Inhibition of Human Erythrocyte Carbonic Anhydrase B by Chloroacetyl Sulfonamides with Labeling of the Active Site*

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SUMMARY

In an attempt to characterize the active site of human erythrocyte carbonic anhydrase B, we have studied the inhibition of this enzyme by several specific sulfonamide inhibitors and their N-chloroacetyl derivatives. In addition, two of the modified inhibitors were found to react irreversibly with the enzyme, resulting in inactivation. Enzymatic activity was determined from its catalytic hydrolysis of p-nitrophenyl acetate. The CO₂-hydrating activity was also determined in some experiments and paralleled the activity determined from the esterase assays.

N-Chloroacetylation at an amino group on the inhibitor far from the sulfonamide group resulted in little change or a decrease in the \( K_I \) values of the two inhibitors tested. Modification at the sulfonamide group abolished the inhibitory ability of two inhibitors, but two others retained their ability to inhibit the enzyme although the \( K_I \) values were higher. These are the only amide \( N \)-substituted sulfonamides that have been clearly shown to inhibit carbonic anhydrase reversibly. These two inhibitors, chloroacetylchlorothiazide and chloroacetylcyclothiazide, also reacted slowly with 1 eq of histidine per molecule of enzyme and caused complete, irreversible inactivation. After removal of the zinc atom from the enzyme, chloroacetylchlorothiazide failed to undergo this specific irreversible reaction. It is thought that the reactive histidine must be at or near the active site.

Carbonic anhydrases (carbonate hydro-lyase, EC 4.2.1.1) have been isolated from several sources, and in several cases

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more than one form of the enzyme has been obtained from a single source. For example, three forms (A, B, and C) have been isolated from human erythrocytes (1-3). The various forms have many properties in common in addition to their primary ability to catalyze the reversible hydration of carbon dioxide. They seem to have about the same molecular weight and over-all conformation (1-8). They contain 1 atom of zinc, which is essential for activity (1, 4). They catalyze the hydrolysis of esters, such as p-nitrophenyl acetate (3, 4, 9-11), and, in addition, the hydration of aldehydes, such as acetaldehyde (12) and pyridine aldehydes (13). They are specifically and powerfully inhibited by several sulfonamides (14 16). This latter property is the one exploited in these studies.

On the other hand, the enzymes differ in their physical, chemical, and catalytic properties and in their ability to bind the sulfonamide inhibitors. Thus, the study and comparison of these enzymes will hopefully result in at least a partial understanding of how the enzymes function in these catalytic reactions, in the specific binding and role of the single metal atom, and in the binding of the inhibitors.

We have prepared \( N \)-chloroacetyl derivatives of several sulfonamide inhibitors and studied their ability to inhibit and react with human erythrocyte carbonic anhydrase B. Two of these reversibly inhibit the enzyme and then react more slowly with a histidine residue to cause complete inactivation. This histidine may, therefore, be at or near the active site and may have some function in the catalytic reactions or in metal binding, or both.

In the companion paper (17) we report that bromoacetic acid and iodoacetamide are also reversible inhibitors which react irreversibly with a histidine residue and inactivate the enzyme.

EXPERIMENTAL PROCEDURE

Methods

Carbonic Anhydrase B—The enzyme was prepared from human erythrocytes by the procedure of Nyman (1).

Enzyme Activity—The activity of carbonic anhydrase was determined from the rate of hydrolysis of 0.4 mM p-nitrophenyl acetate at 25° in Tris-sulfate buffer (pH 7.6, ionic strength 0.075)
plus 1% acetone, by following the absorbance at 400 μm. Tris-
chloride buffer and 4% acetone were used in some of the early
experiments, but this was changed because chloride and acetone
are also inhibitors of enzymatic activity. The ester was dis-
solved in acetone (10 or 40 μm), and 100 or 25 μl were added to
the assay solution (2.50 ml) to start the reaction. The rate of
hydrolysis of the substrate by enzyme plus 1 mM acetazolamide
(Compound I) was subtracted as a blank to correct for non-
specific hydrolysis by the solvent and enzyme. The enzyme
concentration was generally about 3 to 4 μM.

R<sub>r</sub> Values of Sulfonamides—These were determined by thin
layer chromatography with Kieselgel G (Merck) and the solvent
methanol-water (65:25, by volume). The plates were developed with the chloro-iodide-starch method (18), which is positive for compounds with N-H bonds.

Nitrogen—Nitrogen was determined by a micro-Kjeldahl
method.

