Location of Histidine Residues that React with Bromoacetazolamide and N-Bromoacetylacetazolamide in Human and Bovine Carbonic Anhydrases*

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SUMMARY

Human carbonic anhydrase C and bovine carbonic anhydrase B were modified with the affinity label, bromoacetazolamide, and human carbonic anhydrase B with N-bromoacetylacetazolamide. Tryptic peptides from the modified enzymes, containing alkylated histidines, have been isolated by ion exchange and two-dimensional high voltage electrophoresis-paper chromatography. The partial amino acid sequence of the peptide from alkylated bovine enzyme B has been found to be Met-Val-Asn-Gly-His-Ser-Phe-Asn-Val-Glu-Tyr-Asx-Asx(Glx,Asx,Ser)Lys-. In all probability the histidine in this sequence that reacted is His-64. Comparison of the amino acid composition of the tryptic peptides from the two human isoenzymes with the proposed sequences of these enzymes has made it possible to identify histidine-64 in human enzyme C as that reacting with bromoacetazolamide and histidine-67 in human enzyme B as that reacting with N-bromoacetylacetazolamide.

EXPERIMENTAL PROCEDURE

Materials and Reagents—Human and bovine carbonic anhydrases were isolated as reported earlier (12). Bromo[14C]acetazolamide, with a specific activity of 0.35 μCi per pmole, and N-bromo[14C]acetylacetazolamide, with a specific activity of 0.16 μCi per pmole, were prepared in a manner similar to that already described (3, 12). Trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone, three times recrystallized chymotrypsin, and carboxypeptidase A, both treated with diisopropylphosphoro-fluoridate, were obtained from Worthington. Leucine aminopeptidase, type III, was purchased from Sigma. Dowex 50 resin (AG 50W X 8, 200 to 400 mesh), Dowex 1 resin (AG 1-X 2, 200 to 400 mesh) and DEAE-cellulose (Cellex D, high capacity) were obtained from Bio-Rad, California. Phenyl

1 Following the recent suggestion of Andersson et al. (8), we apply throughout this paper the established numbering of residues in human carbonic anhydrase B to human carbonic anhydrase C and bovine carbonic anhydrase B. Thus, residues 64, 67, and 120 in human enzyme C used in this communication correspond to residues 63, 66, and 125 used by the x-ray crystallographers (8).
isothiocyanate, trifluoroacetic acid, and pyridine for sequential degradation (sequential grades) were purchased from Pierce Chemical Company. All other solvents were reagent grade and were redistilled before use.

Preparation and Purification of Bovine Carbonic Anhydrase B and Human Carbonic Anhydrase C Alkylated with Bromo[14C]-acetazolamide—Bovine enzyme B (600 mg, 20 μmoles) was reacted with 1.2-fold molar excess of bromo[14C]acetazolamide (7.22 mg, 24 μmoles) in 0.1 M Tris-chloride buffer at pH 8.7 for 24 hours (12). The reaction mixture was dialyzed against distilled water and freeze-dried. A portion of the dried material (200 mg) was added and the digestion continued until no more NaOH was added and the digestion ceased (5 hours), additional trypsin (10 mg, 0.42 μmole) dissolved in a minimum amount of 0.001 N HCl was added and allowed to digest for 15 hours at 37°. After removal of the insoluble material by centrifugation (31 mg), the pH of the digest was lowered to 3 by addition of acetic acid. A precipitate, amounting to 1 to 2%, was separated by centrifugation and the solution was freeze-dried.

Digestion with Chymotrypsin—The labeled peptide isolated from the tryptic digest of alkylated bovine enzyme B (1 μmole) was hydrolyzed with chymotrypsin (46 mg, 0.02 μmole) in 0.1 M ammonium bicarbonate buffer (1 ml) at pH 7.8 for 2 hours at 37°. The digestion was stopped by adjusting the pH to 4 with acetic acid, then the digest was freeze-dried. Fraction 1 (Fig. 7) peptide T1 (0.5 μmole), isolated from the tryptic digest of alkylated human enzyme B, was cleaved with chymotrypsin. The conditions of the hydrolysis were the same as described above.

Digestions with Carboxypeptidase A and Leucine Aminopeptidase—These were done according to published methods (24, 25). The amino acids liberated by carboxypeptidase A were identified on a Beckman-Spinco analyzer, and the amino acids liberated by leucine aminopeptidase were identified by a combination of high voltage paper electrophoresis at pH 1.9 and paper chromatography using Solvent I.

