Monocarboxamidomethyl Carbonic Anhydrase Purified by Affinity Chromatography*

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SUMMARY

A monocarboxamidomethyl derivative of human erythrocyte carbonic anhydrase B was purified by affinity chromatography. The modified enzyme possesses 3% of the CO₂-hydrating activity and 30% of the esterase activity of the native enzyme. The esterase activity is inhibited by the usual carbonic anhydrase inhibitors although the $K_i$ values are, in general, higher than for native enzyme. The pH dependence of esterase activity for modified enzyme has a $pK_a$ of 8.3 compared to 7.3 for native enzyme.

Zinc dissociates so slowly from modified enzyme that it would take about 3 months to remove 90% of the metal. After half of the metal was removed from the enzyme, pure apoenzyme was separated by affinity chromatography.

Carboxamidomethyl Co²⁺-enzyme was prepared from apoenzyme and its visible spectrum was determined at several pH values. The general shapes of the spectra were similar to those for native Co²⁺-enzyme. However, the major pH-dependent transition has a $pK_a$ of 8.3 compared to 7.3 for native enzyme. In addition, a secondary pH-dependent transition which occurs in native enzyme below pH 7 is absent in the modified enzyme, and a transition with a $pK_a$ of 10.8 occurs in modified enzyme but is absent in native enzyme.

MATERIALS AND METHODS

The insoluble matrix for affinity chromatography of carbonic anhydrase was prepared by coupling p-aminomethylbenzene sulfonamide to Sepharose 4B by procedures similar to those of Cuatrecasas et al. (9). Two large batches were employed. The second batch bound enzyme with a higher affinity than the first batch.

Human erythrocyte carbonic anhydrase B was prepared by chromatography on DEAE-Sephadex (10). It was further purified by affinity chromatography. The second batch bound enzyme with a higher affinity than the first batch.

Human erythrocyte carbonic anhydrase B was prepared by chromatography on DEAE-Sephadex (10). It was further purified by affinity chromatography. The next step removed inactive protein which amounted to about 1% of the total protein. Co²⁺-enzyme was prepared as described before (8), except that the apoenzyme was purified by affinity chromatography.

Carboxamidomethyl carbonic anhydrase B was prepared by reaction with 25 mM iodoacetamide in 50 mM Tris sulfate buffer, pH 7.1, for 16 hours at room temperature in the dark. The reaction was terminated by dialysis in the cold. Carboxamidomethyl carbonic anhydrase B was prepared by reaction with 2 mM sodium bromoacetate at pH 7.1 for 8 hours at room temperature followed by affinity chromatography under conditions as given for chromatography of carboxamidomethyl enzyme in Fig. 1B.

The concentration of enzyme in solution was determined from its absorbance at 280 nm using a value of 48,900 $M^{-1} cm^{-1}$ as the extinction coefficient (11).

* This work was supported by Grant GB-26444 from the National Science Foundation.

1 Unpublished results. Details will be reported later.
The visible spectra of Co²⁺-enzymes and the esterase activity were determined as described previously (8).

CO₂-hydrating activity was assayed at 3⁰ by the method of Wilbur and Anderson (12). A small volume of enzyme solution (0.1 ml) was added to 3 ml of buffer (25 mM sodium barbital-10 mM H₂SO₄, pH 8.2) plus 2 drops of 0.1% sodium bromothymol blue solution. The rest of the assay and calculation of activity were as by Rickli et al. (18).

Samples for amino acid analysis were hydrolyzed in 6 N HCl at 110° for 22 hours. The HCl was removed by flash evaporation and the samples were oxidized with performic acid. Amino acid analyses were done with a Beckman 120C analyzer by Dr. J. Ryan, Department of Medicine, University of Miami School of Medicine.

Iodoacetamide, bromoacetate, and p-nitrophenyl acetate were purified as described previously (8).

Most of the p-aminomethylbenzene sulfonamide used in these studies was Sulfamylon hydrochloride, a generous gift from Dr. S. Archer at the Sterling-Winthrop Research Institute, Rensselaer, New York; some was from Aldrich. Both were used without purification and gave the same value of $K_i$ for carbonic anhydrase B.