Amino Acid Analyses—Amino acids were analyzed (19) after
hydrolysis of the protein for 20 hours at 110° in 6 N HCl.
Carboxymethylhistidine was determined on samples which had been
oxidized with performic acid (20) before hydrolysis.

Synthesis of Modified Sulfonamides

Chloroacetazolamide—This compound (Compound III, 5-
chloroacetamido-1,3,4-thiadiazole-2-sulfonamide) was synthe-
sized by heating 5-amino-1,2,4-thiadiazole-2-sulfonamide (Com-
 pound II; Lederle Laboratories, American Cyanamid, Lot
5416R-29; R<sub>r</sub> 0.65; 0.9 g, 5 mmoles) to reflux for a few minutes
with chloroacetic anhydride (1.9 g, 12 mmoles) and chloroacetic
acid (2 g). After cooling, the product was recrystallized from
water. The yield was 88%; m.p. about 235° (reported m.p.
236-240° (21)); R<sub>r</sub> 0.76.

N-Chloroacetylchlorothiazide—Preparation of this derivative (Compound
VII; RF 0.76; 0.74 g, 2.5 mmoles) in 2 N sodium hydroxide (5 ml), adding
chloroacetyl chloride (0.57 g, 5 mmoles), and purifying the prod-
cuct as described above for Compound VIII. The yield was
86%; m.p. 250°; R<sub>r</sub> 0.65 for the dried sample and 0.3 for the wet
sample;

C<sub>10</sub>H<sub>18</sub>NO<sub>6</sub>S
Calculated: N 5.7%
Found: N 5.7%

N-Chloroacetylchlorothiazide—Preparation of this derivative (Compound
XII, 6-chloro-3,4-dihydro-3-(5-norbornen-2-yl)-7-N-chloro-
acetamido-1,2,4-benzothiadiazine 1,1-dioxide) was accomplished by dissolving
chlorothiazide (Compound IX; Lot 281592, Lilly; Compound XI;
R<sub>r</sub> 0.86) by the procedure used for Compound X. Thin layer
chromatography of the precipitate (84% yield, m.p. 270°) showed
some unreacted chlorothiazide plus another component (R<sub>r</sub> 0.36)
along with the main product (R<sub>r</sub> 0.60). The product was dis-
solved in cold aqueous sodium bicarbonate, precipitated with
hydrochloric acid, washed with water, and dried. This material
still contained small amounts of impurities.

C<sub>9</sub>H<sub>15</sub>N<sub>3</sub>O<sub>3</sub>S<sub>2</sub>
Calculated: N 9.0%
Found: N 8.7%

RESULTS

Synthesis—Ultraviolet and infrared spectra (notably the
position of the carbonyl absorption bands) and nuclear magnetic
resonance spectra of the compounds in Table I were in agreement
with the structures we have written, except for the nuclear
magnetic resonance spectrum of Compound XII. In this case,
the configuration at the optically active carbon atoms around the
bond to the norbornenyl group is complicated by the presence of the
endo and exo forms. Compound XI is probably about 80% endo and 20% exo.2 Furthermore, about 20% of the preparation of
Compound XII is N-chloroacetylated at a second position,
probably at nitrogen in position 4. The presence of these various
forms and derivatives of Compound XI may affect some of the
results of this paper quantitatively, but the qualitative conclu-
sions will not be seriously affected, because Compounds XI and
XII were present at nearly the same concentration as the en-
zyme. Hence the results must be due primarily to the major
component of these preparations.

Further evidence about the position of chloroacetylation was
provided from the conditions required for synthesis of the modi-
fied inhibitors. Under acidic conditions, substitution at the sul-
fonamide group was more difficult than at amino groups. Com-

2 We are indebted to Dr. H. Buzs, Eli Lilly and Company, for
the interpretation of the nuclear magnetic resonance spectrum of
Compound XI leading to this conclusion.
**TABLE I**

Inhibition of carbonic anhydrase B by sulfonamide inhibitors and their N-chloroacetyl derivatives

The dissociation constants for reversible inhibition ($K_r$) of these compounds were determined with the esterase activity at 25° in Tris-sulfate buffer, pH 7.6, and 4% acetone. The sample of Diamox contained about 1.5 eq of sodium and 1 eq of water to give an equivalent weight of about 274.