Partial Acid Hydrolysis—The labeled peptide separated from the tryptic digest of alkylated bovine enzyme B (0.3 μmole) was dissolved in 0.03 N HCl in 30% acetic acid (0.8 ml) and kept in an evacuated sealed tube at 105° for 16 hours. The hydrolysate was evaporated to dryness under reduced pressure and separated on paper using high voltage electrophoresis at pH 6.4 and chromatography in Solvent II.

Ion Exchange Chromatography—The mixture of peptides produced by trypsin and chymotrypsin hydrolysis was fractionated by either one or both of the following ion exchangers: Dowex 1-X2, and Dowex 50-X2. The methods of preparing the resin and the buffers employed were essentially the same as those recommended by Schroeder (28). The specific conditions will be described where these methods were used.

Sequence Determination—The method of Gray and Smith (26) was used for sequential Edman degradation, with the exception that the anilinothiazolinones initially formed were hydrolyzed to the corresponding amino acid with constant boiling HCl in an evacuated sealed tube at 150° for 22 hours according to the method of Van Orden and Carpenter (27). The amino acids so formed were identified on the analyzer. Occasionally the peptides remaining after Edman cycles were hydrolyzed to identify the amino acids removed by the degradation.

Amide Assignments—This was performed either by converting the initially formed anilinothiazolinones to the corresponding phenylthiohydantoinamines according to the method of Edman and Berg (28) or by leucine aminopeptidase digestion. Phenylthiohydantoinamines were identified by thin layer chromatography in Solvent 5 followed by Solvent 4 of Jeppsson and Sjöquist (29).
The flow rate from the mixing chamber to the column was set at 144 ml per hour. The bars indicate the fractions that were pooled. Amino acids liberated by leucine aminopeptidase were identified by two-dimensional paper electrophoresis chromatography (pH 1.9, Solvent II).

## RESULTS

### Purification of Bovine Carbonic Anhydrase B Alkylated with Bromo[\(^{14}\)C]acetazolamide

Alkylated bovine enzyme B was separated from unreacted enzyme by the use of DEAE-cellulose chromatography as reported earlier (12) (Fig. 1). Fraction I was found to be identical with the native enzyme. Fraction II contained 0.93 eq of \(^{14}\)C-labeled reagent covalently attached to the enzyme. After acid hydrolysis the alkylated enzyme yielded 0.92 eq of His (3-CM) and the loss of 0.88 eq of histidine. The amount of this inhibitor required for complete inhibition was, however, less than 0.1 mole per mole of Fraction II. This observation indicated that Fraction II after the first chromatography still contained 4 to 5% of native enzyme. It is obvious that Fraction II eluted from the first column. This observation indicated that Fraction II after the first chromatography still contained 4 to 5% of esterase activity of the native enzyme and not any residual activity in the alkylated enzyme was responsible for the esterase activity of Fraction II eluted from the first column. Our previous report (7) concerning the residual esterase activity of the alkylated enzyme thus requires modification; bovine enzyme B modified with bromoacetazolamide is inactive.\(^3\)

\(^3\) James W. Wells in our laboratories found that Fraction II eluted from the first DEAE-cellulose column can be inhibited by acetazolamide. The amount of this inhibitor required for complete inhibition was, however, less than 0.1 mole per mole of Fraction II. This observation prompted us to reinvestigate the residual activity of the modified enzyme. See also Footnote 3.

### Purification of Labeled Tryptic Peptide—Initially we attempted to isolate the labeled peptide from the tryptic digest of bromo-\(^{14}\)C]acetazolamide alkylated bovine carbonic anhydrase B using a Dowex 50 column. Although we succeeded in separating the digest into 17 well resolved fractions, the radioactivity was spread all over the effluent. Similar observations were reported by Bradbury (9) and Henkart (30). With a Dowex 1 column 94% of the radioactivity applied to the column was recovered in a single fraction that was eluted with 2 N acetic acid (Fig. 3). This fraction was purified by a combination of high voltage paper electrophoresis and paper chromatography (pH 6.4, Solvent I) to obtain a radioactive peptide (yield 50.4%), the amino acid composition of which is presented in Table I. As expected from the elution pattern from the Dowex 1 column (Fig. 1), this peptide migrated quickly to the anode. No other peptide could be detected on the paper by radioautography, indicating that essentially all the radioactivity incorporated into the enzyme was present in this single peptide.

