Some Characteristics of Human, Bovine, and Horse Carbonic Anhydrases As Revealed by Inactivation Studies*

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SUMMARY

Two representatives of the low (human Enzyme B and horse Enzyme B) and two representatives of the high (bovine Enzyme B and human Enzyme C) catalytic activity forms of erythrocyte carbonic anhydrase were reacted with bromoacetate and bromoacetazolamide with the aim of elucidating structural differences at their active sites.

The dissociation constants of the reversible inhibition of bromoacetate and bromoacetazolamide have been determined. It was found that the constants for bromoacetate showed 25- to 30-fold differences between the low and high activity forms of the enzyme, but only 1.1- to 6-fold differences within one activity type. The dissociation constants of the enzyme-bromoacetazolamide complex were almost the same for the two groups.

Several lines of evidence suggest that bromoacetazolamide inactivates both types of enzymes with simultaneous alkylaion of a histidine (or histidines) at or near the active site at the 3-nitrogen position. The rate of alkylaion is, however, markedly different for the two types. Stoichiometric amounts of this inhibitor partially alkylate a histidine of the high catalytic activity forms, but do not react with the low catalytic activity forms. At higher inhibitor concentrations both types react, but the high activity forms react significantly faster.

By contrast, bromoacetate at low concentrations inactivates exclusively the low catalytic activity forms with the carboxymethylation of a histidine at the 3-nitrogen position. Evidence is presented that this reaction for the horse enzyme occurs at or near the active site. At higher inhibitor concentrations the high catalytic activity forms also became inactivated, but this reaction is nonspecific. The half-times of the inactivation by bromoacetate were also determined. These values for the high catalytic activity enzymes were found to be 36 times greater than could be explained on a basis of enzyme-inhibitor complex concentration. It is suggested that a difference in the conformation of the active site, or a steric hindrance brought about by an amino acid side chain sufficiently close to the reactive histidine, would account for the observed differences in the rates of alkylaion and inactivation.

The irreversible inactivation of human carbonic anhydrase B (carbonate hydro-lyase, EC 4.2.1.1) by haloacetic acids and bovine Enzyme B by bromoacetazolamide has recently been shown (1-3). The inactivation was found to occur in both cases by a modification of 1 eq of histidine residues at the 3-nitrogen position (1-3). It has also been shown that the inactivation is preceded by the formation of an enzyme-inhibitor complex, indicating that the inactivation occurs via the active site (1-3). These investigations have revealed that bromoacetate at a concentration that almost completely inactivated human B does not inactivate human C (2) or bovine B carbonic anhydrases (3). The latter two enzymes differ from the previous one in that they catalyze several times faster the reversible hydration of CO2 as well as the hydrolysis of esters (4).

It is the purpose of the present paper to describe further investigations of the covalent interaction of bromoacetate and bromoacetazolamide with human, bovine, and horse carbonic anhydrases and to consider the implications of the resulting data on the relationship between the chemical reactivity of the active site histidine and the catalytic activity of carbonic anhydrase isoenzymes.

MATERIALS AND METHODS

Preparation of Carbonic Anhydrases—Crude human carbonic anhydrases were prepared from red blood cells.1 The conditions for hemolysis, as well as the preparation of crude carbonic anhydrase by chloroform-ethanol treatment, were similar to those described by Armstrong et al. (5) with the exception that the dialyzed supernatant was freeze-dried. A column measuring 4 x 40 cm was packed with DEAE-cellulose (previously washed according to Peterson and Sober (6)) and equilibrated with 0.01 M Tris chloride buffer at pH 8. To the column 800 mg of crude carbonic anhydrase were applied and eluted at 5° with a concave gradient that was developed by two metering pumps. The flow rate from the reservoir (which contained 0.1 M Tris chloride buffer, pH 8) to the mixing chamber (containing 2500 ml of 0.01 M Tris chloride buffer, pH 8) was 80 ml per hour. The flow rate from the mixing chamber to the column was set at 230 ml per hour. The effluent was continuously monitored with the aid of an Isco ultraviolet analyzer (model UA 2).

