Covalent Labeling of the Active Site of Human Carbonic Anhydrase B with N-Bromoacetylacetazolamide*

(Received for publication, November 26, 1971)

SHOW-CHU C. WONG,† STEPHEN I. KANDEL,‡ MARIANNE KANDEL, AND ALLAN G. GORNALL

From the Faculty of Pharmacy and Department of Pathological Chemistry, University of Toronto, Toronto 181, Canada

SUMMARY

Two or possibly three equivalents of N-bromoacetylacetazolamide interact with two histidine residues located in the active site region of human carbonic anhydrase B. The most rapid reaction occurs at the 1-nitrogen of a histidine residue and this is followed by a slower alkylation of the 3-nitrogen of the same histidine and the 3-nitrogen of a second histidine.

From a reaction mixture of N-bromoacetylacetazolamide and the enzyme, a species was isolated that contained approximately 1 eq of reagent covalently bound to the 1-nitrogen of a histidine (or histidines). This alkylated enzyme is active toward p-nitrophenyl acetate, but shows a pH dependence which is different from that of the native enzyme. At pH 6 the esterase activity is 95%, and levels off at pH 7.6 to about 35% relative to the native enzyme. The $K_m$ of the alkylated enzyme is almost identical with that of the native enzyme. These observations indicate that the histidine that reacted fastest with the reagent does not participate in the catalysis.

Like the native enzyme alkylated human carbonic anhydrase B reacts with bromoacetate at the 3-nitrogen position of a histidine in the active site region. The partially purified dialkylated enzyme has a residual esterase activity at pH 7.6 of less than 0.8%. This raises the possibility that the histidine that reacts with bromoacetate plays a role in the catalysis.

Human carbonic anhydrase B (carbonate hydro-lyase, EC 4.2.1.1) is one of three major isoenzymes isolated from human erythrocytes. It is a single chain protein containing 1 zinc ion which is essential for the catalysis and the binding of inhibitors (1, 2). It catalyzes the reversible hydration of CO$_2$ (3) and certain carbonyl compounds (4), as well as the hydrolysis of esters (5) and of fluorodinitrobenzene (6). Although the rate of the catalysis is markedly different for the different substrates, the pH rate profiles for most of the known catalytic hydration or hydrolysis reactions are sigmoid with an inflection point around neutrality (4-7). This indicates that a group in the enzyme with a pK around 7 is required in the basic form for catalysis.

Several attempts have been made recently to identify this group. It is believed that the catalysis of CO$_2$ hydration is dependent upon a water molecule bound to the zinc ion at the active site (8-13). The ionization of this water molecule and the subsequent nucleophilic attack by the resulting OH group on the carbon dioxide molecule, along with an accompanying proton transfer, would be the basis of the catalytic mechanism and the observed pH dependence of the reaction. It seems obvious, however, that this simple mechanism cannot account for the extremely high catalytic rate of CO$_2$ hydration. The side chain of a basic amino acid was postulated by Wang (14) to be hydrogen bonded to the zine-bound OH ion to enhance its nucleophilicity and to facilitate the proton transfer. The imidazole group of histidine seemed to be the most likely candidate for such a role since its pK is closest of that of all amino acids to the pH of the group found to be essential for catalysis. A histidine was also implicated in the catalytic mechanism of Pocker and Stone (15).

Affinity labeling of human carbonic anhydrase B by Bradbury (10) and Whitney et al. (16, 17) led to the alkylation at the 3-nitrogen position of two sequentially remote histidines. Similarly, we have found that bromoacetazolamide specifically and covalently interacts with the 3-nitrogen of a histidine residue of bovine carbonic anhydrase B (18). The enzymes alkylated by bromoacetate and bromoacetazolamide, however, retain 5 to 15% of the original esterase activity of the native enzyme. This observation was taken as evidence that the histidines that reacted with these reagents, although located in the active site region, are not essential for the catalysis. On the other hand, the enzyme alkylated by N-chloroacetylchloothiaside is completely inactiv (16). It is believed, however, that this is not the result of the modification of a histidine, but rather is due to the presence of the bulky group covalently bound at the active site (13). The modification of all lysine residues in the bovine enzyme B (19) and in the human enzyme B (17) did not lead to

---

* This work was supported by Grant MA3057 and Grant MT369 from the Medical Research Council of Canada.
† This work comprises part of a thesis submitted to the University of Toronto, Toronto, Canada, for the degree of Doctor of Philosophy. Present address, Department of Biology, Brookhaven National Laboratory, Upton, Long Island, New York 11973.
‡ To whom inquiries may be addressed at the Faculty of Pharmacy, University of Toronto.
any significant loss in the activity of these proteins. Similar results were obtained also when tert-butylmethane was used to modify one tyrosine residue in the bovine enzyme and three in the human enzyme (20). Consequently, any critical role for these residues can be ruled out. Khalifah (13) recently proposed a mechanism for the CO₂ hydration reaction that does not involve any amino acid side chain.

In order to map the active site of carbonic anhydrase further, with the aim of identifying an amino acid that is directly or indirectly involved in the catalysis, we prepared N-bromoaacetetylacetazolamide and studied its reaction with human carbonic anhydrase B. The results of these experiments are reported here.

**EXPERIMENTAL PROCEDURE**

**Materials and Reagents—**Acetazolamide (Lot CL-6063) was supplied by Lederle Laboratories through the courtesy of Mr. S. R. Davis. Bromoacetic acid (Eastman) and thioglycolic acid (British Drug Houses, Toronto) were distilled under reduced pressure prior to use. Urea (British Drug Houses, Toronto) was recrystallized twice from 95% ethanol and n-propylphenyl acetate (Eastman) three times from acetate-water (9:1 v/v). Bromo(1-)Clacetic acid was obtained from New England Nuclear Corporation (23 mCi per mmole). DEAE-cellulose (Cellex-D, high capacity) was purchased from Bio-Rad, Richmond, California. Peroxide-free tetrahydrofuran was obtained by distilling tetrahydrofuran (Fisher Reagent) from sodium borohydride. Acetone and ethyl acetate (Fisher Reagent) were kept with anhydrous potassium carbonate overnight and then distilled at their respective boiling points. Other solvents were reagent grade and redistilled prior to use. Crude mixture of bovine carbonic anhydrases was purchased from Worthington and horse blood from Woodlawn Farms, Guelph, Ontario. Human red blood cells were supplied through the courtesy of Dr. J. I. Crookston from the blood bank at Toronto General Hospital.