### Amino Acid Sequence Studies of Tryptic Peptide—Four steps of Edman degradation established the NH\(_2\)-terminal sequence of...
TABLE I
Amino acid composition of peptides obtained by tryptic (T) and chymotryptic (C) cleavage of bovine carbonic anhydrase B and human carbonic anhydrase C alkylated with bromoacetazolamide and human carbonic anhydrase B alkylated with N-bromoacetylacetazolamide

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Bovine enzyme B</th>
<th>Human enzyme C</th>
<th>Human enzyme B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>6.60 (6 or 7)</td>
<td>2.00 (2)</td>
<td>4.15 (4)</td>
</tr>
<tr>
<td>Serine</td>
<td>1.68 (2)</td>
<td>0.61 (1)</td>
<td>0.00 (1)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>2.10 (9)</td>
<td>2.00 (8)</td>
<td>3.08 (3)</td>
</tr>
<tr>
<td>L-Carboxymethyl histidine</td>
<td>1.00 (1)</td>
<td>1.00 (1)</td>
<td>1.00 (1)</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.00 (1)</td>
<td>1.00 (1)</td>
<td>1.00 (1)</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.20</td>
<td>1.00 (1)</td>
<td>1.00 (1)</td>
</tr>
<tr>
<td>3-Carboxymethyl histidine</td>
<td>1.00 (1)</td>
<td>1.00 (1)</td>
<td>1.00 (1)</td>
</tr>
<tr>
<td>Valine</td>
<td>2.02 (2)</td>
<td>1.00 (1)</td>
<td>1.16 (1)</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.80 (1)</td>
<td>0.65 (1)</td>
<td>0.66 (1)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.00 (1)</td>
<td>1.00 (1)</td>
<td>1.00 (1)</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.20 (1)</td>
<td>1.00 (1)</td>
<td>1.00 (1)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.70 (1)</td>
<td>1.00 (1)</td>
<td>0.50 (2)</td>
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<tr>
<td>Phenylalanine</td>
<td>0.99 (1)</td>
<td>0.66 (1)</td>
<td>2.08 (2)</td>
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<tr>
<td>Lysine</td>
<td>1.00 (1)</td>
<td>1.00 (1)</td>
<td>0.90 (1)</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.00 (1)</td>
<td>1.00 (1)</td>
<td>1.00 (1)</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.90 (1)</td>
<td>0.66 (1)</td>
<td>1.01 (1)</td>
</tr>
</tbody>
</table>

*Not corrected for destruction during hydrolysis.

**The presence of the -Ile-Ile bond causes the low yield even after 48 hours of hydrolysis.

Fig. 3 (left). Fractionation of the tryptic digest of alkylated bovine carbonic anhydrase B. Chromatography was performed on a column (1.8 × 150 cm) of Dowex 1-X2 maintained at 37°C. A linear gradient from 2000 ml of buffer (made up with 45 ml of N-ethylmorpholine, 60 ml of α-nicotine, 30 ml of pyridine adjusted to pH 9.4 with acetic acid in a volume of 3 liters) to 2000 ml of 0.5 N acetic acid was employed. At fraction 150 the elution was continued with 2 N acetic acid. The flow rate was 111 ml per hour, and 23.5-ml fractions were collected. Ninhydrin analysis was performed on aliquots from alternate fractions after alkaline hydrolysis using a Technicon Peptide Analyser. Radioactivity was measured on 0.5-ml samples taken from each fraction. The bar indicates the fractions that were pooled.