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Chemical name</th>
<th>Structure</th>
<th>Weight</th>
<th>$K_r \times 10^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Acetazolamide (Diamox)</td>
<td><img src="#" alt="Structure" /></td>
<td>222.3</td>
<td>0.4</td>
</tr>
<tr>
<td>II</td>
<td>Aminothiadiazole</td>
<td><img src="#" alt="Structure" /></td>
<td>180.1</td>
<td>0.4</td>
</tr>
<tr>
<td>III</td>
<td>Chloroacetazolamide</td>
<td><img src="#" alt="Structure" /></td>
<td>256.7</td>
<td>0.6</td>
</tr>
<tr>
<td>IV</td>
<td>Sulfanilamide</td>
<td><img src="#" alt="Structure" /></td>
<td>172.2</td>
<td>19</td>
</tr>
<tr>
<td>V</td>
<td>N-Chloroacetyl-sulfanilamide</td>
<td><img src="#" alt="Structure" /></td>
<td>248.7</td>
<td>0.4</td>
</tr>
<tr>
<td>VI</td>
<td>N,N'Dichloroacetysulfanilamide</td>
<td><img src="#" alt="Structure" /></td>
<td>325.2</td>
<td>&gt;1,000 (if any)</td>
</tr>
<tr>
<td>VII</td>
<td>p-Toluensulfonamide</td>
<td><img src="#" alt="Structure" /></td>
<td>171.2</td>
<td>0.5</td>
</tr>
<tr>
<td>VIII</td>
<td>N-Chloroacetyl-p-toluensulfonamide</td>
<td><img src="#" alt="Structure" /></td>
<td>247.7</td>
<td>&gt;10,000 (if any)</td>
</tr>
<tr>
<td>IX</td>
<td>Chlorothiazide</td>
<td><img src="#" alt="Structure" /></td>
<td>295.8</td>
<td>20</td>
</tr>
<tr>
<td>X</td>
<td>N-Chloroacetylcyclothiazide</td>
<td><img src="#" alt="Structure" /></td>
<td>372.3</td>
<td>500</td>
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<tr>
<td>XI</td>
<td>Cyclothiazide</td>
<td><img src="#" alt="Structure" /></td>
<td>389.9</td>
<td>0.6</td>
</tr>
<tr>
<td>XII</td>
<td>Chloroacetylcyclothiazide</td>
<td><img src="#" alt="Structure" /></td>
<td>466.4</td>
<td>65</td>
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</tbody>
</table>

Compounds VII and IX were recovered unchanged after heating with an excess of chloroacetic anhydride, whereas the amino nitrogens of Compounds II and IV were acylated under these conditions. Compounds VIII and X could only be synthesized in alkaline aqueous solutions, where dissociation of a proton from the sulfonamide nitrogens occurs to form the nucleophiles RSO₂NH⁻.

As would be expected, the chloroacetyl group of Compound X is converted to glycine in concentrated aqueous ammonia during 3 days at room temperature, followed by evaporation to
dryness under vacuum and acid hydrolysis in 6 N HCl at 110° for 18 hours.

Reversible Inhibition—The $K_I$ values for these inhibitors are given in Table I. These were calculated on the assumption that inhibition is noncompetitive with the substrate, since the substrate concentration is only about 0.1 $K_m$ and since previous reports on sulfonamide inhibition of carbonic anhydrase have shown that the inhibition appears to be noncompetitive (3). It should also be pointed out that the $K_I$ values of sulfonamide inhibitors increase with increasing pH (8).

Substitution at the sulfonamide groups, to give Compounds VI, VIII, X, and XII, results in a marked increase in the $K_I$ values or the disappearance of inhibitory capability. Determination of the $K_I$ values for these modified inhibitors was complicated by the presence of small amounts of the corresponding strongly inhibitory unmodified inhibitor. Since, in the case of Compounds VI and VIII, the $K_I$ of the unmodified inhibitor was lower than the concentration of enzyme, the inhibition followed a different equation and was determined at several inhibitor and enzyme concentrations. The inhibition could be accounted for by the presence of about 1.2% unmodified inhibitor in the preparation of Compound VIII. Recrystallizations of these preparations did not affect the results from Compound VI, but did lower the amount of unmodified inhibitor required to account for the inhibition by the preparation of Compound VIII to only 0.06%. Data showing the lack of inhibition by Compound VIII are given in Table II. The possibility that Compounds VI and VIII can inhibit at high concentrations is not ruled out, but it is difficult to test because of the limits of solubility and purification.