**Preparation of Carbonic Anhydrases—**Human, bovine, and horse carbonic anhydrases were separated as previously described (18).

**Zinc-free and Carboxymethyl Human Carbonic Anhydrase B—**These enzyme species were prepared according to Lindskog and Mainsterm (21) and Whitney et al. (17), respectively. Zinc analysis was carried out by atomic absorption spectroscopy (Perkin Elmer, model 300).

**Thin Layer Chromatography—**Analytical and preparative thin layer chromatography was done on precoated silica gel plates with fluorescent indicator F-254 (0.25 mm and 2 mm layer thickness, respectively, Merck Brinkmann Instrument (Canada) Rexdale, Ontario). The solvent system was ethyl acetate-acetone-water (60:40:4). Aromatic sulfonamides were detected by short wave length ultraviolet light.

**Synthesis of N-Acetylacetazolamide (Fig. 1)—**N-Acetylacetazolamide was prepared by acetylation of acetazolamide (300 mg, 1.1 mmoles) with acetic anhydride (1 ml) in the presence of concentrated sulfuric acid (3 drops) as a catalyst. The reaction mixture was kept at 95-100° for 60 min, then extracted with carbon tetrachloride (2 x 10 ml) and triturated with cold water (2 x 5 ml). The solid residue was recrystallized twice from 50% tetrahydrofuran. N-Acetylacetazolamide was obtained in a 47% yield, m. p. 230-231°.

**Preparation of Bromoacetic Anhydride—**Bromoacetic anhydride was prepared according to the method of Brauns (22). Pure bromoacetic anhydride was distilled between 145 and 148° at 20 mm, m.p. 37-39° (literature m.p. 41°) (22).

**Synthesis of N-Bromoacetacetazolamide (Fig. 1)—**N-Bromoacetacetazolamide was prepared by bromoacetyllating acetazolamide (1.11 g, 5 mmoles) with bromoacetic anhydride (6.5 g, 25 mmoles) in the presence of concentrated sulfuric acid (5 drops) as a catalyst. The reaction mixture was kept at 96-100° for 50 min with occasional swirling. A semisolid mixture was produced and extracted with carbon tetrachloride (3 x 10 ml) which was subsequently removed with a Pasteur pipette. The remaining precipitate was suspended and triturated with water at 0° (2 x 5 ml), then filtered through a fine sintered glass funnell. The residue was dissolved in a minimum amount of 90% tetrahydrofuran and vacuum filtered. Part of the solvent was removed on a rotary evaporator under reduced pressure until crystals formed and carbon tetrachloride (30 ml) was added to complete the precipitation. After filtration the precipitate was dried in a desiccator over P₂O₅ under vacuum prior to further purification.

**Purification of N-Bromoaacetacetazolamide by Preparative Thin Layer Chromatography—**The above reaction mixture (80 to 100 mg) was dissolved in a minimum amount of 95% tetrahydrofuran and applied to a preparative precoated silica gel plate. The plate was developed by allowing the solvent to run to the top. Approximately 4 hours were needed for each run and 2 to 4 plates were used simultaneously. The band corresponding to N-bromoaacetacetazolamide was scraped off and extracted with 95% acetone (4 x 15 ml). After centrifugation the supernatant was collected and evaporated to dryness on a rotary evaporator. The residue collected from 4 plates (400 mg of reaction mixture) was suspended in 1-butanol (8 ml) and ethyl acetate (40 ml) and extracted with 0.01 N HCl (3 x 6 ml). The solvent was evaporated on the rotary evaporator until heavy crystals precipitated and then 2 volumes of chloroform were added to complete the precipitation. Crystals were collected by filtration, dried and dissolved in peroxide-free tetrahydrofuran (30 ml), and filtered through a fine sintered glass funnel to remove any insoluble contaminants. 1-Butanol (5 ml) was added to the filtrate and the mixture was evaporated again to 4 to 5 ml. The crystalline mixture so obtained was kept in a freezer (−20°) for 20 min then filtered and washed with cold anhydrous acetone.
Reaction mixture contained 1 x 10^{-6} M to 3 x 10^{-4} M enzyme, p-nitrophenyl acetate in the absence of the enzyme. The change at 348 nm on a Gilford recording spectrophotometer was determined at 24°C using p-nitrophenyl acetate as a substrate. Enzyme activity was determined at 24°C using p-nitrophenyl acetate in 0.075 M Tris sulfate buffer at pH 7.6, plus 0.8% acetone, unless otherwise indicated in the legends to the various tables and figures.

Preparation and Separation of Human Carbonic Anhydrase B Alkylated with N-Bromosuccinimide (Alkylated Enzyme)—Human enzyme B (300 mg, 10 μmoles) was reacted with 10-fold molar excess (34.32 mg, 100 μmoles) N-bromosuccinimide in 0.1 M phosphate buffer (300 ml) at pH 7.2 for 7 hours at 24°C. The reaction mixture was dialyzed against distilled water and freeze-dried.

A portion of the dried material (200 to 300 mg) was then applied to a DEAE-cellulose column (2.5 x 50 cm) previously equilibrated with 0.02 M Tris chloride at pH 8.4. Elution was begun with this buffer at a flow rate of 210 ml per hour. After the first peak had emerged completely, the elution was continued with sodium chloride in linear gradient from 0 to 0.5 M in 0.02 M Tris chloride buffer at pH 8.4 using the same flow rate. The reservoir contained 1000 ml of buffer and 0.5 M sodium chloride and the mixing chamber contained 1000 ml of buffer. The effluent was monitored at 250 nm (Isco, model UA 2).