Two well separated fractions were obtained. The faster

1 The cells were supplied through the courtesy of Dr. J. H. Crookston from the blood bank at Toronto General Hospital.

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Moving fraction (400 to 553 ml, Fraction I) gave two bands on starch gel corresponding to Enzymes B and C (50 to 60 mg). The slower moving fraction (690 to 986 ml, Fraction II) was pure Enzyme B (350 to 400 mg). Fraction I was dialyzed against distilled water and freeze-dried; a total of 200 mg of this fraction was applied to a DEAE-Sephadex A-50 column (2.5 x 25 cm) which was equilibrated with 0.05 M Tris chloride buffer at pH 8 and 5°C. The column was eluted with the same buffer at 5°C with a flow rate of 20 ml per hour. Two fractions were obtained. Fraction I (91 to 169 ml) was pure human C carbonic anhydrase (92 mg); Fraction II (182 to 260 ml) was pure human B (36 mg).

Horse blood was purchased from Woodlyn Farms, Guelph, Ontario. Crude horse carbonic anhydrases were prepared as described by Furth (7); 500 mg of crude enzyme mixture, which still contained considerable amounts of hemoglobin, were chromatographed on a DEAE-cellulose column, 4 x 40 cm, at pH 8 as described above for the separation of crude human carbonic anhydrases. Two major fractions were obtained. The starch gel electrophorogram indicated that the fastest moving fraction (450 to 640 ml, Fraction I, 35 mg) contained horse Enzyme C and two unidentified proteins. The slower moving fraction (660 to 1040 ml, Fraction II, 80 mg) was impure horse Enzyme B (on starch gel, in addition to Enzyme B, a faint unidentified band was seen). After dialysis against distilled water and freeze-drying, 50 mg of Fraction II were rechromatographed on a DEAE-cellulose column, 1 x 60 cm, with the same gradient as was used for the separation of crude horse carbonic anhydrases. The mixing chamber contained 204 ml of 0.01 M Tris chloride buffer at pH 8. The flow rate to the mixing chamber was set at 8 ml per hour and to the column at 25 ml per hour. The major peak (146 to 175 ml) was starch gel electrophoretically pure horse Enzyme B (31 mg).

For bovine carbonic anhydrases, 800 mg of crude bovine enzyme (Worthington) was separated into three fractions on a DEAE-cellulose column (4 x 40 cm) previously equilibrated with 0.01 M Tris chloride buffer at pH 8. Elution was achieved by a linear gradient from 0.01 to 0.1 M at pH 8 with a flow rate of 260 ml per hour. The volume of the buffer in the reservoir was 1 liter. The first major fraction (1040 to 1300 ml, Fraction I, 350 mg) was starch gel electrophoretically pure bovine Enzyme B; the second fraction (1387 to 1586 ml, 70 mg) was impure Enzyme A. At this point the gradient was replaced by 0.1 M Tris chloride at pH 8 to elute a third fraction (1820 to 2028 ml, 61 mg) which was still enzymatically active. On starch gel, however, this gave three bands: two corresponded to Enzymes B and A and the third one migrated between the two.

Zinc-free Human Carbonic Anhydrase C—This was prepared according to Lindskog (8) at pH 5.5. After 12 days of dialysis against o-phenanthroline followed by dialysis against distilled water, the supernatant was separated from the denatured protein by centrifugation and freeze-dried. Zinc analyses were carried out by atomic absorption spectroscopy (Perkin Elmer, model 308). Alkylated Human B and Bovine B Carbonic Anhydrases—Carboxymethylated human Enzyme B was prepared by a slight modification of the method of Whitney, Nyman, and Malström (2). The enzyme was reacted with a 60-fold molar excess (1.8 x 10^-2 M) of bromoacetate in 0.1 M Tris chloride buffer at pH 7.6 for 96 hours at 24°C. Alkylated bovine Enzyme B was prepared and purified by DEAE-cellulose chromatography as reported previously (3).

Inhibitors and Reagents—Bromoacetazolamide was prepared as reported (3). Commercial bromoacetate acid (Eastman) was redistilled under reduced pressure before use. 1-14C-Bromoacetate acid was purchased from New England Nuclear and diluted with freshly distilled bromoacetate acid to obtain a specific activity of 0.8 mCi per mmole. Urea was recrystallized from ethanol, p-nitrophenyl acetate (Eastman) twice from acetone-water. Dimethylformamide was redistilled under reduced pressure. DEAE-cellulose (high capacity) was purchased from Bio-Rad and DEAE-Sephadex A-50 from Pharmacia (Canada). All other chemicals were reagent grade and were used without further purification.