Fig. 4 (right). Fractionation of the chymotryptic digest of the peptide containing the radioactive label isolated from the tryptic digest of alkylated bovine carbonic anhydrase B. Chromatography was performed on a Dowex 50-X2 column (0.9 × 60 cm). The column was maintained at 37°C. The elution was initiated with 0.2 N acetic acid at pH 3.1. At fraction 25 the elution was continued with a linear gradient from 0.2 N pyridine acetate to 2.0 N pyridine acetate buffer, pH 4.9. The reservoir and the mixing chamber each contained 250 ml of buffer. The flow rate was 15 ml per hour and 2.5-ml fractions were collected. Ninhydrin analysis was performed on aliquots from alternate fractions after alkaline hydrolysis using a Technicon Peptide Analyser. Radioactivity was measured on 0.5-ml samples taken from each fraction. The bars indicate the fractions that were combined. From alternate fractions 0.3 ml was removed for ninhydrin analysis after alkaline hydrolysis and 0.1-ml samples were taken from each fraction for radioactivity measurement.
bovine carbonic anhydrase in a ratio of 1:0.92. One cycle of Edman degradation yielded glycine. The acid hydrolysate of the aqueous soluble fraction gave 3-carboxymethyl histidine on the analyzer, thus establishing the sequence of this peptide as Gly-His (modified).

**Fractionation of Chymotryptic Peptides from Tryptic Peptide—**
The chymotryptic digest of the tryptic peptide was fractionated on a Dowex 50 column (Fig. 4) with 81.6% recovery of radioactivity applied to the column. Of this, 70% appeared in Fraction II. Only Fraction III (C3) proved to be pure, as indicated by its amino acid composition (Table I). Fraction II (C2) was purified further by two-dimensional high voltage paper electrophoresis and paper chromatography (pH 6.4, Solvent I). Radioautography indicated two radioactive peptides with equal anodic mobility. Amino acid analysis showed that both peptides had identical amino acid compositions and they corresponded to the NH₂-terminal segment of the tryptic peptide. The two spots were eluted and when combined represented 37% of the radioactivity applied to the paper. Fraction III (C3) proved to be the COOH-terminal segment of the tryptic peptide.

**Radioautography indicated two radioactive peptides with equal anodic mobility.**

Amino acid analysis showed that both peptides had identical amino acid compositions and they corresponded to the NH₂-terminal segment of the tryptic peptide. The two spots were eluted and when combined represented 37% of the radioactivity applied to the paper. Fraction III (C3) proved to be the COOH-terminal segment of the tryptic peptide. The amino acid composition of peptides C2 and C3 accounted for that of the parent tryptic peptide (Table I).

**Amino Acid Sequence Studies of Peptide C2—** After the third and fourth Edman degradations the initially formed anilinothiohydantoin derivatives were converted to corresponding phenylthiohydantoin derivatives (26) and analyzed by thin layer chromatography (29). In both cases an asparagine derivative was identified. From this and from the four-step Edman degradation of the intact tryptic peptide the NH₂-terminus was identified as Met-Val-Asn-Asn-. Treatment of peptide C2 with carboxypeptidase A for 24 hours released phenylalanine and serine in a ratio of 1:0.55, establishing the COOH-terminal sequence as Ser-Phe. The amino acid sequence of peptide C2 is then Met-Val-Asn-Asn-Gly-His(modified)-Ser-Phe. The amino acid sequence of peptide C3 is then Met-Val-Asn-Gly-Ile-(modified)-Ser-Phe.

**Amino Acid Sequence Studies of Peptide C3—**
Four steps of the Edman cycle established the following sequence for the NH₂-terminal segment: Asx-Val-Glx-Tyr-. This sequence was confirmed by amino acid analysis of the residual peptide after the fourth cycle, which is shown in Table II. After two further Edman degradations the amino acid analysis of the residual peptide indicated the loss of 1.45 eq of aspartic acid residue (Table II). The 5th and 6th amino acids from the NH₂-terminus were also identified as aspartic acid by amino acid analysis of the acid hydrolysate of the phenylhydantoin derivatives formed at the fifth and sixth Edman cycle. Leucine aminopeptidase digestion for 3 hours of the intact peptide C3 released asparagine, valine, and glutamic acid, which were identified by two-dimensional high voltage electrophoresis-paper chromatography. These data gave the following NH₂-terminal sequence of peptide C3, Asn-Val-Glu-Tyr-Asn-. Due to the lack of sufficient C3 peptide no further sequence studies were attempted. The partial sequence of the tryptic peptide from bovine enzyme B is presented in Table III.