Another portion of the reaction mixture (150 mg) was applied to a preparative isoelectric focusing column (model 8102, 440-ml internal volume LKB Producter AB, Stockholm) and run according to the procedure of Svensson (25). Carrier ampholytes (Ampholine, LKB Producter AB, Stockholm) in the pH range 5 to 8 were used. The separation was achieved at 5°C for 72 hours using a constant voltage power supply at 350 to 1000 volts depending on the current. At no time did the power exceed 5 watts. The column was emptied at a rate of 2 ml per minute and 5-ml fractions were collected. The absorbance at 280 nm and the pH were measured for each fraction prior to the combining of appropriate fractions and removal of Ampholine and sucrose by dialysis. Proteins were recovered by freeze drying.

Preparation and Separation of N-Bromosuccinimide-Alkylated Human Carbonic Anhydrase B Alkylated with Bromoacetic Acid (Diarylket Enzyme)—Human enzyme B alkylated with N-bromosuccinimide and purified by DEAE-cellulose chromatography (78 mg, 2.6 μmoles) was further reacted with 40-fold molar excess of bromoacetate (14.3 mg, 101 μmoles) in Tris sulfate buffer (8.7 ml) at pH 7.6 for 24 hours at 24°C. Buffer and excess reagent were removed by dialysis. The freeze-dried protein (30 mg) was applied to an LKB isoelectric focusing apparatus (440-ml internal volume). The separation was carried out as described above with the exception that a pH gradient between 5 and 7 was used.

Starch Gel Electrophoresis—This was performed according to the procedure of Ricki et al. (26).

Amino Acid Analyses—Protein samples (1 to 2 mg) were hydrolyzed in constant boiling glass-distilled hydrochloric acid (0.5 to 1 ml) in evacuated, sealed tubes for 22 hours at 110°C. Alkylated protein samples were oxidized prior to acidic hydrolysis (18, 27). Tryptophan was determined according to the method of Matsubara and Sasaki (28). Amino acid analyses were performed with a Beckman-Spinco automatic amino acid analyzer according to the method of Stackman et al. (29).

Measurement of Radioactivity—Radioactive counting was carried out in a Packard Tri-Carb liquid scintillation spectrometer (model 3375). Dried protein samples were dissolved in 1 ml of Soluene (Packard Instrument Co.). Counts were corrected for quenching by means of an external standard.
RESULTS

Isolation of Pure N-Bromoacetylacetazolamide—The reaction of acetazolamide with bromoacetyl anhydride gave N-bromoacetylacetazolamide in good yield but the product contained, in addition to unreacted acetazolamide, some N-bromoacetyl-bromoacetazolamide and N-acetylacetazolamide. These last two derivatives probably resulted from an intramolecular transacylation and they were prepared separately in order to facilitate their identification as by-products of this reaction. The reaction mixture was separated by preparative thin layer (2 mm) chromatography; when re-run on thin layer (0.25 mm) N-bromoacetylacetazolamide moved as a single spot even when 70 μg of the final product were applied. In this way acetazolamide impurity, even as little as 0.01%, would have been detected in the purified N-bromoacetylacetazolamide.

Stability of N-Bromoacetylacetazolamide in Various Buffers It was shown by silica gel thin layer chromatography that N-bromoacetylacetazolamide is rapidly converted to acetazolamide in 0.1 M Tris sulfate buffer (Tham, Fisher Scientific or British Drug Houses AnaLaR) at pH 7.2 and 8.7 and room temperature. After 1 hour no detectable amount of N-bromoacetylacetazolamide could be found in the incubation mixture. The decomposition of the reagent was confirmed by showing that human carbonic anhydrase B (3.3 × 10⁻⁴ M), when reacted for 8 hours with N-bromoacetylacetazolamide (3.3 × 10⁻⁴ M) that had been kept in 0.1 M Tris sulfate buffer at pH 7.2 for 23 hours, formed only trace amounts of carboxymethylhistidine derivative. Under identical conditions, in 0.1 M phosphate buffer, 0.55 eq of His(1-Cm) was found in the hydrolysate.

No decomposition of N-bromoacetylacetazolamide could be observed in thin layer chromatograms when exposed to 0.1 M ammonium sulfate buffer at pH 0.2 for as long as 7 hours, or in 0.1 M phosphate buffer in the pH range 5.2 to 8.2 for 48 hours.

In 0.1 M acetate buffer at pH 5.2 a different type of decomposition was observed. On thin layer chromatograms a gradual increase in N-acetylacetazolamide corresponded to a decrease in N-bromoacetylacetazolamide, a conversion that could be explained by an intramolecular transacylation mechanism. The formation of acetazolamide in Tris sulfate buffer might be due to some catalytic contaminant in this buffer. Hydrolytic or ammonolytic cleavage cannot be the cause, since the compound appears to be stable in ammonium sulfate and phosphate buffer even at alkaline pH. The fast decomposition of N-bromoacetylacetazolamide in Tris and acetate buffer prompted us to use phosphate buffer in these studies, whenever it seemed necessary, in spite of the known inhibitory effect of this buffer (10).

Apparent Ionization Constants of Acetazolamide Derivatives—Potentiometric titrations of N-acetylacetazolamide and N-bromoacetylacetazolamide yielded two pK values for each compound (Table I). The two dissociable protons are located in each case at the acetamido and sulfonamido groups, respectively. The assignment of the apparent pK values is based on both structural considerations and literature data (30).

Specificity of Interaction of N-Bromoacetylacetazolamide with Human Carbonic Anhydrase B The results in Table II show that a 10-fold molar excess of N-bromoacetylacetazolamide, when reacted with human enzyme B at pH 7.2 for 24 hours, yielded histidines alkylated at the l-nitrogen, the 3-nitrogen, and the 1,3-nitrogen positions. Under identical conditions, neither the native enzyme in the presence of 8 μM urea nor the zinc-free enzyme reacted, thereby indicating that the native structure of the active site is necessary for the reaction. The observation that acetazolamide, a potent reversible inhibitor of carbonic anhydrases, protects against these reactions provides further evidence that the reactions take place at or near the active site. α-Haloacetates are known to interact with human carbonic anhydrase B with the formation of 1 eq of 3-carboxymethylhistidine in the active site region (10, 17). The carboxymethyl human enzyme B so formed does not react with N-bromoacetylacetazolamide. Results shown later indicate that this observation cannot be taken as evidence that the two reagents react with the same histidine. Nevertheless, it seems that the carboxymethyl group hinders the approach of the reagent toward the active site histidines either sterically or by charge repulsion. Finally, the fact that human enzyme C does not react under the same conditions (Table VIII) can also be considered evidence for the specificity of the reaction with human enzyme B.