RESULTS

Purification of Carboxamidomethyl Carbonic Anhydrase—The reaction of iodoacetamide with carbonic anhydrase B was stopped when, on the basis of loss of esterase activity, about two-thirds of the enzyme was modified. Affinity chromatography was employed to separate the modified enzyme from unreacted enzyme. Results of chromatography of 100 mg of enzyme are shown in Fig. 1A. One or 2% of the protein emerged at the breakthrough volume of the column. This protein had little activity and was probably denatured enzyme. Carboxamidomethyl enzyme was retarded somewhat and emerged about 60 ml later. The peak of carboxamidomethyl enzyme was severely skewed and some of the modified enzyme was eluted at the front of the second buffer (160 ml). This was closely followed by a sharp peak of unreacted enzyme.

The proteins in the two major peaks were characterized by amino acid analysis and activities. Amino acid analysis of the modified enzyme gave a value of 0.99 residue of telecarboxamidomethyl histidine per mole of enzyme whereas there was none detected in unreacted enzyme. Specific CO₂-hydrating activities of modified enzyme and unreacted enzyme were 890 and 12,900, respectively, compared with 12,800 for native enzyme. Specific esterase activities at pH 7.6 in terms of $A_{280}$, for 1 μM enzyme were 0.023 and 0.207 for modified and unreacted enzymes, respectively, compared to 0.208 for native enzyme. Therefore, unreacted enzyme is identical with native enzyme and carboxamidomethyl enzyme has 3% CO₂-hydrating activity and 11% esterase activity compared with native enzyme.

I wish to make two comments on the separation and shape of the last two peaks in Fig. 1A. The reason the two peaks are separated is that the pH of the eluant does not decrease immediately as the second buffer emerges. First it increased to about 8.3 (because of the higher ionic strength) and then gradually decreases to pH 6 in about 40 to 50 ml. The modified enzyme is eluted by the acetate and perchlorate above pH 8, but unreacted enzyme is not eluted until the pH decreases.

The elution behavior of the modified enzyme can be markedly changed by varying the conditions of chromatography. The experiment in Fig. 1A was carried out at room temperature. At 4⁰, the carboxamidomethyl enzyme is completely bound by the column in 5 mM Tris sulfate-22 mM Na₂SO₄, pH 7.6. After 100 ml, the buffer was changed to 10 mM sodium phosphate-20 mM Na₂SO₄, pH 6.5. The flow rate was about 20 ml per hour and the temperature was 4⁰. This run was stopped before elution of unreacted enzyme.

The above experiments were carried out with a preparation of affinity gel which has relatively low affinity for carbonic anhydrase. A column of gel with high affinity was also used (Fig. 1B). The modified enzyme is completely retained by the column in Tris buffer at pH 7.6 and is eluted in a sharp peak in 10 mM sodium phosphate-20 mM Na₂SO₄, pH 6.5.

Preparation of Carboxamidomethyl Co²⁺-enzyme—Only about half of the zinc was removed from carboxamidomethyl enzyme when it was dialyzed against p-phenanthroline for 3 weeks. In order to remove the remaining Zn²⁺-enzyme from apoenzyme, the preparation was chromatographed on a column of gel with high affinity (Fig. 2). The apoenzyme emerged at the breakthrough volume of the column and the carboxamidomethyl enzyme was not eluted until the buffer was changed to 10 mM sodium phosphate-20 mM Na₂SO₄, pH 6.5.

Co²⁺-carboxamidomethyl enzyme was prepared from apoenzyme by adding 1 eq of CoSO₄ per mole of enzyme. The rate of association of Co²⁺ with the active site was slow. About 1 hour was required for complete binding to occur.
FIG. 2. Separation of carboxamidomethyl carbonic anhydrase B from its apoenzyme. A preparation of apoenzyme of carboxamidomethyl enzyme (100 mg) was run on the same column of affinity gel used in Fig. 1B. The starting buffer was 10 mM Tris sulfate-20 mM Na₂SO₄, pH 7.1, with 0.05 mM EDTA. After 180 ml, the buffer was changed to 10 mM sodium phosphate-20 mM Na₂SO₄, pH 6.5.