**Isolation and Identification of ¹⁴C-labeled Peptide from Human Carbonic Anhydrase C Alkylated with Bromo[¹⁴C]aceticazolamide**

A ¹⁴C-labeled peptide was isolated from the tryptic digest of alkylated human enzyme C by ion exchange chromatography. The radioactive fraction eluted from Dowex 1 resin (Fig. 5) was pooled and freeze-dried (yield 67%) and applied to a Dowex 50 column. Of the radioactivity applied to this column 77% was recovered in a single peak (Fig. 6). In a separate experiment the tryptic digest from approximately 5 mg of the alkylated enzyme was applied to paper and subjected to high voltage electrophoresis and chromatography (pH 6.4, Solvent I). A single spot was identified by radioautography. Its position on the paper was almost identical to that of the single radioactive peptide from the tryptic digestion of the alkylated bovine enzyme. The amino acid composition of the radioactive peptide isolated by the two different methods was found to be the same, indicating that essentially all the radioactivity resides in one peptide, as presented in Table I. The amino acid analysis showed some uncertainty concerning the number of aspartic acid and glycine residues. Nevertheless, it was in good harmony with the amino acid composition of the proposed sequence of human enzyme C between residues 58 and 76 (1). Göthe and Nyman (7) recently isolated a tryptic peptide from human enzyme C containing histidine-64. They reported an amino acid composition for this peptide which is closer to that presented in Table I than to the proposed sequence between residues 58 and 76 presented in Table III.

**Isolation and Identification of ¹⁴C-labeled Peptide from Human Carbonic Anhydrase C Alkylated with N-Bromo[¹⁴C]acetazolamide**

**Purification of ¹⁴C-labeled Tryptic Peptide—** The tryptic digest of human carbonic anhydrase B modified with N-bromo[¹⁴C]-

---

**Table I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Aspartic acid</th>
<th>Serine</th>
<th>Glutamic acid</th>
<th>Valine</th>
<th>Tyrosine</th>
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<tr>
<td>0</td>
<td>4.16</td>
<td>0.80</td>
<td>2.00</td>
<td>1.27</td>
<td>1.05</td>
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<tr>
<td>4</td>
<td>2.84</td>
<td>0.77</td>
<td>1.00</td>
<td>0.11</td>
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</tr>
<tr>
<td>6</td>
<td>1.39</td>
<td>0.80</td>
<td>1.00</td>
<td>0.16</td>
<td>0.17</td>
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</table>

**Table II**

<table>
<thead>
<tr>
<th>Edman degradation of peptide C3 from bovine carbonic anhydrase B</th>
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<tbody>
<tr>
<td>Step</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>6</td>
</tr>
</tbody>
</table>

**Table III**

<table>
<thead>
<tr>
<th>Amino acid sequence of tryptic peptides containing the reactive histidines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human carbonic anhydrase B past 60 Glu-Ile-Asn-Val-His-Asp-Glu-Asp-Val-Asn-Asn-Asp-Asp-Ser</td>
</tr>
<tr>
<td>Human carbonic anhydrase C past 60 Asn-Leu-Ile-Asn-Ile-Gly-Asp-Asp-Val-Asp-Asn-Asn-Asp-Ser</td>
</tr>
</tbody>
</table>

* These sequences are taken from earlier reports (1,8)
* Histidine that reacted with N-bromacetylcetazolamide.
** Histidine that reacted with bromoacetazolamide.
acetylacetazolamide was fractionated on a Dowex 1 column (Fig. 7). Of the radioactivity applied to the column, 85% was recovered in two fractions. Fraction I (T1) contained 39% and Fraction II (T2) contained 61% of the radioactivity. The radioactive peaks emerged from the column at positions similar to those of the radioactive peaks from the tryptic digests of alkylated bovine enzyme B and human enzyme C, indicating that in all three enzymes the same segment of the primary structure contained the reactive histidine. On the basis of the amino acid composition of peptides T1 and T2 (Table I) and the now known sequence of human enzyme B (8), we assigned peptide T1 to a stretch of the primary structure between residues 58 and 76 and peptide T2 to a stretch between residues 65 and 76 (Table III). Even after 48 hours of acid hydrolysis only 1 eq. of isoleucine was found in peptide T1, although there is an -Ile-Ile- sequence in the corresponding part of the primary structure of human enzyme B (1). Four steps of Edman degradation of peptide T1, however, confirmed the reported sequence as Glu-Ile-Ile-Asp-. The presence of 61% radioactivity and the corresponding amount of 1-carboxymethyl histidine in the acid hydrolysate of peptide T2 indicated that the modification of human enzyme B with N-bromoacetylacetazolamide occurred at least partially at histidine 67.

