer in a linear gradient from 0.01 M to 0.1 M at pH 8.0 was employed (8).

Bromoacetazolamide—This compound was synthesized from 2-amino-1,3,4-thiadiazole 5-sulfonamide, which in turn was prepared from acetazolamide as follows. Acetazolamide (4 g) was refluxed with 1 HCl (20 ml) for 2 hours. Sodium bicarbonate was added to the hydrolysate until it became alkaline. The precipitate that formed after keeping the reaction mixture at 4° for 2 hours was filtered and washed to neutrality with cold water to obtain the free base. After two recrystallizations from water, 2.4 g of product were obtained (74%), m.p. 223-224°.

\[\text{C}_6\text{H}_4\text{N}_2\text{O}_2\text{S}_2\text{Br} (202.0) \text{ Compound I} \]

Calculated: C 13.31, H 2.23, N 31.09%

Found: C 13.49, H 2.44, N 30.88%

To a solution of Compound I (900 mg, 5 mmoles) and bromoacetic acid (765 mg, 5.5 mmoles), in 50 ml of peroxide-free tetrahydrofuran, was added dicyclohexylcarbodiimide (1.072 g, 5.2 mmoles). The reaction mixture was kept at room temperature for 4 hours. The dicyclohexylurea that precipitated was filtered, washed with tetrahydrofuran (5 ml), and the solvent was evaporated from the filtrate under reduced pressure. The residue was dissolved in a minimum of ethyl acetate, which was extracted first with 1 HCl (20 ml), then with water (3 X 10 ml). The ethyl acetate layer was dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure. The crystals that remained were recrystallized twice from tetrahydrofuran-chloroform (1:2). A yield of 1.3 g of bromoacetazolamide (72%) was obtained; m.p. 217-219°.

\[\text{C}_6\text{H}_4\text{N}_2\text{S}_2\text{O}_2\text{Br} (301.1) \]

Calculated: C 15.94, H 1.67, N 18.00, Br 26.55

Found: C 16.09, H 1.86, N 18.49, Br 26.50

14C-Bromoacetazolamide—This was prepared in the same manner as for bromoacetazolamide. Bromoacetic acid-14C with a specific activity of 10.1 mC per mmole was obtained from the Radiochemical Centre, Amersham, England. An amount containing about 17 μC was diluted with unlabeled bromoacetic acid (181 mg) to obtain a specific activity of ap-

* We are indebted to Cynamin of Canada Limited for kindly supplying acetazolamide.

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Table I

<table>
<thead>
<tr>
<th>Time after adding bromoacetazolamide</th>
<th>Irreversible inhibition</th>
<th>Time after adding bromoacetazolamide</th>
<th>Irreversible inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs</td>
<td>%</td>
<td>hrs</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>50.0</td>
<td>4</td>
<td>67.3</td>
</tr>
<tr>
<td>1</td>
<td>58.4</td>
<td>8</td>
<td>87.5</td>
</tr>
<tr>
<td>1</td>
<td>63.0</td>
<td>24</td>
<td>91.5</td>
</tr>
</tbody>
</table>

A pH slightly higher than 8.0 was used here and throughout this study since we found that the irreversible inactivation is pH dependent. The highest percentage of inactivation was reached at around pH 8.5. At lower pH values the reaction is significantly slower. Above pH 8.5 the inactivation is practically the same.

proximately 12.7 μC per mmole. The reaction mixture contained 216 mg (1.2 mmoles) of Compound I and 181 mg (1.3 mmoles) of bromoacetic acid-14C. The final product showed a specific activity of 10.69 μC per mmole and this was unchanged after five recrystallizations.

Inactivation Studies—In the presence of bromoacetazolamide, bovine carbonic anhydrase B was quickly and almost completely inactivated. The enzyme was allowed to react at room temperature with bromoacetazolamide for varying lengths of time and at different concentration ratios of inhibitor to enzyme. Unreacted inhibitor and buffer were then removed and the enzyme was recovered and assayed according to the procedure of Wilbur and Anderson (9). The effect of time, at a fixed ratio of inhibitor to enzyme, is shown in Table I. It can be seen that an early, rather rapid irreversible inactivation is followed by a slower inactivation that reaches 90% in 8 to 100%.

Table II

<table>
<thead>
<tr>
<th>Experimental mixture</th>
<th>Concentration of bromoacetazolamide</th>
<th>Irreversible inhibition</th>
<th>Irreversible binding of 14C-bromoacetazolamide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>moles/mole enzyme</td>
<td>%</td>
<td>moles/mole inhibited enzyme</td>
</tr>
<tr>
<td>I</td>
<td>0.5</td>
<td>51.9</td>
<td>0.9</td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>81.4</td>
<td>0.9</td>
</tr>
<tr>
<td>III</td>
<td>2</td>
<td>85.7</td>
<td>1.0</td>
</tr>
<tr>
<td>IV</td>
<td>3</td>
<td>89.8</td>
<td>1.2</td>
</tr>
<tr>
<td>V</td>
<td>20</td>
<td>91.3</td>
<td>1.0</td>
</tr>
<tr>
<td>VI</td>
<td>100</td>
<td>94.4</td>
<td>2.1</td>
</tr>
</tbody>
</table>

a These figures were calculated as follows:

\[
\text{Molar specific radioactivity of enzyme} \times 100
\]

\[
\text{Molar specific radioactivity of inhibitor} \times 100
\]
zyme was increased to 100:1. The gradual formation of these starch gel electrophoresis (Fig. 1).