FIG. 3. pH dependence of the visible spectrum of carboxamidomethyl Co²⁺-carbonic anhydrase B. The concentration of enzyme was 410 μM and the solvent was 25 mM Na₂SO₄. Below pH 9.5, the solution was titrated with 0.2 M NaOH. Higher pH was reached with 1 M NaOH. The spectra were recorded at 25°C.

Spectral Properties of Carboxamidomethyl Co²⁺-enzyme—The spectrum of carboxamidomethyl Co²⁺-carbonic anhydrase was determined at several pH values and the results are shown in Fig. 3. The initial pH of this solution of Co²⁺-enzyme was 6.5 and the spectrum at that pH was nearly the same as at pH 7.1. In another experiment the initial pH was 6.0, and little change in the spectrum occurred between pH 6.0 and 7.1. It is evident that no significant transition occurs between pH 6 and 7.

Carboxamidomethyl Co²⁺-enzyme becomes somewhat unstable as the pH is increased above pH 9. This instability is evident from a time-dependent decrease in absorbance between 500 and 650 nm and an increase in absorbance below 500 nm. These time-dependent effects are slow enough to be minimized by working rapidly. Small time-dependent effects were noted in the curve at pH 11.4 in Fig. 3, but they are not large enough to have a significant effect on the results.

The spectra for native and modified Co²⁺-enzymes at pH 6.0 and 11.4 are presented in Fig. 4. At low pH, the absorbance for the modified Co²⁺-enzyme around 650 nm is higher than for the native Co²⁺-enzyme. At high pH, the most notable differences are the lower absorbance around 640 nm and the higher absorbance around 585 and 520 nm.

The pH dependence of these spectral changes is determined by following the increase in absorbance near 640 nm as the pH is increased. The results for native and modified Co²⁺-enzymes are shown in Fig. 5. The results were fitted with theoretical titration curves with a pK of 7.3 for native enzyme and a pK of 8.3 for the major transition of the modified enzyme. A second transition at higher pH is evident in the case of the modified enzyme, and a curve (the dashed line) with a pK of 10.8 was drawn through these points. The absorbance of the native enzyme increased slightly in this pH region, but this is a small general increase over the entire spectrum in contrast to the large change in form which occurs in the spectrum of the modified enzyme.

Esterase Activity of Carboxamidomethyl Carbonic Anhydrase—As noted above, the carboxamidomethyl enzyme is 17% as active as native enzyme at pH 7.6. The pH dependence of this residual activity was investigated and the results are presented in Fig. 5. The pH dependence of esterase activity, like that of absorbance at 643 nm for carboxamidomethyl Co²⁺-enzyme, follows the theoretical titration curve with a pK of 8.3. The esterase activity observed for the modified enzyme at high pH amounts to about 30% of the activity of native enzyme at the same pH.

Inhibition of the residual esterase activity by several inhibitors was determined at pH 7.6. In addition, inhibition of native and carboxymethyl enzymes by these inhibitors was also studied for purpose of comparison. (Table I.)

The Kᵢ values for carboxamidomethyl enzyme are generally higher than for native enzyme. However, there is no significant change in Kᵢ for acetazolamide, and the Kᵢ for bromoacetate is
lower than that for native enzyme. The effect of the carboxy-
methyl group is qualitatively the same as the carboxamidomethyl
group, but the effect of the negatively charged group is about five
times as large.

Some of the $K_i$ values for native and carboxymethyl enzyme
which have been published (8, 14) differ significantly from the
values reported here. In such cases, the $K_i$ was checked using
fresh inhibitor solutions and different enzyme preparations.

**DISCUSSION**

Reaction of iodoacetamide with human erythrocyte carbonic
anhydrase B results in the introduction of a perturbing group at
the active site. The reagent reacts with a histidyl residue (His
204) which is located near the active site (1) to give a modified
enzyme which has lower catalytic activity and lower affinity for
most inhibitors. The modified enzyme was studied in an attempt
to explain the effects of the added carboxamidomethyl group and
gain insight into the chemical nature of the active site.