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>pK' (-CO-NH-)</th>
<th>pK' (-SO₂-NH-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetylacetazolamide</td>
<td>8.16 ± 0.02</td>
<td>3.28 ± 0.01</td>
</tr>
<tr>
<td>N-Bromoacetylacetazolamide</td>
<td>8.10 ± 0.02</td>
<td>3.08 ± 0.02</td>
</tr>
</tbody>
</table>

### Table II

**Specificity of the reaction of human carbonic anhydrase B with N-bromoacetylacetazolamide**

The enzyme (5.3 × 10⁻⁴ M) and N-bromoacetylacetazolamide (3.3 × 10⁻⁴ M) were previously incubated with the enzyme for 30 min prior to the addition of the N-bromoacetylacetazolamide. Following the reaction, buffer and unreacted reagent were removed by dialysis against distilled water and the carboxymethylhistidine content of the lyophilized protein was determined.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>His(1-Cm) formed</th>
<th>His(2-Cm) formed</th>
<th>His(1,3-Cm) formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native enzyme plus N-bromoacetylacetazolamide</td>
<td>0.72</td>
<td>0.15</td>
<td>0.18</td>
</tr>
<tr>
<td>Zinc free enzyme plus N-bromoacetylacetazolamide</td>
<td>0</td>
<td>Trace</td>
<td>0</td>
</tr>
<tr>
<td>Native enzyme + urea (8 M) plus N-bromoacetylacetazolamide</td>
<td>Trace</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Native enzyme + acetazolamide plus N-bromoacetylacetazolamide</td>
<td>0</td>
<td>Trace</td>
<td>0</td>
</tr>
<tr>
<td>Carboxymethyl human enzyme B</td>
<td>1.02</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Carboxymethyl human enzyme B plus N-bromoacetazolamide</td>
<td>Trace</td>
<td>1.06</td>
<td>0</td>
</tr>
</tbody>
</table>

* This enzyme preparation contained less than 0.05 g atom of zinc per molecule of enzyme.
exceeds the rate of inactivation. As the reaction proceeds further this ratio increases, reaching a value of 127 after 48 hours with the simultaneous formation of two other alkylated histidine species. It appears that while a relatively fast alkylation at the 1-nitrogen position leads to a partial inactivation the two slower secondary alkylations cause a much greater degree of inactivation. However, the relative contribution of these two latter reactions is not known.

In Table IV we have tabulated the number of equivalents of alkylated histidines that formed in 24 hours with different concentrations of the reagent. When this concentration was increased from 10-fold to 20- and 100-fold molar excess, the amount of 1-alkylated enzyme gradually decreased with a parallel increase in 1,3-dialkylated enzyme. The total of the two derivatives approximates and does not exceed 1 eq per molecule of enzyme. Based on these observations it seems likely that the reagent at first reacted with the 1-nitrogen of a histidine residue, followed by a second alkylation of the same histidine at the 3-nitrogen position to form dialkylated histidine. Further evidence for the sequential formation of a dialkyalted histidine residue is presented in Table VII.

**pH Dependence of Reaction**—Fig. 2 shows the amount of His(1-Cm) formed in 4 hours as a function of pH. The rate was maximal at a pH near neutrality and decreased at both lower and higher pH. In order for the reaction to proceed, the interacting histidine should be present in unprotonated form since the reaction can be considered to take place via the nucleophilic attack of the 1-nitrogen of the histidine on the electrophilic carbon atom of the N-bromoacetyl group of the reagent. Based on this concept one might expect that the rate of alkylation would increase with an increase in pH up to a limiting value after which it would remain constant. The increase in formation of alkylated enzyme between pH 5.6 and 7 probably reflects the deprotonation of the histidine that reacted. The reason for the decrease in the rate above pH 7.0 is less obvious. We can rule out the possibility that the decomposition of N-bromoacetylacetazolamide at alkaline pH causes the decreased rate, since the reagent after being kept at pH 8 for 24 hours is still equally capable of reacting at pH 7.2. N-Bromoacetylacetazolamide possesses two ionizable hydrogens. While dissociation of the acetamido proton (pK = 8.1, Table I) is unlikely to contribute to the electrophilicity of the methylene carbon of the N-bromoacetyl group, the dissociation of the sulfonamido proton might. However, the
pK of this group was found to be 3.1 (Table I) which is far too low to affect the reaction between pH 7 and 8.

Since the rate of the reaction is likely controlled by the concentration of the reversible enzyme-inhibitor complex, a rapid decrease in the affinity of the enzyme for the reagent at alkaline pH would considerably decrease the rate of the reaction. A decreased binding of sulfonamides and anionic inhibitors at alkaline pH values has been reported (7, 15, 31). However, the decrease in rate at alkaline pH might also be due to the influence of a residue located close to the reactive histidine such that the former should be present in protonated form to facilitate the reaction by enhancing the nucleophilicity of the latter. Our data are not sufficient to distinguish between these two possibilities.