**Purification of N°C-labeled Chymotryptic Peptide from Peptide T1**

Since peptide T1 contained 1 eq. of histidine in addition to 1 eq of modified histidine, it was necessary to cleave it to smaller fragments to establish whether all the radioactivity was located at histidine 67, or part of it at histidine 64. Peptide T1 was therefore hydrolyzed with chymotrypsin and the hydrolysate separated on paper by the combination of high voltage electrophoresis and chromatography (pH 6.4, Solvent I). Two peptides with almost equal anodic mobility were identified by radioautography and eluted from the paper by 30% acetic acid (yield 28%). The amino acid composition of both peptides (Table I) corresponded to the sequence of residues 67 to 76 (Table III), showing that N-bromoacetylacetazolamide reacted exclusively with histidine 67.

**DISCUSSION**

The complete amino acid sequence of human enzyme B has become known recently. It was possible therefore to identify the histidine in this enzyme that reacted with N-bromoacetylacetazolamide simply on the basis of the amino acid composition of a tryptic and chymotryptic peptide that contained the alkylated histidine. In Table III we present the amino acid sequence of this enzyme between residues 58 and 76 as reported by Andersson et al. (8). Histidine 67 that reacted with N-bromoacetylacetazolamide is indicated by an asterisk.

The amino acid sequence of human carbonic anhydrase C is less well known. Based on the known sequential homology between human enzymes B and C, as well as the electron density map of human enzyme C, a partial amino acid sequence has been proposed for human enzyme C (1). This sequence between residues 58 and 76 is also presented in Table III. The amino acid composition of the tryptic peptide that contained the histidine alkylated with bromoacetazolamide is in complete agreement with this proposed sequence. The residue that reacted with bromoacetazolamide is tentatively assigned in the proposed sequence as histidine-64 and is indicated by a double asterisk.

The amino acid sequence of bovine enzyme B is known to only a very limited extent (1). On the basis of the known sequential homology among mammalian erythrocyte carbonic anhydrases (1), it seems justifiable to assign the sequence of the tryptic peptide that contain the alkylated histidine to a segment
between residues 59 and 76. This segment contains histidine-64 (indicated with a double asterisk in Table III) which reacted with bromoacetazolamide.

We reported earlier that bromoacetazolamide reacts significantly faster with human enzyme C and bovine enzyme B than with human enzyme B and horse enzyme B (6). The first two enzymes belong to the high activity forms and the latter two enzymes to the low activity forms of carbonic anhydrase. While the present studies indicate clearly that histidine-64 reacted with bromoacetazolamide in the two high activity forms, we do not know yet whether this reagent also reacted with histidine-64 in human enzyme B, or with another histidine at the active site region. Nevertheless it is clear that both groups of enzymes have a histidine residue in a sequentially homologous position, as we predicted (histidine-64) and that these histidines have a markedly different reactivity toward bromoacetazolamide (6). Göthe and Nyman (7) reported recently an observation that is somewhat similar to ours. They found that bromopyruvate alkylates histidine-64 in human enzyme C but in human enzyme B histidine-200 reacts instead. We defer further interpretation of these interesting observations until an investigation of the nature of the covalent interaction of human B enzyme with bromoacetazolamide, as well as the identification of the alkylated histidine in the primary structure, are completed in our laboratories.

It is tempting to assign some functional role to histidine-64, particularly since there is homology around it in the three carbonic anhydrases and since bovine enzyme B is completely inactive when alkylated with bromoacetazolamide at histidine-64. The evidence, however, is not convincing. Bromoacetazolamide is a reversible inhibitor of carbonic anhydrase. One molecule of the reagent while reversibly bound to the enzyme results in complete inhibition. If the covalently bound bromoacetazolamide binds to the active site in such a way as to present the bromine in close proximity to histidine-64, no role in the catalysis. On the other hand, the presence of histidine-64 in all three enzymes and the high homology around it is such a coincidence. The high residual activity of human enzyme C alkylated with bromopyruvate at the $\delta$-nitrogen of histidine-64 clearly indicates that this is the case, and also that this residue is not essential for the catalysis (7).