Enzyme Assays—CO2 hydratase activity was measured by the Wilbur-Anderson method at 4°C. Specific activity units were defined by the equation given earlier (9). The enzyme was determined with p-nitrophenyl acetate as substrate (10), following the optical density change at 348 mp (Zeiss PMQ II or Gilford recording spectrophotometer) (5). The reaction cuvette contained 0.6 x 10^-8 M to 1 x 10^-4 M p-nitrophenyl acetate in 0.025 M Tris sulfate buffer plus 0.8% acetone at pH 7.6 and 24°C.

Determination of Inhibition Constants—The dissociation constants (K) for reversible inhibition of bovine carbonic anhydrase and bromoacetazolamide were determined by the method of Dixon (11) by esterase activity, assuming that the inhibition is noncompetitive.

Starch Gel Electrophoresis—This was done by reported procedures. For the human and bovine enzymes, pH 8.9 (9) and, for the horse enzyme, pH 8.6 (7) were used.

Amino Acid Analysis—Protein samples were evacuated, treated with 6 N HCl, sealed, and hydrolyzed for 22 hours at 110°C. Since cystine overlaps His(3-Cm) the modified human and horse enzymes were oxidized prior to acid hydrolysis (12) with performic acid 30-fold in excess of that required to oxidize the cystine and methionine in the modified proteins. Amino acid analysis of the hydrolysates was performed by the method of Spackman, Stein, and Moore (13). A synthetic mixture of carboxymethyl histidines (14) was used to identify the newly formed peaks in the hydrolysates. The integration constant given by Cropsey, Stein, and Moore (14) was used for the quantitative determination of His(3-Cm) and His(1-Cm). Molar ratios of amino acids were calculated on the basis of glycine equal to 20 for bovine B, 16 for human B, 22 for human C, and 23 for horse B carbonic anhydrases.

Determination of Radioactivity—All of the counting was carried out in a Packard Tri-Carb liquid scintillation counter (model 2075). Protein samples were finely suspended in 0.1 ml of dimethylformamide and then dissolved in 1 ml of Hyamine. Absolute count rates were determined by either the internal or external standard method.

RESULTS

Carbonic Anhydrase Preparations—In recent years bovine, human, and horse erythrocyte carbonic anhydrases have been obtained in highly purified form, usually by ion exchange chromatography (5, 7, 15-17). The preparations of pure bovine B, human B, human C, and horse B carbonic anhydrases described

The abbreviations used are: His(3-Cm), 3-carboxymethyl histidine; His(1-Cm), 1-carboxymethyl histidine.
Inhibition constants of bromoacetate and bromoacetazolamide—estimation of equilibrium concentration of enzyme-inhibitor complex

The inhibition constants ($K_i$) of bromoacetate and bromoacetazolamide were determined in 0.025 M Tris sulfate buffer + 0.8% acetone at pH 7.6 by esterase activity and a Dixon plot. The enzyme concentration in the reaction mixture was 6.6 X $10^{-7}$ M for human C and bovine B carbonic anhydrases, 1.3 X $10^{-4}$ M for human Enzyme B, and 4 X $10^{-5}$ M for horse Enzyme B. $p$-Nitrophenylacetate concentration was 8 X $10^{-4}$ M for horse Enzyme B and 4 X $10^{-4}$ M for the other enzymes. The concentration of the enzyme-inhibitor complex was calculated from Equation 1 when the bromoacetate concentration was 2 X $10^{-6}$ M (20-fold molar excess) and the bromoacetazolamide concentration was 4 X $10^{-6}$ M (1.2-fold molar excess).