Separation of Alkylated Human Enzyme B (Alkylated Enzyme)

-In view of the effects of alkylation shown in Tables II and III, it was of interest to study the properties of this form of the enzyme. To avoid multiple alkylation, the reaction mixture contained a 10-fold molar excess of N-bromoacetylacetazolamide and the incubation period was limited to 7 hours at room temperature. The result of the chromatographic separation of this mixture on DEAE-cellulose is shown in Fig. 3. Fraction I was found to be identical with the unreacted native enzyme. Fraction III was not characterized although its acidic hydrolysates contained His(1-Cm), His(3-Cm), and His(1,3-di-Cm) indicating that even under the controlled conditions specified above the reaction is not confined to the 1-nitrogen position of one histidine. After acid hydrolysis, Fraction II yielded 0.91 eq of His(1-Cm) with a corresponding loss of 0.89 eq of histidine. In all other respects the amino acid content (including tryptophan) of this hydrolysate was identical with that of the native enzyme. When both the reaction and the separation were repeated under identical conditions using 14C-labeled N-bromoacetylacetazolamide, Fraction II contained 0.88 eq of covalently bound reagent. Therefore, Fraction II is human carbonic anhydrase B alkylated with this reagent at the 1-nitrogen position of one or more histidine residues. During electrophoresis on starch gel at pH 8.9 (Fig. 5) this alkylated enzyme migrated...
Binds bromoacetate and acetazolamide. The inhibition constant was 9.2 mM (a) and 5.3 mM (b) for bromoacetate. The inhibition constant of acetazolamide was determined on the assumption that the inhibition mechanism is non-competitive (1). $K_i$ values for both the native and alkylated enzymes were found by the Dixon plot (Fig. 6) to be 5.3 and 0.2 mM, respectively when measured in 0.025 M phosphate buffer at pH 7.6. The inhibition constant of acetazolamide was calculated from Equation 1.

$$K_i = \frac{(E_i - EI)(I_i - EI)}{(EI)}$$

Acetazolamide combines almost stoichiometrically with carbonic anhydrases, thereby determining the lower limit of the concentration of acetazolamide in the reaction mixture. If the enzyme concentration exceeds the inhibitor concentration, the value of $EI$ obtained from Equation 2 may fall within the range of experimental error and in this way zero or even negative values may be obtained for the $K_i$. On the other hand, the sensitivity of the spectrophotometer determines the upper limit of the concentration of the inhibitor, since the value of $V_i$ rapidly approaches zero as the inhibitor concentration exceeds that of the enzyme. Thus, the nature of the interaction between acetazolamide and carbonic anhydrase severely restricts the useful range of inhibitor concentration.

In Table V are listed the $K_i$ values calculated from Equation 1 for the native and alkylated enzymes at pH 7.6 in 0.025 M phosphate buffer. We found, for example, that at equimolar concentrations of the native enzyme and acetazolamide, this value decreased in the inhibitor concentration. The $K_i$ at 0.21 mM inhibitor concentration, as calculated from Equation 1, was found to be 0.3 mM for the native and 0.19 mM for the alkylated enzyme. At an inhibitor concentration of 0.96 mM, these values decreased to 0.11 mM and 0.05 mM, respectively. The concentration dependence of the inhibition constant is possibly due to the presence of a minute amount of acetazolamide in our N-bromoacetylecetazolamide preparation.

<table>
<thead>
<tr>
<th>Concentration of inhibitor (mM)</th>
<th>Native enzyme</th>
<th>Alkylated enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.64</td>
<td>0.25</td>
<td>0.04</td>
</tr>
<tr>
<td>5.28</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>7.92</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>10.56</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>13.20</td>
<td>0.44</td>
<td></td>
</tr>
</tbody>
</table>

Table V

Inhibition of esterase activity of native and alkylated human carbonic anhydrase B by acetazolamide

The esterase activity was determined at pH 7.6 in 0.025 M phosphate buffer. Concentrations of the native and alkylated enzymes were 2.66 x $10^{-4}$ M and 5.33 x $10^{-5}$ M, respectively.
was somewhat lower than that obtained at higher concentrations of the inhibitor. Our value for the native enzyme, 0.35 μM and that of Armstrong et al. 0.25 μM (5) agree reasonably well, although the two are not directly comparable due to differences in the reaction conditions. The value obtained for the alkylated enzyme at equimolar concentrations of acetazolamide and enzyme is 0.04 μM, which is approximately one-ninth that for the native enzyme. Even at this inhibitor concentration, the activity remaining was only 3.1%, and therefore we did not attempt to estimate $K_i$ values at higher inhibitor concentrations.

In Table VI we present the Michaelis constants ($K_m$) and catalytic constants ($k_{cat} = V_{max}/E_0$) which were determined by the Lineweaver-Burk plot at three pH values. A typical plot for the native and alkylated enzymes at pH 7.6 is shown in Fig. 7. The values of $K_m$ for the native enzyme at pH 6, 6.8, and 7.6 vary between 5.6 and 6.9 mM. These values are close to the value (6 mM) obtained by Verpoorte et al. (33), but considerably smaller than that (20 mM) obtained by Whitney (34). The corresponding values for the alkylated enzyme are 6.1 mM and 6.4 mM, respectively. These variations probably do not indicate a variation of $K_m$ with pH but rather reflect the experimental uncertainties involved in the use of p-nitrophenyl acetate as substrate (5). A pH-independent binding of p-nitrophenyl acetate to the native enzyme has been reported (33, 34).

**Esterase and CO$_2$ Hydration Activity of Alkylated Enzyme—**The alkylated enzyme possesses residual esterase and CO$_2$ hydration activity, although the values show some variation depending on the method of separation. Samples separated on a DEAE-cellulose column possess 32 to 35% residual esterase activity, while those isolated by electrophoresis retain only 25 to 28% residual esterase activity when assayed at pH 7.6. Values for CO$_2$ hydration activity are 40% and 34%, respectively. The lower value of the electrophoretically purified sample did not increase after filtration through a Sephadex G-50 column to remove traces of ampholine which might still be present even after exhaustive dialysis. The observed discrepancy could be due to the presence of some native enzyme in the chromatographically purified sample. A partial loss of enzymatic activity during electrophoresis appears to be ruled out since the specific catalytic activity of the unreacted enzyme reisolated from the reaction mixture by electrophoresis (Fig. 4, Fraction II) is identical with that of the native enzyme.

Fig. 8 illustrates the variation with pH of the esterase activity of both the native and alkylated enzymes. The observed activity at pH 9.2 was taken as 100%. The resulting plot for the native enzyme is sigmoid and the pH dependence is approximately 7.6. Our value for the pH, although slightly higher than earlier reported values (17, 33) agrees reasonably well. The same plot for the alkylated enzyme is not sigmoid, indicating that a more complex relationship exists between pH and activity. This complexity is readily seen in the plot of the residual enzyme activity (modified/native) versus pH, which is also included in Fig. 8. The activity of the alkylated enzyme at pH 6 is 95% that of the native species. It then decreases sharply with the increase in pH, leveling off at around pH 7.6. This observation indicates that although the activity of the alkylated enzyme, like that of the native enzyme, increases with increasing pH, this increase is greater for the native than for the alkylated enzyme. Obviously from this plot we cannot obtain accurately the pK of the catalytically essential group in the alkylated enzyme, although it seems that the value has shifted downward relative to that of the native enzyme.