Complete inactivation of human enzyme C with bromoacetazolamide versus the partial inactivation of this enzyme with bromopyruvate by modification of the same histidine residue at the same nitrogen position demonstrates the difficulties inherent in the interpretation of an observed complete inactivation brought about by an affinity labeling reagent. This is particularly true when the reagent like bromoacetazolamide is a "fully reversible" type of inhibitor.

X-ray crystallography of human carbonic anhydrase C has revealed that in addition to three histidine residues that are liganded to the essential Zn atom, there are two other histidine residues in the active site region (32). One is histidine-64, the other histidine-129. The identification of histidine-64 at the active site by affinity labeling with bromoacetazolamide is in complete agreement with data provided by x-ray crystallographers. It conflicts, however, with their prediction that histidine-129 and not histidine-64 should react with bromoacetazolamide. This prediction was based on the electron density map of the human enzyme C-acetazolamide complex (10). Acetazolamide is structurally similar to bromoacetazolamide (3), the only difference being the replacement of a hydrogen atom of the methyl group in acetazolamide by a bromine in bromoacetazolamide. According to the electron density map the acetylamino nitrogen of acetazolamide is hydrogen bonded to the $\delta$-nitrogen of histidine-129, and the methyl group of the acetylamino moiety, which carries the bromine in bromoacetazolamide, is in close proximity to histidine-129 (10). Model-building studies seem to substantiate this prediction (33). We have to assume that either histidine-64 moved closer to the bromoacetyl group or the bromoacetyl group moved closer to histidine-64 for the reaction to occur. Another possibility is that the crystal structure of human enzyme C-acetazolamide complex is different from the solution structure of the same complex.

Only human carbonic anhydrase B has histidine-67 that reacted with $\Delta$-bromoacetylacetazolamide, which explains the complete inactivity of human enzyme C and bovine enzyme B toward this reagent. The sequentially homologous amino acids in the human C and bovine B enzymes are glutamine (32) and asparagine, respectively. This fact and the high residual activity of the modified enzyme makes it unlikely that this histidine, at least at the 1-nitrogen position, has a critical functional role in the catalysis. Some role other than functional is still possible, however, and we made earlier one observation that seems to lend support to this idea (3). We found that $K_a$, which in this case is probably equal to $K_i$ (34), is identical for both the native and the histidine-67 alkylated enzyme, indicating that the decreased activity is likely due to the actual bond making and breaking process (3). The replacement of a basic amino acid like histidine by an uncharged amino acid like asparagine and glutamine, although not an homologous replacement, is not a drastic change in a structural sense. All three of these amino acid side chains are potentially capable of forming a hydrogen bond, for example to the catalytically essential group in the enzyme, and thus holding it in the proper orientation.

X-ray crystallography has also revealed the presence of an organized water structure in the active site cavity, consisting of 9 water molecules (32). These water molecules are linked to each other and to the essential Zn atom by a network of hydrogen bonds. It is now assumed that one of the water molecules of the organized structure is the interacting species in hydrolytic and hydration reactions catalyzed by carbonic anhydrase (35). We are postulating that by hydrogen bonds to the organism...
water structure, the 64th amino acid residue, which is histidine in all three enzymes, as well as the 67th, which is histidine in human enzyme B (8), glutamine in human enzyme C (32), and asparagine in bovine enzyme B, would keep this organized water structure in the proper orientation for maximum catalytic activity. The electron density map of human carbonic anhydrase C has indeed indicated the possibility of a hydrogen bond between this water structure and histidine-64 and glutamine-67 (32). Modification of either of these amino acid residues could interfere with the hydrogen bonding, resulting in a less favorable orientation of the interacting water molecules and, consequently, a decrease in catalytic rate.

Histidine-67 has also been modified with N-chloroacetylchlorothiazide by Whitney et al. (2). The reaction, however, occurred at the 3'-nitrogen position and the modified enzyme was found to be completely inactive. The difference in the nature of the two alkylated enzyme species can easily be explained by the structural difference of the two reagents. N-Chloroacetylchlorothiazide, in addition to the chloroacetylated sulfonamide moiety, possesses a second sulfonamide group that after covalent bond formation can still be liganded to the essential Zn ion, replacing the Zn bound water molecule and resulting in complete inactivation. N-Bromoacetylacetazolamide no longer has such a group. This explanation, however, does not rule out the possibility that the 3'-nitrogen of histidine-67 may have some role in the catalysis.

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REFERENCES