| Inhibitor          | Carbonic anhydride | $K_i$ | Inhibitor concentration | Equilibrium concentration of enzyme-inhibitor complex |
|--------------------|--------------------|-------|-------------------------|------------------------------------------------------|
|                    |                    | $M$   | $M$                     | %                                                   |
| Bromoacetic acid   | Bovine B           | 2.7 X $10^{-1}$ | 2 X $10^{-6}$ | 30.7 |
|                    | Human C            | 2.3 X $10^{-3}$ | 2 X $10^{-6}$ | 0.86 |
|                    | Human B            | 9 X $10^{-3}$ | 2 X $10^{-6}$ | 18.18 |
|                    | Horse B            | 5.6 X $10^{-2}$ | 2 X $10^{-6}$ | 3.45 |
| Bromoacetazolamide | Bovine B           | 1.2 X $10^{-5}$ | 4 X $10^{-6}$ | 97.1 |
|                    | Human C            | 1.1 X $10^{-5}$ | 4 X $10^{-6}$ | 97.0 |
|                    | Human B            | 1.3 X $10^{-6}$ | 4 X $10^{-6}$ | 96.9 |
|                    | Horse B            | 3.4 X $10^{-6}$ | 4 X $10^{-6}$ | 92.2 |

Table II

Inactivation of human, bovine, and horse carbonic anhydrases by bromoacetazolamide

Carbonic anhydrases were dissolved in 0.1 M Tris sulfate buffer at pH 7.6 or pH 8.7 (1 mg of enzyme per ml of buffer) and a 1.2- or 20-fold molar excess (4 X $10^{-5}$ M or 6.6 X $10^{-4}$ M) of bromoacetazolamide was added. The reaction proceeded for 24 hours at room temperature; the mixture was then dialyzed against distilled water and freeze-dried. Activities were determined by the Wilbur-Anderson method with the CO$_2$ hydration reaction.

| Enzyme          | pH | Concentration of bromoacetazolamide | His(3-Cm) formed | Irreversible inhibition |
|-----------------|----|------------------------------------|-----------------|-------------------------|
|                 |    | M/mole enzyme                      | residues/molecule | %                       |
| Bovine B        | 7.6| 1.2                                | 0.56            | 64.2                    |
| Human C         | 7.6| 1.2                                | 0.56            | 60.1                    |
| Human B         | 7.6| 1.2                                | Nil             | Nil                     |
| Horse B         | 7.0| 1.2                                | Traces          | 10.2                    |
| Bovine B        | 7.6| 20                                 | 0.78            | 84.6                    |
| Human C         | 7.6| 20                                 | 0.72            | 81.3                    |
| Human B         | 7.6| 20                                 | 0.31            | 36.4                    |
| Horse B         | 7.6| 20                                 | 0.46            | 51.3                    |
| Bovine B        | 8.7| 1.2                                | 0.51            | 64.6                    |
| Human C         | 8.7| 1.2                                | 0.46            | 52.3                    |
| Human B         | 8.7| 1.2                                | Nil             | Nil                     |
| Horse B         | 8.7| 1.2                                | Nil             | 8.0                     |
| Bovine B        | 8.7| 20                                 | 0.92            | 94.6                    |
| Human B         | 8.7| 20                                 | 0.25            | 31.2                    |

In this paper were obtained by modifications of known procedures. This was necessary because some of the published methods did not give in our hands electrophoretically pure enzyme samples. The modified procedures have been repeated in two laboratories several times and have proved highly reproducible. The specific catalytic activities expressed in Wilbur-Anderson units were 40,000 to 50,000 for bovine B, 8,000 to 9,000 for human B, 35,000 to 45,000 for human C, and 2,000 for horse B enzymes. Each enzyme migrated as a single band on starch gel, and its amino acid content agreed well with reported data (5, 7, 18).

Zinc-free Human Carbonic Anhydrase C—Our zinc-free human C preparation contained less than 0.05 g atom of zinc per mole and was almost completely inactive. Upon the addition of 1 g atom of zinc the enzyme regained its original activity.

Alkylated Enzymes—The carboxymethylated human carbonic anhydrase B contained 1 eq of His(3-Cm) and indicated 4 to 5% esterase activity. Bovine Enzyme B alkylated with bromoacetazolamide was isolated as a chromatographically pure sample which, on starch gel at pH 8.9 migrated as a single band toward the anode ahead of the native enzyme. It contained 0.9 eq of His(3-Cm) and possessed 4 to 5% esterase activity.

The CO$_2$ hydration property of the two alkylated enzymes could not be determined with sufficient accuracy by the Wilbur-Anderson method to tell whether they possessed any residual activity.