**Covalent Interaction of Alkylated Enzyme with Bromoacetate and N-Bromoacetylecetazoamide—**The alkylated enzyme reacts with bromoacetate and N-bromoacetylecetazoamide in a complex manner (Table VII). The acid hydrolysate of the purified enzyme alkylated with N-bromoacetylecetazoamide contained 0.91 eq of His(1-Cm) and after a period of 24 hours in the presence of bromoacetate this value decreased to 0.52 eq with the simultaneous formation of 0.39 eq of His(1,3-di-Cm). The latter value almost completely accounts for the loss of His(1-Cm) initially present, indicating that the same histidine reacted with...
derivatives is identical with the amount of His(l-Cm) found in the presence of 0.22 eq of His(l,3-di-Cm). The total of these two initially present dropped to 0.72 eq with the concurrent appearance of acetazolamide at the l-nitrogen position. A slow carbosymethylation at the S-nitrogen position of the bromoacetate (17). This faster reaction would be followed by histidine of the native enzyme known to yield His(Y-Cm) with alkylated enzyme which is likely identical with the reactive acetate at first reacted with the 3-nitrogen of a histidine of the alkylation mixture.

The concentration of the native enzyme in the reaction mixture was $6 \times 10^{-3} M$ between pH 6.0 and 6.8 and $3 \times 10^{-4} M$ between pH 7.2 and 9.2. The observed activities of both enzymes at pH 9.2 were taken as 100% and other values are related to this. 

To rationalize these observations, we postulate that the bromoacetate at first reacted with the 3-nitrogen of a histidine of the alkylated enzyme which is likely identical with the reactive histidine of the native enzyme known to yield His(3-Cm) with bromoacetate (17). This faster reaction would be followed by a slow carbosymethylation at the 3-nitrogen position of the histidine that had originally reacted with N-bromoacetylacetazolamide at the 1-nitrogen position.

As expected, N-bromoacetylacetazolamide also reacted with the alkylated enzyme (Table VII). The 0.91 eq of His(1-Cm) initially present dropped to 0.72 eq with the concurrent appearance of 0.22 eq of His(1,3-di-Cm). The total of these two derivatives is identical with the amount of His(1-Cm) found in the alkylated enzyme, which confirms the multiple interaction of N-bromoacetylacetazolamide with the native enzyme (Tables III and IV) as well as the ability of this reagent to alkylate both nitrogens of the same histidine residue. We do not know whether the presence of 0.2 eq of His(3-Cm) (Table VII) arises from the alkylation of the histidine that reacted with bromoacetate or whether it is a result of the alkylation of yet another histidine. Our observation that the carboxymethyl human enzyme B does not react with N-bromoacetylacetazolamide (Table II) strengthens though it does not prove the hypothesis that the same histidine is involved in both cases. Acetazolamide protects against these alkylations (Table VII) supporting our earlier suggestion that both reactive histidines are in the active site region.

**FIG. 8.** pH dependence of the cattexlase activity of native and alkylated human carbonic anhydrase B. Assays were carried out in 0.025 M bis-Tris sulfate (from pH 6 to 8.6) and Tris sulfate (from pH 7.2 to 9.2) buffer as described under "Experimental Procedure." The concentration of the native enzyme in the reaction mixture was $6 \times 10^{-3} M$ between pH 6.0 and 6.8 and $3 \times 10^{-4} M$ between pH 7.2 and 9.2. The observed activities of both enzymes at pH 9.2 were taken as 100% and other values are related to this. o, esterase activity of native enzyme; w, esterase activity of alkylated enzyme; ■, residual esterase activity of alkylated enzyme.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Concentration of the reagent</th>
<th>Concentration of the enzyme</th>
<th>Alkylated histidines formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native enzyme</td>
<td>0.3</td>
<td>0.91</td>
<td>None</td>
</tr>
<tr>
<td>Alkylated enzyme</td>
<td>0.3</td>
<td>0.53</td>
<td>1.05</td>
</tr>
<tr>
<td>Alkylated enzyme</td>
<td>0.3</td>
<td>0.39</td>
<td>0.39</td>
</tr>
<tr>
<td>Alkylated enzyme</td>
<td>0.3</td>
<td>6</td>
<td>0.33</td>
</tr>
<tr>
<td>Alkylated enzyme</td>
<td>0.03</td>
<td>31.2</td>
<td>0.14</td>
</tr>
<tr>
<td>Alkylated enzyme</td>
<td>0.03</td>
<td>0.72</td>
<td>0.20</td>
</tr>
<tr>
<td>Alkylated enzyme</td>
<td>0.33</td>
<td>0.33</td>
<td>Trace</td>
</tr>
<tr>
<td>Alkylated enzyme</td>
<td>0.33</td>
<td>0.33</td>
<td>Trace</td>
</tr>
<tr>
<td>Alkylated enzyme</td>
<td>0.33</td>
<td>0.39</td>
<td>None</td>
</tr>
</tbody>
</table>

**Table VII**

Covalent interaction of alkylated enzyme with bromoacetate and N-bromoacetylacetazolamide

The reaction mixture contained native, carboxymethyl, and N-bromoacetylacetazolamide alkylated human carbonic anhydrase B and various reagents at the concentrations indicated. The interaction with bromoacetate was carried out in 0.1 M Tris sulfate buffer at pH 7.6 and that with N-bromoacetylacetazolamide in 0.1 M phosphate buffer at pH 7.2. The concentration of bromoacetate in the reaction with the alkylated enzyme was increased relative to that with the native enzyme to compensate for its decreased affinity (Fig. 6). After keeping the mixture for 24 hours at room temperature, the buffer and unreacted reagent were removed by dialysis against distilled water and the protein was then freeze-dried and analyzed for its alkylated histidine content.
the main product, contained some other unidentified protein species.