Inhibition by the preparations of Compounds X and XII was even more difficult to evaluate, and the values of $K_I$ in Table I are only estimates. However, the inhibition cannot be completely accounted for by the presence of unmodified inhibitor. Strong confirmation that these compounds are reversible inhibitors is provided by the fact that they are irreversible inhibitors.

Irreversible Inactivation—The results with these modified inhibitors are shown in Fig. 1. Compounds III, V, VI, and VIII cause no significant loss of activity whereas Compounds X and XII do. In the case of Compounds III and V, there is binding to the enzyme, but no irreversible inactivation. Apparently they are bound in a way which does not allow interaction with modifiable groups on the enzyme. Compounds VI and VIII probably do not bind to the enzyme (at least they are not inhibitors) and do not react with the enzyme to any significant extent. However, Compounds X and XII apparently do bind to the enzyme in a way which allows the chloroacetyl group to react with the enzyme and cause irreversible inactivation. The ability to catalyze the hydration of CO$_2$ is also lost during the reaction. After acid hydrolysis of the inactivated enzyme, 1 eq of 3’-carboxymethylhistidylhistidylhistidine is found (acid hydrolysis results in cleavage of the sulfonamide N-acyl bond to leave only the carboxymethyl group attached to histidine). Fig. 1 also shows the amount of 3’-carboxymethylhistidylhistidine found after 4 days of reaction with these compounds.

In order to test the specificity of this binding and inactivation, metal-free carbonic anhydrase B was allowed to react with 1 mm Compound X for 4 days. Only 0.1 eq of 3’-carboxymethylhistidine was found. In another experiment, the enzyme was first allowed to react with bromoacetic acid to specifically carboxymethylate 1 histidine at the 3’ nitrogen (17). After 4 days of reaction with Compound X, the amount of 3’-carboxymethylhistidine increased from 0.88 to 1.10 eq.

**Discussion**

Since carbonic anhydrase, like chymotrypsin, catalyzes the hydrolysis of p-nitrophenyl acetate, we attempted to apply to this enzyme some of the reagents and techniques which had been useful in many studies of the active site of chymotrypsin. How-

**Table II**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>$E_o = 3.08 \mu M$</th>
<th>$E_o = 1.54 \mu M$</th>
<th>$E_o = 0.72 \mu M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_I$</td>
<td>$R \times 10^1$</td>
<td>$K_I$</td>
<td>$R \times 10^1$</td>
</tr>
<tr>
<td>1.0</td>
<td>0.87 2 6.8 4.7</td>
<td>2.0</td>
<td>0.68 2 4.4 6.0</td>
</tr>
<tr>
<td>2.0</td>
<td>0.35 2 3.1 6.5</td>
<td>3.0</td>
<td>0.50 2 6.9 3.1</td>
</tr>
<tr>
<td>3.0</td>
<td>0.45 2 4.4 6.0</td>
<td>4.0</td>
<td>0.55 2 6.9 3.1</td>
</tr>
</tbody>
</table>

**Fig. 1.** Inactivation of carbonic anhydrase B by N-chloroacetyl-p-toluenesulfonamide (Compound VIII).
ever, in general there seems to be little in common between the properties of the active sites of these two enzymes. One equivalent of diisopropyl fluorophosphate (23) or diphenylcarbamyl chloride (24) reacts at the active site of chymotrypsin and causes complete inactivation, but these reagents do not seem to interact with carbonic anhydrase (10). Cyanate readily inactivates chymotrypsin (25), but it did not cause any irreversible inactivation of carbonic anhydrase, although it is a good reversible inhibitor. Several sulfonyl fluorides (26) and a sulfonyl chloride (27) can sulfonate the active site of chymotrypsin, but methyl- and benzzenesulfonyl chlorides were instead found to be fairly good substrates of carbonic anhydrase.

Although the reagents which were used successfully with chymotrypsin did not react with carbonic anhydrase, valuable results were obtained with the technique of using reagents which have specificity for the active site plus a group capable of reacting with an amino acid side chain (28, 29). Many sulfonamides are known to inhibit carbonic anhydrase strongly. We added a chloroacetyl group to some of these inhibitors and tested their ability to inhibit or react with the enzyme.