Reversible Inhibition In Table I are shown the inhibition constants of bromoacetate and bromoacetazolamide at pH 7.6 for the four enzymes. For bromoacetate this constant varies between 9 X $10^{-4}$ M for human Enzyme B (which possesses low catalytic activity) and 2.7 X $10^{-1}$ M for bovine Enzyme B (which possesses high catalytic activity). This variation corresponds to a 30-fold difference in the binding of bromoacetate by these two enzymes. It is interesting to note that the binding of this reagent to bovine B and human C enzymes, both having high catalytic activity, is almost the same, whereas the difference in binding between the two low activity forms (human B and horse B) is approximately 6-fold. The constants for bromoacetazolamide are of the same order of magnitude. The variation between the two extreme values is 3-fold.

Irreversible Inactivation by Bromoacetate and Bromoacetazolamide at Different Molar Concentrations—Table II shows that at pH 7.6 a 1.2-fold molar excess bromoacetazolamide partially inactivated bovine B and human C carbonic anhydrases (high activity), with a concomitant alkylation of a histidine at the 3 nitrogen position. Under identical conditions neither human B nor horse B enzymes (low activity) reacted at all.

In Table III it can be seen that bromoacetate had an opposite effect. Although a 20-fold molar excess of this reagent at pH 7.6 brings about 50 to 60% inactivation with human B and horse B enzymes, with the simultaneous formation of 3-carboxymethyl histidine, neither bovine B nor human C enzymes showed any significant inactivation or carboxymethylation.

If the concentration of bromoacetazolamide relative to the enzyme was increased to 20-fold (Table II), all four carbonic anhydrases showed inhibition and alkylation, although the quantitative aspects of the reaction were significantly different. The high catalytic activity forms yielded an almost inactive enzyme and approximately 0.8 eq of alkylated histidine; the low activity forms revealed only 0.30 to 0.46 eq of alkylated histidine and a corresponding inactivation. Similarly, a 60-fold
TABLE III
Inactivation of human, bovine, and horse carbonic anhydrases by bromoacetate

The reaction conditions were the same as described in the legend of Table II with the exception that a 20- or 60-fold molar excess (6.6 X 10^-4 M and 2 X 10^-3 M) of 14C-bromoacetate was used.

| Enzyme     | pH | Concentration of bromoacetate | Hi's(3-Cm) formed | Irreversible inactivation | Incorporation of 14C-bromoacetate |
|------------|----|-------------------------------|------------------|---------------------------|----------------------------------|
| Bovine B   | 7.6| 20 Nil                         | Nil              | 0.04                      |
| Human C    | 7.6| 20 Nil                         | Nil              | 0.11                      |
| Human B    | 7.6| 20 0.58                        | 51.6             | 0.68                      |
| Horse B    | 7.6| 20 0.55                        | 56.8             | 0.45                      |
| Bovine B   | 7.6| 60 0.08                        | 10.6             | 0.12                      |
| Human C    | 7.6| 60 10.9                        | 19.9             | 0.99                      |
| Human B    | 7.6| 60 0.82                        | 96.3             | 0.99                      |
| Horse B    | 7.6| 60 0.86                        | 92.1             | 0.96                      |
| Bovine B   | 8.7| 20 Nil                         | Nil              | Nil                       |
| Human B    | 8.7| 20 0.86                        | 50.4             | Nil                       |

*Not tested.

Irreversible inactivation of human, bovine, and horse carbonic anhydrases by bromoacetate

The observed values of alkylation tabulated in Table II for equal molar concentrations of bromoacetazolamide should also reflect the reactivity of the active site histidine, for at these concentrations the enzyme-inhibitor complex concentrations are nearly identical.

Half-Time of Inactivation by Bromoacetate—The rate of inactivation of carbonic anhydrases with bromoacetate at pH 7.6 was also determined. The half-time of the reactions at 18 X 10^-3 M concentration of inhibitor (60-fold molar excess), tabulated in Table V, is consistent with the results presented above. Bromoacetate inactivates all four carbonic anhydrases; the half-times of the reaction are, however, remarkably different.

The observed half-times of human B and horse B enzymes were 18 and 70 min, respectively. For bovine B and human C enzymes the half-times were 5 and 4 days and not 3.2 and 2.7 hours as one would expect if the difference in binding would account for the difference in the rate of inactivation.
A kinetic study with bromoacetazolamide to determine the half-time of the reaction was unsuccessful. A recently revealed pseudoirreversible binding of acetazolamide and bromoacetazolamide made it impossible to separate the reversible and irreversible binding of these reagents by the usual dilution techniques. Further details of this phenomenon will be given elsewhere.