After acid hydrolysis of Fraction I, 0.92 eq of His(1-Cm) and 1.04 eq of His(3-Cm) were found with a simultaneous loss of 2.1 eq of histidine. The esterase activity of the dialkylated enzyme at pH 7.6 was less than 0.8% relative to the native enzyme.

Alkylation preferentially human enzyme C and bovine enzyme B, which represent low activity forms of this enzyme. By contrast, bromoacetazolamide was found to alkylate preferentially human enzyme B and horse enzyme 13, which represent high activity forms (18). As Table VIII shows, the amount of alkylation exceeded 2 eq, clearly demonstrating that a trialkylated species is possible that the alkylated enzyme reacts with an additional equivalent of N-bromoacetylacetazolamide to form either His(1,3-di-Cm) or His(3-Cm) but not both. Thus, a dialkylated species, and as a consequence the second molecule, when bound, is capable of bridging to a reactive nucleophilic side chain.

Although a third molecule of N-bromoacetylacetazolamide can apparently be accommodated in the active site region, it is possible that the alkylated enzyme reacts with an additional equivalent of N-bromoacetylacetazolamide to form either His(1,3-di-Cm) or His(3-Cm) but not both. Thus, a dialkylated but not a trialkylated protein would be formed. If either the reaction time or the reagent concentration could be increased to the point where the total amount of covalently linked acetylacetazolamide exceeded the limit of 2 eq per enzyme molecule, it would help to rule out this possibility. Alternatively, the purification and characterization of the various reaction products would also permit a final decision to be made. Neither of these approaches is within the scope of this investigation.

In contrast the reaction of the purified N-bromoacetylacetazolamide alkylated enzyme with bromoacetate led to a reaction mixture in which the sum of the covalently bound reagents did exceed 2 eq, clearly demonstrating that a trialkylated species can be formed. The protection against alkylation at both positions by acetazolamide suggests that these alkylation occurred in the active site region.

**DISCUSSION**

The complex nature of the interaction of carbonic anhydrases with inhibitors is well documented. The active site of bovine enzyme B is capable of interacting with 2 molecules of bromoacetazolamide (23). Human enzyme B binds 1 molecule of bromoacetate, which reacts with a single histidine and in so doing shifts away from the original binding site of the noncovalent enzyme-inhibitor complex, thereby making the reconstituted site available for binding another molecule of bromoacetate (17, 34).

The covalent interaction of N-bromoacetylacetazolamide with human carbonic anhydrase B is confined to the active site region, although in the over all reaction two histidine residues and 2 or possibly 3 molecules of reagent are involved. If a mechanism similar to that for bromoacetate operates for N-bromoacetylacetazolamide the observed differences could be explained by differences in the structure of the two inhibitors. A second molecule of bromoacetate may fail to react simply because the distance between the binding point and a nucleophilic amino acid side chain is longer than the reagent itself. N-Bromoacetylacetazolamide is a considerably larger molecule and as a consequence the second molecule, when bound, is capable of bridging to a reactive nucleophilic side chain.

A second molecule of bromoacetate may fail to react simply because the distance between the binding point and a nucleophilic amino acid side chain is longer than the reagent itself. N-Bromoacetylacetazolamide is a considerably larger molecule and as a consequence the second molecule, when bound, is capable of bridging to a reactive nucleophilic side chain.

Although a third molecule of N-bromoacetylacetazolamide can apparently be accommodated in the active site region, it is possible that the alkylated enzyme reacts with an additional equivalent of N-bromoacetylacetazolamide to form either His(1,3-di-Cm) or His(3-Cm) but not both. Thus, a dialkylated but not a trialkylated protein would be formed. If either the reaction time or the reagent concentration could be increased to the point where the total amount of covalently linked acetylacetazolamide exceeded the limit of 2 eq per enzyme molecule, it would help to rule out this possibility. Alternatively, the purification and characterization of the various reaction products would also permit a final decision to be made. Neither of these approaches is within the scope of this investigation.

In contrast the reaction of the purified N-bromoacetylacetazolamide alkylated enzyme with bromoacetate led to a reaction mixture in which the sum of the covalently bound reagents did exceed 2 eq, clearly demonstrating that a trialkylated species can be formed. The protection against alkylation at both positions by acetazolamide suggests that these alkylation occurred in the active site region.

**TABLE VIII**

Covalent interaction of N-bromoacetylacetazolamide with horse carbonic anhydrase B, human carbonic anhydrase C, and bovine carbonic anhydrase B

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction time</th>
<th>Alkylated histidine formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>His(1-Cm)</td>
</tr>
<tr>
<td>Human B</td>
<td>8</td>
<td>0.55</td>
</tr>
<tr>
<td>Horse B</td>
<td>8</td>
<td>None</td>
</tr>
<tr>
<td>Human C</td>
<td>8</td>
<td>None</td>
</tr>
<tr>
<td>Bovine B</td>
<td>8</td>
<td>None</td>
</tr>
<tr>
<td>Human B</td>
<td>24</td>
<td>0.79</td>
</tr>
<tr>
<td>Horse B</td>
<td>24</td>
<td>None</td>
</tr>
<tr>
<td>Human C</td>
<td>24</td>
<td>None</td>
</tr>
<tr>
<td>Bovine B</td>
<td>24</td>
<td>None</td>
</tr>
</tbody>
</table>

**Fig. 9.** Isoelectric focusing of the reaction products of N-bromoacetylacetazolamide-alkylated human carbonic anhydrase B with bromoacetate. After the removal of buffer and excess reagent by dialysis 25 mg of freeze-dried protein were applied to an LKB electofocusing column. Conditions were the same as in Fig. 4 with the exception that a pH gradient between 5 and 7 was used. The bars indicate the fractions that were pooled.
From the foregoing it would appear that N-bromoacetylacetazolamide interacts in the same way with both the native and alkylated enzymes. Bromoacetate on the other hand reacts only once with the native enzyme (17) but twice with the alkylated species to form dialkylated and trialkylated enzymes.