**Carboxamidomethyl Co²⁺-enzyme**—Co²⁺-enzymes are prepared
by removing the zinc ion to give apoenzyme followed by addition
of Co²⁺. Whereas the zinc can be removed from native enzyme
almost completely by dialysis against α-phenanthroline for 5 to
10 days, less than half of the zinc was removed from carbox-
amidomethyl enzyme during 3 weeks of dialysis. It may be
questioned why zinc dissociates from carboxamidomethyl enzyme
so slowly. The proposed mechanism for removing the metal is
via a ternary complex of enzyme-metal-chelator (1). One possi-
ability is that the carboxamidomethyl group could slow the re-
moval of metal by inhibiting formation of the ternary complex.
A second possibility is that the perturbing group sterically hinders
the entrance and exit of the metal ion. A third possibility is
that there may be a change in enzyme conformation which accom-
panies the entrance and exit of the metal and that the added
carboxamidomethyl group hinders this change in conformation.
The first possibility appears incorrect since the rate of association
of metal ion (without chelator) with the modified apoenzyme is
at least an order of magnitude slower than with native apoen-
zyme.

It is interesting to compare the exchange rate of metal ion and
carboxamidomethyl enzyme with that of another similar modified
enzyme, carboxymethyl carbonic anhydrase B. Although the
carboxamidomethyl group is located at the same site (5) as the car-
boxamidomethyl group and the two groups are about the same
size, the metal ion exchange rate for carboxamethyl enzyme ap-
pears to be about the same as for native enzyme.

**Spectral Properties of Co⁺⁺-enzyme**—The shape of the spectral
curves, the pK of the major pH-dependent transition, and the
type of secondary pH-dependent transitions are all changed to
some extent upon introduction of the carboxamidomethyl group.

Above pH 11, the spectrum of the carboxamidomethyl Co⁺⁺
enzyme is similar to that of the native Co⁺⁺-enzyme and nearly
the same as those for carboxymethyl Co⁺⁺-enzyme (8, 15) and,
surprisingly, Co⁺⁺-carbonic anhydrase C (11, 15). At low pH, the
shape of the spectra for native and carboxamidomethyl Co⁺⁺-enzymes
are about the same but the absorbance of the modified enzyme is
higher.

The difference in absorbance at low pH is due to the presence
of a secondary pH-dependent transition in native Co⁺⁺-enzyme
which is absent in the modified enzyme. This secondary transi-
tion causes an over-all increase in absorbance of the visible spec-
trum of native Co⁺⁺-enzyme as the pH increases from 6 to 7 (8).
This secondary transition is also observed in the carboxamethyl
Co⁺⁺-enzyme (8).

In contrast, at high pH the carboxamidomethyl Co⁺⁺-enzyme
undergoes a secondary pH-dependent transition which is not ob-
served in the native or carboxymethyl enzymes. This transition
has a pK of 10.8 and causes marked changes in the shape of the
spectral curve (note the differences between the spectra at pH
10.0 and 11.4 in Fig. 3). Without this secondary transition, the
spectrum of the carboxamidomethyl enzyme at high pH would
not be so similar to those of the native or carboxymethyl en-
zymes.

The pH-dependent secondary transition which occurs in the
carboxamidomethyl enzyme reflects changes in the orientation
or environment of ligands to the cobaltous ion in the active site.
The basis for this transition must be the titration of one or more