Specificity of Inactivation—Table VI indicates that human C carbonic anhydrase had lost approximately 81% of its activity when it reacted with a 20-fold molar excess of bromoacetazolamide at pH 7.6. As a result of this inactivation, 0.7 eq of histidine became alkylated at the 3-nitrogen position. Under identical conditions the metal-free apoenzyme yielded only 0.1 eq of alkylated histidine. When 1 eq of Zn++ was added to this metal-free enzyme preparation 92% of its usual activity was regained. It is well documented that Zn++ is an essential part of all of the known mammalian erythrocyte carbonic anhydrases and is necessary for the binding of sulfonamide inhibitors. The lack of any significant reaction of bromoacetazolamide with the zinc-free enzyme is a good indication that it is first bound to the active site and then forms a covalent bond with a histidine at or close to the active site. Furthermore, if the native enzyme is reacted under identical conditions, but in the presence of 6 M urea, the formation of alkylated histidine drops to 0.2 eq, confirming the specificity of the reaction. When 1.2-fold molar excess of the reagent was used (Table II) a good correlation was found between irreversible inactivation and His(3-Cm) formation. The mere fact that a 20-fold molar excess of bromoacetate did not bring about any significant inactivation and alkylation in 24 hours (Table III) can also be considered as evidence for the specificity of the reaction of human C enzyme with bromoacetazolamide.

In order to determine the active site-directed nature of the irreversible inactivation of horse carbonic anhydrase B with bromoacetate a different course was followed. The rate of the reaction was determined at 9 mM and at 45 mM concentrations of the reagent (Fig. 1) and a comparison of the estimated half times indicated a “rate saturation effect” (19). The rate of the reaction increased by a factor of only 3.3 and not by a factor of 5, which would be expected if the covalent bond formed outside the active site by a bimolecular mechanism. From Equation 1 the calculated value for the rate saturation effect is 3.2, which agrees well with the observed value. The good stoichiometry of carboxymethylation, inhibition, and 14C-bromacetate incorporation substantiates the view that the reaction occurred via the active site (Table III).

The specificity of the reactions of human carbonic anhydrase B with bromoacetazolamide and of bovine Enzyme B with bromoacetate was also studied. Carboxymethylated human Enzyme B was reacted with 20-fold molar excess of bromoacetazolamide and it was found that the initial value of His(3-Cm) (1 eq) did not increase significantly (1.07 eq), indicating that the His(3-Cm) formed from human Enzyme B with bromoacetazolamide occurs at the active site. By contrast, bromoacetate at 1.8 X 10^-2 M concentration (60-fold molar excess) did react with monoalkylated bovine Enzyme B. The initial value of His(3-Cm) (0.9 eq) increased to 1.2 eq, indicating that at this high concentration bromoacetate reacts with a histidine or histidines outside the active site. The nonspecific nature of the reaction is also indicated by the significant nonequivalence of His(3-Cm) formation and inactivation, which is shown in Table V. Bovine Enzyme B when it was 50% inactivated with bromoacetate contained 0.53 eq of His(3-Cm) and 0.13 eq of His(1-Cm). The 0.33 eq excess of His(3-Cm) relative to the inactivation almost completely accounts for the His(3-Cm) formed from the monoalkylated enzyme with bromoacetate. A similar nonspecificity of the reaction of human Enzyme C with bromoacetate is seen in Table V.

**FIG. 1.** Inactivation of horse carbonic anhydrase B by bromoacetate. The reaction mixture contained 3 X 10^-4 M enzyme, and 9 X 10^-4 M and 4.5 X 10^-2 M bromoacetate in 0.1 M Tris sulfate buffer at pH 7.6. Aliquots, 0.1 ml, were withdrawn at appropriate intervals and diluted to 7 ml and the esterase activity was determined.

**TABLE VI**

| Experiment                              | Inactivation | His(3-Cm) formed |
|----------------------------------------|--------------|-----------------|
| Native enzyme plus bromoacetazolamide  | 81.3         | 0.72            |
| Zinc-free enzyme plus bromoacetazolamide plus 1 g atom Zn++ | 8.0          | 0.1             |
| Native enzyme plus 6 M urea plus bromoacetazolamide | ...a        | 0.2             |

* Not tested.