As is evident from Table I, chloroacetylation of amino groups which are far from the sulfonamide group (Compounds I and IV) results either in little change or in a decrease in $K_I$. Substitution at the sulfonamide group (to give Compounds VI, VIII, X, and XII), on the other hand, causes a drastic increase in $K_I$. Previous reports have made it questionable whether carbonic anhydrase can really be inhibited by substituted sulfonamides. Mamm and Keilin (14) tested the inhibition of carbonic anhydrase by several sulfonamides and concluded that an unsubstituted sulfonamide (RSO$_2$NH$_2$) was required for inhibition. Krebs (15) did find some inhibition by substituted sulfonamides, but the $K_I$ was 100 to 1000 times higher than for the unsubstituted compound. Maren (30) and Maren and Wiley (31) also found some inhibition by preparations of substituted sulfonamides, but concluded that this was due to contamination by small amounts of unsubstituted inhibitor. The results obtained here with Compounds X and XII show that they can inhibit, although the $K_I$ is much higher than that of the corresponding unsubstituted inhibitor.

At the concentrations used in these experiments, the chloroacetyl group on the modified inhibitors is not reactive enough to result in much nonspecific reaction with groups on the enzyme. Two of the modified sulfonamides which can act as reversible inhibitors, namely Compounds X and XII, are able to react irreversibly with a histidine side chain and to inactivate the enzyme completely. This is believed to be due to a binding of Compounds X and XII to the protein in a way which places the chloroacetyl group next to a histidine residue on the enzyme. This would greatly accelerate the reaction and thus lead to the specific modification of the side chain. It is highly probable that Compounds X and XII bind at the active site and, if so, the histidine is then at or near the active site. There are several reasons for believing this.

1. The fact that Compounds X and XII inhibit the enzyme when they bind reversibly and cause complete inactivation after reacting with the histidine strongly suggests that the binding occurs at the active site.

2. It is almost certain that zinc is part of the active site. Studies have shown that the metal is required for the strong binding of sulfonamides (32), even though the over all conformation of the enzyme does not change very much upon removal of the metal (33, 34). The failure to obtain the specific irreversible binding of Compound X to the metal-free enzyme indicates that the metal also is required for reversible binding in the case of an inhibitor with a substituted sulfonamide group. This points toward a similar mode of binding for substituted and unsubstituted sulfonamides.

3. The replacement of the zinc ion of the native protein with divalent cobalt gives a catalytically active protein with an absorption spectrum in the visible region (32). The binding of a sulfonamide inhibitor to the Co(II)-enzyme is accompanied by drastic changes in the visible absorption spectrum; this strongly indicates a binding of the inhibitor near the metal. The Co(II)-enzyme seems to constitute a suitable system in general for studying changes in the neighborhood of the metal, but this has not yet been utilized in the case of Compound X.

4. The model of human carbone anhydrase C which has been constructed from 5.5-A x-ray crystallographic data shows that sulfonamide inhibitors bind near the zinc atom in a crevice which is probably in the active site region (35). It is also interesting that Compound X could easily fit into this crevice so as to place the chloroacetyl group near the zinc. Because of the similarity of protein structure and sulfonamide inhibition between the two enzymes, it is possible that the inhibitors are bound to the B form of carbonic anhydrase in a similar fashion. It must be noted that Compounds X and XII could be regarded as having two substituted sulfonamide groups, the second one in the heterocyclic ring. In the unmodified inhibitors, the unsubstituted sulfonamide group undoubtedly has a very important role for the binding and orientation of the molecule. In the case of the modified compounds, a similar decision cannot be made at present, and this means that the orientation of Compounds X and XII when bound to the enzyme need not necessarily be similar to that of the corresponding unmodified inhibitor.

It is interesting, however, that Compound XI binds more strongly than Compound IX, and the derivative of Compound XI binds more strongly than the derivative of Compound IX. That is, the norbornenyl group strengthens the binding of both Compound XI and its derivative. This suggests that substitution of the sulfonamide group may not greatly change the binding position and orientation.

5. Bromoacetate, iodoacetamide, and Compounds X and XII have quite different physical properties, but all reversibly inhibit enzymatic activity and inactivate the enzyme by reacting with
the 3' nitrogen of a histidine (17). After bromoacetate has modified a single histidine residue, Compound X no longer reacts with the enzyme at a significant rate. A simple explanation for this competitive behavior is that the compounds react with the same histidine. Another explanation would be that they react with different histidines and that the modification with bromoacetate causes a change in the binding site for Compound X. The latter possibility is supported by preliminary results from primary structure investigations on modified enzyme, which we hope will also provide further information on the structure of the active site.

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REFERENCES
Inhibition of Human Erythrocyte Carbonic Anhydrase B by Chloroacetyl Sulfonamides with Labeling of the Active Site
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