To reconcile this apparent contradiction, we suggest that N-bromoacetylacetazolamide induces a local conformational change at the active site of the enzyme making a second histidine sterically available for the subsequent reaction with bromoacetate. Aromatic sulfonamide and iodide-induced local conformational changes have been postulated recently by King and Roberts (36) on the basis of nuclear magnetic resonance spectroscopic data.

The presence of two sequentially removed histidines in the active site region has been revealed by affinity labeling with α-haloacetates and N-chloroacetylchlorothiazide. α-Haloacetates reacted with histidine residue number 204 from the amino terminus (36, 37), and the histidine that reacted with N-chloroacetylchlorothiazide was located between residues numbers 65 and 73 (37).

Bromoacetate at pH 7.6 binds to the alkylated enzyme with a $K_i$ of 9.2 mM, which is of the same order of magnitude as the $K_i$ of this inhibitor for the native enzyme (5.3 mM). Furthermore, the reaction with both the native and alkylated enzymes occurs at the 3-nitrogen position of a histidine residue. These observations suggest that bromoacetate reacted with the same histidine in both native and alkylated enzymes and consequently the histidine that reacted fastest with N-bromoacetylacetazolamide and is modified in the monoalkylated enzyme is not histidine$_{204}$. The assignment of the position of the fastest reacting histidine in the primary structure must wait until the sequence around it is known.

The properties of the alkylated enzyme resemble in many respects those of the native species. It still catalyses the hydration of CO$_2$ and the hydrolysis of p-nitrophenyl acetate. The pH profile of the ester hydrolysis catalysed by the modified enzyme is qualitatively similar to that of the native enzyme, but the apparent pH of the activity linked group is shifted to a lower pH value. At pH 6.0 the residual esterase activity of the modified enzyme is 95% of that of the native enzyme. As the pH is increased, the residual activity decreases rapidly, leveling off around neutrality at about 55%. This demonstrates that the decrease in the pH of the catalytically essential group accounts at least in part for the decrease in residual activity at neutral and alkaline pH after modification of the histidine.

Like the native enzyme, the alkylated species still binds bromoacetate or acetazolamide. While the affinity of bromoacetate has decreased, the affinity of acetazolamide has increased as a result of the alkylation. Such a decrease and increase in the affinity of inhibitors has been reported previously for carboxic anhydrases that have been modified at the histidine$_{204}$ position (34, 38). At three pH values (6.0, 6.8, and 7.6) the $K_m$ of the alkylated enzyme is almost identical to that of the native enzyme, indicating that the former enzyme binds substrate with the same affinity as does the latter. The modified enzyme reacts with bromoacetate to alkylate a second histidine in the active site region, this time at the 3-nitrogen position. This second histidine residue is probably identical with histidine$_{204}$ in the native enzyme that reacts with bromoacetate (36, 37). The dialkylated enzyme so formed is almost completely inactive.

All of these observations indicate that the histidine residue that reacted with N-bromoacetylacetazolamide, although it must be located in the active site region, is not a catalytically essential amino acid. By discounting this histidine as an essential group, we lend support to the hypothesis that the group in the enzyme that controls the catalysis and ionizes around neutrality is not a histidine but likely the zinc-bound water molecule itself (6, 10, 12, 13).

An observation that must not be overlooked, however, is that there are at least two histidines in the active site region. While the chemical modification of one does not result in a complete loss of activity the chemical modification of two histidines does. This suggests that one or the other of these histidines does participate in the catalysis. Upon the alkylation of one histidine the enzyme can compensate for it by using a sterically neighboring histidine. For the following reasons we prefer to assign this participating role in the native enzyme to histidine$_{204}$ which reacted with bromoacetate. The residual activity of the bromoacetate alkylated enzyme never exceeds 15% (17, 34). Furthermore, this modified enzyme binds p-nitrophenyl acetate with the same affinity as does the native enzyme, thereby indicating that the decreased activity is not due to a decreased binding of the substrate, but rather to a decrease in $V_{\text{max}}$ (34).

The participation of histidine$_{204}$ in the catalysis is not necessarily functional, but rather it could well be structural. X-ray crystallography of human enzyme C has revealed that there are two histidine residues in the active site region that are probably bonded to 2 water molecules, which in turn are hydrogen bonded to several other water molecules, including the one that is liganded to the zinc ion (12). Khalifah (13) has proposed that one of the water molecules in such an ordered water structure would function both as a hydrogen donor and acceptor, thus catalyzing the CO$_2$ hydration reaction. Assuming a similar water structure in human enzyme B, it is postulated that the alkylation of histidine$_{204}$ would, either directly or through an induced local conformational change, interfere with this structure, resulting in a less favorable orientation of the participating water molecules. The enzyme would then continue to function but with a reduced activity.

No functional role can be assigned to the histidine that reacted with N-bromoacetylacetazolamide, since at pH 6 the native and alkylated enzymes are kinetically almost indistinguishable. However, in the enzyme alkylated at histidine$_{204}$, it could be responsible for holding the participating water molecules in the less favorable orientation suggested above. On further alkylation to form a dialkylated species the water structure would collapse completely, resulting in an almost nonfunctioning enzyme.

While this interpretation of our results is attractive in that it assigns role to histidine$_{204}$, the almost complete lack of activity of the dialkylated enzyme would be due to a different type of conformational change, or could be the result simply of steric blocking of the active site region and the resultant prevention of substrate binding. We still lack sufficient information to exclude these alternatives.

Acknowledgments—We thank Mrs. L. Beysovec and Mr. A.
Nagy for their excellent and enthusiastic contributions to this research project.

REFERENCES

Covalent Labeling of the Active Site of Human Carbonic Anhydrase B with \(N\)-Bromoacetylacefazolamide

Show-Chu C. Wong, Stephen I. Kandel, Marianne Kandel and Allan G. Gornall


Access the most updated version of this article at [http://www.jbc.org/content/247/12/3810](http://www.jbc.org/content/247/12/3810)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/247/12/3810.full.html#ref-list-1](http://www.jbc.org/content/247/12/3810.full.html#ref-list-1)