**DISCUSSION**

Carbonic anhydrase is present in mammalian erythrocytes in multiple molecular forms. Most of the species studied contain at least two major isoenzymes, one possessing specific catalytic activity 4 to 10 times higher than the other (7, 9, 20). From bovine erythrocytes two major forms have been isolated; both possess the same high specific catalytic activity and it is believed that they are conformers (18). It seemed reasonable to assume that the differences in catalytic activity of the various enzymes reside in the chemical structure of their active sites. In the last 2 years three laboratories have reported that there is a chemically reactive histidine at or close to the active site of...
human Enzyme B and bovine Enzyme B (1-3). Although at present there are serious doubts about the significance of this histidine in catalysis (2, 21), we reasoned that a probe of its chemical reactivity might give some clue to the differences in the active sites of carbonic anhydrase isoenzymes. This reasoning found some support from the following observations. Whitney et al. (2) as well as Bradbury (21) reported that haloacetates rapidly inactivate the low catalytic activity form of human iso-enzymes (human B) with the labeling of 1 eq of histidine at the 3-nitrogen position. Whitney et al. (2) also reported briefly that in one experiment human Enzyme C, which possesses high catalytic activity, did not react with bromoacetate. Concurrent with this investigation we (3) found that bovine Enzyme B, which like human C possesses high catalytic activity, does not react with haloacetates even when 20-fold molar excess of this reagent is used, but does react with bromoacetazolamide. We demonstrated that this reaction occurs at or close to the active site of this enzyme with the alkylation of 1 eq of histidine at the 3-nitrogen position.

In order to study further the chemical reactivity of the active site histidine of the two types of carbonic anhydrase isoenzymes, bromoacetate and bromoacetazolamide were reacted with bovine B and human C (high activity forms) and human B and horse B (low activity forms). We selected these enzymes as typical representatives of the two types of carbonic anhydrase isoenzymes since they are the most readily available and the best characterized. The data presented in this paper show conclusively that a difference exists between the chemical reactivity of a histidine of the high and the low catalytic activity forms of mammalian carbonic anhydrases when tested with bromoacetate and bromoacetazolamide, respectively.

In order to make these differences meaningful it was necessary to prove that the histidines that reacted with the two reagents are located at or close to the active site. This requirement seems to have been fulfilled in the case of the reaction of haloacetate and human carbonic anhydrase B (1, 2, 21), as well as in the case of the reaction of bromoacetazolamide and bovine Enzyme B (3). Based on the evidence given in this paper we believe that the reactions of bromoacetate with horse Enzyme B and of bromoacetazolamide with human B and human C enzymes also occur via the active site. By contrast, the reaction of bovine B and human C enzymes with bromoacetate was nonspecific at the concentration used, while the low activity human B enzyme reacted specifically with this reagent.

Our results provide a basis for the following deduction. In the case of high catalytic activity enzymes, although bromoacetate reacts with a histidine that is at or close to the active site, it is also capable of reacting with a histidine (or histidines) outside the active site. The latter reaction, however, does not affect enzyme activity. The results also indicate that bromoacetazolamide is a better reagent for labeling the active site of carbonic anhydrases possessing high catalytic activity.

Although bromoacetate at 45 mM concentration inactivates horse Enzyme B relatively rapidly, the inactivation is not complete. The enzyme reached 96% inactivation in about 5 hours, and this value did not increase further even after 24 hours. Moreover, a chromatographically purified alkylated bovine Enzyme B, migrating as a single band on starch gel electrophoresis, retains 4 to 5% of its esterase activity. A small residual esterase activity was found previously by Whitney et al. (2) and Bradbury (21) for carboxymethylated human Enzyme B. Our results suggest that a residual esterase activity is a general phenomenon of mammalian erythrocyte carbonic anhydrases, after alkylation at the active site histidine.

In the reactions described here a combination must occur initially between the enzyme active site and the inhibitor, which then reacts with a properly oriented histidine. The rate of inactivation would be controlled by the concentration of the enzyme-inhibitor complex, which in turn depends on both the concentration and the inhibition constant of the inhibitor.