**TABLE I**

Inhibition of native and modified carbonic anhydrases

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ Values</th>
<th>Native enzyme</th>
<th>Cam enzyme</th>
<th>Cam/active</th>
<th>Cm enzyme</th>
<th>Cm/active</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>mM</td>
<td></td>
<td></td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td>Acetazolamide</td>
<td>0.0005</td>
<td>0.0004</td>
<td>1.3</td>
<td>0.0017</td>
<td>6</td>
<td>6</td>
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<tr>
<td>Sulfamylon</td>
<td>0.0059</td>
<td>0.75</td>
<td>130</td>
<td>5.0</td>
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<td>850</td>
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<tr>
<td>Sulfanilamide</td>
<td>0.0004</td>
<td>0.37</td>
<td>40</td>
<td>2.0</td>
<td>210</td>
<td>210</td>
</tr>
<tr>
<td>Chloride</td>
<td>15</td>
<td>110</td>
<td></td>
<td>7 400</td>
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<td>27</td>
</tr>
<tr>
<td>Bromide</td>
<td>3.6</td>
<td>17</td>
<td>5</td>
<td>60</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Iodide</td>
<td>0.6</td>
<td>1.8</td>
<td>3</td>
<td>9</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Bromoacetate</td>
<td>4.4</td>
<td>2.5</td>
<td>0.6</td>
<td>21</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Idoacetamide</td>
<td>25</td>
<td>80</td>
<td>3</td>
<td>280</td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>
groups of the enzyme with a pK near 10.8. Groups most likely to be involved would be tyrosyl or lysyl side chains. Since the pK is in the normal region for tyrosine, the effect is probably not due to ionization of a buried tyrosyl residue.

One other major difference between the spectral properties of native and carboxamidomethyl Co\textsuperscript{2+}-enzymes is the pK of the major pH-dependent transition. The major transition for native enzyme has a pK of 7.3, and this is shifted to 8.3 for carboxamidomethyl enzyme. In comparison the major transition for carboxymethyl enzyme has a pK of 9.2 (8). The explanation given for the increase in pK for carboxyethyl enzyme was that it was due to the added negative charge which suppressed the dissociation of the proton from the group which causes this transition (8). In light of the fact that introduction of the uncharged carboxamidomethyl group causes the pK to shift from pH 7.3 to 8.3, the presence of the charge can only explain part of the shift in pK for carboxamidomethyl enzyme. This conclusion was also reached by Taylor and Burgen on the basis of kinetic evidence (16).

The pH dependence of esterase activity of carboxamidomethyl enzyme has the same pK as the major transition of the spectrum of the Co\textsuperscript{2+}-enzyme. This is also the case with native (3, 11, 14) and carboxymethyl (8, 15) enzymes. This implies that the pH dependence of these two properties is due to the ionization of the same group in the active site of the enzyme.

Inhibition of Esterase Activity—Iodoacetamide appears to react with carbonic anhydrase B while it is bound as a reversible inhibitor. As a reversible inhibitor, iodoacetamide completely inhibits enzymic activity and competes with the binding of other inhibitors such as chloride and sulfanilamide (8). After reaction, the carboxamidomethyl group would be expected to lie near the reversible binding site and stop enzymic activity and the binding of inhibitors. In fact, the modified enzyme has substantial esterase activity and this is inhibited by the usual carboamide inhibitors. This shows that the iodoacetamide must move at least part way out of its reversible binding site upon reaction with His 204. A similar conclusion was reached for the reaction of bromoacetate with His 204 (8, 15).

Although inhibitors do bind to the modified enzyme, the carboxamidomethyl group influences their binding affinities. For instance, the $K_i$ for sulfanilamide is 40 times higher for modified enzyme than for native enzyme. This effect does not seem to be simply a steric one since $K_i$ for acetazolamide is not affected significantly even though this inhibitor is as large as sulfanilamide.

The anionic inhibitor whose binding is least affected is bromoacetate. This is not unreasonable since it was previously shown that bromoacetate and iodoacetamide can both bind to the native enzyme at the same time (8).

**Effects of Carboxamidomethyl Group**—It is apparent that the carboxamidomethyl group perturbs many different properties of the active site. This includes changes in the pK of groups on the enzyme activity, the affinity of inhibitors, the rates of association and dissociation of metal from the active site, and spectral properties of the Co\textsuperscript{2+}-enzyme. All of these changes cannot be explained by steric hindrance due to the added group. Some of the effects could be due to changes in conformation of the enzyme which might accompany the modification reaction. The optical rotatory dispersion curve of carboxymethyl enzyme is the same as for native enzyme, so large changes in conformation are unlikely in that case (4). Perhaps there are small local changes in conformation in the active site region. Changes in polarity or solvent structure in the active site are likely, especially since the dissociation of a proton from a solvent molecule (water bound to the metal) is probably responsible for the major pH-dependent transition (1, 17).

**REFERENCES**