At low bromoa&azokmide concentrations, only 1 mole of the various compounds to the enzyme was 20. Otherwise the experimental conditions were the same as described in Table II. With 14C-bromoa&azokmide it was possible to determine the equivalents of inhibitor bound irreversibly by the inhibited enzyme. Although the rate of inactivation was affected very little by raising the inhibitor concentration there was a distinct change in binding. At low bromoa&azokmide concentrations, only 1 mole of inhibitor was bound to 1 mole of inhibited enzyme. At bromoa&azokmide concentration ratios in excess of 20, 2 eq of inhibitor were bound by the inhibited enzyme, and this did not increase further even when the molar ratio of inhibitor to enzyme was increased to 100:1. The gradual formation of these two irreversibly inhibited enzyme species could be followed by starch gel electrophoresis (Fig. 1).

Further evidence that the irreversible inactivation occurred at or near the active site is provided in Table III. Under identical conditions neither bromoacetic acid nor iodoacetamide inhibited the enzyme or reacted significantly with it, although these compounds possess similar or stronger halogen activity than bromoa&azokmide.

Bromoacetalozolamide did not combine irreversibly with the zinc-free protein; after addition of Zn++, full enzyme activity was restored. This confirmation of the fact that Zn++ is indispensable to the active site (12), and our observation that zinc is required for irreversible binding of bromoa&azokmide to include strong evidence that this irreversible binding does not occur outside the active site.

It can be seen from this table also that the effect of chloroa&azokmide is not as great as that of bromoa&azokmide, probably because bromine is a better leaving group than chlorine.

From a reaction mixture containing equal molar quantities of 14C-bromoa&azokmide and bovine carbonic anhydrase B we succeeded in separating two protein species as follows. A column (2.5 x 25 cm) of DEAE-cellulose was equilibrated with 0.01 M Tris-chloride buffer at pH 8. Elution was carried out at 5° with a concave gradient that was developed by two metering pumps. The flow rate from the reservoir (containing 264 ml of 0.13 M Tris-chloride buffer, pH 8) to the mixing chamber (containing 780 ml of 0.01 M Tris-chloride buffer, pH 8) was 54 ml per hour. The flow rate from the mixing chamber to the column was set at 144 ml per hour. The effluent was monitored at 280 nm. The faster moving component was identical with the native enzyme; the slower moving one was completely inactive, electrophoretically pure, and contained 0.92 eq of 14C-bromoa&azokmide.

The two enzyme species thus obtained were hydrolyzed and subjected to amino acid analysis by the accelerated procedure of Spackman (13). Comparison of the amino acid composition of the two proteins indicated that irreversible inactivation was accompanied by the disappearance of 0.88 eq of histidine and the simultaneous formation of a substance showing a peak corresponding to 0.9 eq of 3-carboxymethylhistidine (14). This can be interpreted as indicating that during irreversible inactivation bromoa&azokmide reacted with the 3 nitrogen of histidine.

The experiments described above appear to have established that bromoa&azokmide irreversibly inactivates the enzyme via the active site. It would be expected however that only 1 inhibitor molecule would be attached to 1 molecule of inhibited enzyme, since it has been shown (12) that carbonic anhydrase possesses one active site. The observation of the formation of two inhibited enzyme species requires further explanation. It is evident that the active site of the enzyme reacts first with 1 molecule of inhibitor, but the attachment of the 2nd molecule of inhibitor seems also to be specific. It is postulated that formation of a complex, or a covalent bond, by the 1st molecule of inhibitor, alters the three-dimensional structure of the enzyme and makes another amino acid available to react with a 2nd molecule of inhibitor. A random reaction is most unlikely since negligible radioactivity was found in the enzyme when it was incubated with 14C-bromoa&azokmide or with 14C-iodoacetamide. Particularly noteworthy is the fact that the metal-free enzyme did not show any significant radioactivity when incubated with a 20 molar excess of 14C-bromoa&azokmide.

Table III

<table>
<thead>
<tr>
<th>Enzyme preparation and substance tested</th>
<th>Irreversible inhibition</th>
<th>Irreversible binding of 14C compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc-free 14C-Bromoa&amp;azokmide; after removal of excess reagent, 1 g atom of Zn++ replaced</td>
<td>0</td>
<td>0.03</td>
</tr>
<tr>
<td>Native 14C-Bromoa&amp;azokmide</td>
<td>0</td>
<td>0.15</td>
</tr>
<tr>
<td>14C-Iodoacetamide</td>
<td>0</td>
<td>0.13</td>
</tr>
<tr>
<td>Bromoa&amp;azokmide</td>
<td>91.3</td>
<td>*</td>
</tr>
<tr>
<td>Chloroa&amp;azokmide</td>
<td>20.0</td>
<td>*</td>
</tr>
</tbody>
</table>

* Not measured.

REFERENCES
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