A considerable difference was found in the rate of inactivation and alkylation of one particular isoenzyme with bromoacetate and bromoacetazolamide. Human carbonic anhydrase B with bromoacetate at 1.8 x 10^{-3} M concentration, when 66.6% of the enzyme was reversibly complexed, yielded 1 eq of His(3-Cm) (Table V), while with bromoacetazolamide at 6.6 x 10^{-4} M concentration, when 96% of the enzyme was present in combined forms, it yielded only 0.61 eq of His(3-Cm) (Table II). This indicates that bromoacetate reacts with human Enzyme B at least 3 times faster than bromoacetazolamide, although the concentration of enzyme-bromoacetate complex is only two-thirds the concentration of the enzyme-bromoacetazolamide complex. A direct comparison of the rate of alkylation can, however, be misleading, for the relative electrophilicity of the two reagents is not known at this time. A comparison of the rates of alkylation of the high catalytic activity isoenzymes by the two reagents would appear to be difficult since at a concentration of bromoacetate that yields measurable amounts of His(3-Cm) the reaction is nonspecific for the high activity enzymes.

A striking difference was also found between the two types of isoenzymes in the rate of alkylation by either reagent when compared at equal molar concentrations of the reagents (Tables II and III) and at near equal equilibrium concentrations of enzyme-inhibitor complex (Table IV). This is a clear indication that a difference in chemical reactivity, and not in binding, causes the difference in rates of alkylation.

As Tables II, III, and V indicate, the remarkable isoenzyme specificity of the inhibitor holds only if the inhibitor concentration is kept low. At higher inhibitor concentrations both reagents react with the four enzymes, although the rates of the reactions are significantly different. This observation indicates that an almost complete lack of the reaction at lower concentrations cannot be due to the lack of a sterically available histidine at or around the active site.

Bradbury (21) found a pK of 5.8 for the reactive histidine of human carbonic anhydrase B. The pK of the reactive histidine of bovine Enzyme B is not known at the present time. We have found (3) that the inactivation and alkylation of the active site histidine of this enzyme reach a maximum around pH 8. Although these results might indicate a difference in the pK of the reactive histidines of the two enzymes, it is conceivable that at pH 8.7, at which pH the differences in the reaction are equally significant, the histidine of either enzyme will be present largely in unprotonated form and can react as a nucleophile. Based on
this consideration one can probably rule out the possibility that the difference in rates of alkylation is due to a difference in the pK of the active site histidines.

One explanation for our observations would be that the relative orientation of the reagents and the enzymatic nucleophile within the enzyme-inhibitor complex would be different for the two groups of enzymes. This difference might indicate a difference in the conformation of the active sites. A steric hindrance brought about by an amino acid sufficiently close to the active site histidine could, however, account for the observed difference as well.

The present data are barely enough to permit a statement that the differences in the rates of alkylation of the high and low catalytic activity forms of human, bovine, and horse carbonic anhydrases with bromoacetate and bromoacetazolamide are directly related to their catalytic activity. Further experimentation on the role of the histidine that became alkylated will be necessary before such a conclusion can be established. Even if this histidine has no direct role in the catalysis, its position relative to the bond-making and -breaking process could influence the rate of catalysis.

Striking similarities of the kinetic parameters within one group of isoenzymes and dissimilarities of the same parameters between the two types of isoenzymes have been found previously and discussed recently by Furth (7). Based on these observations and particularly on the extent of the homology of the amino acid sequence of a large COOH-terminal fragment of human C and bovine B enzymes, and a less complete homology between human C and human B, Nyman, Str€ed, and Westermark (22) suggested that in the evolution of carbonic anhydrases a gene duplication occurred at an early stage, followed by a divergent evolution of the two genes. Evidence for the control of the human isoenzymes by two separate genes has been provided by Tashkin (23). Our results are in accord with such a hypothesis.

Several selective irreversible inhibitors of isoenzymes have become known recently, particularly from the work of Baker (24) and his associates. In these studies the specificity was deliberately attained by positioning the covalent bond forming group outside the active site, where a difference between the amino acid sequence of the isoenzymes is more likely to occur. Our investigation shows that the structure of the active site of closely related isoenzymes, in which the amino acid sequence of the active site is likely to be the same, can be sufficiently different to obtain selective irreversible inhibition.

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