Effects of Two Ionizing Groups on the Active Site of Human Carbonic Anhydrase B*

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Investigation of some pH-dependent properties of human erythrocyte carbonic anhydrase B indicate that the active site is influenced by at least two charged groups. The properties studied include the pH dependence of inhibition of native, monocarboxamidomethyl, and monocarboxymethyl enzymes by iodide ion and the pH dependence of the visible spectra of the cobalt derivatives of these enzymes.

One ionizing group has a pKₐ of about 7.3 in the native enzyme, 8.2 in the carboxamidomethyl enzyme, and 9.0 in the carboxymethyl enzyme. It has a major influence on activity and anion inhibition, and on the visible spectra of the cobalt enzymes. A second group has a pKₐ of about 6.1 in native and modified enzymes. When zinc is at the active site, the secondary group in its acidic form decreases the Kᵢ for I⁻. With the carboxamidomethyl and carboxymethyl enzymes, the Kᵢ decreases by about an order of magnitude. However, if cobalt is substituted for zinc in the modified enzymes, this group does not influence the Kᵢ for I⁻ and the binding of I⁻ does not influence the pKₐ of the spectral transitions caused by ionization of this secondary group. In the case of nonalkylated Co⁺⁺-enzyme, another ionizing group with a pKₐ of about 6.2 prevents the binding of I⁻ at low pH. These results show that the active site is altered when cobalt is substituted for zinc in carbonic anhydrase B.

An important test in probing the active site of an enzyme is the study of the pH dependence of specific properties of the active site. Many approaches have been applied to carbonic anhydrase B from human erythrocytes to get this type of information. These include the pH dependence of activity and its inhibition (1-8), binding of inhibitors (9-12), chemical modification (6-8), the visible spectrum of the Co⁺⁺-enzyme (4, 6, 13, 14), and NMR spectra of histidine protons (15-20). Most of the active site properties have been analyzed in terms of the simplest case where only one ionizing group influences them. However, the pH dependence of CO₂-hydrating activity (1, 2) and the visible spectrum of the Co⁺⁺-enzyme (6) did not follow a simple titration curve. In addition, the pH dependence of inhibition by bromoacetate suggested that more than one ionizing group influenced the binding of this anion (6).

This paper presents further evidence that there is more than one ionizing group which influences the active site of this enzyme. A major part of the work is on the pH dependence of anion binding to native and two modified forms of carbonic anhydrase B. The modified enzymes are the monocarboxamidomethyl and monocarboxymethyl derivatives which are prepared by reaction with iodoacetamide and iodoacetate or bromoacetate, respectively (6, 7, 21, 22). These alkylating agents are affinity reagents which bind at the active site as reversible inhibitors and then proceed to react with the 3'-nitrogen of histidine 200 (7, 23, 24). The modified enzymes have been purified and shown to have residual activity (6, 7, 22, 25). The sedimentation coefficient and optical rotatory dispersion of the carboxymethyl enzyme were the same as for the native enzyme (7) indicating that no large conformational changes occurred. X-ray diffraction studies (26) determined that histidine 200 is a surface residue in the active site region, and is located within 7 A of the zinc ion which is at the base of the active site cavity.

MATERIALS AND METHODS
Carbonic anhydrase B was prepared from human erythrocytes and employed to prepare Co⁺⁺+, Cam¹ and Cm-enzyme derivatives (22). CamCo⁺⁺ enzyme was prepared from Co⁺⁺-enzyme by reaction at room temperature with 25 mM iodoacetamide at pH 7 for 24 hours. CmCo⁺⁺-enzyme was prepared by reacting Co⁺⁺-enzyme with 1 mM bromoacetate at pH 7 for 5 hours. Each was then titrated to about pH 8 and immediately chromatographed on columns (1.8 x 80 cm) of Sulfamylan-Sepharose (25) at 4° equilibrated with 5 mM Tris sulfate/22 mM Na₂SO₄, pH 7.6, and eluted with 10 mM sodium phosphate/20 mM Na₂SO₄, pH 6.5. It was observed that although these modified enzymes were retarded, they slowly moved down the column in starting buffer. Thus, after eluting the sample, the column was prepared by reaction with iodoacetamide and iodoacetate or bromoacetate, respectively (6, 7, 21, 22). These alkylating agents are affinity reagents which bind at the active site as reversible inhibitors and then proceed to react with the 3'-nitrogen of histidine 200 (7, 23, 24). The modified enzymes have been purified and shown to have residual activity (6, 7, 22, 25). The sedimentation coefficient and optical rotatory dispersion of the carboxymethyl enzyme were the same as for the native enzyme (7) indicating that no large conformational changes occurred. X-ray diffraction studies (26) determined that histidine 200 is a surface residue in the active site region, and is located within 7 A of the zinc ion which is at the base of the active site cavity.

The abbreviations used are: Cam, carboxamidomethyl; Cm, carboxymethyl; bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxy-methyl)aminomethane.

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washed with not more than 100 ml of starting buffer before changing to the second buffer to bring the modified enzyme off in a sharp peak. The reactive reagents and inactive protein came off at the front of the elution pattern. Unmodified enzyme remained bound to the affinity gel and was eluted later. The fractions containing modified enzyme were pooled and concentrated by vacuum dialysis. The modified enzymes were dialyzed against three changes of deionized water during a total of about 6 hours.

Alkylated Co**+-enzymes were prepared by modifying the Co**+-enzymes because it is very difficult to remove zinc from the alkylated enzymes. The modified Co**+-enzymes prepared by alklylation of the Co**+-enzyme or by alkylating and then changing the metal have only one modified histidine, behave the same on affinity chromatography, and have the same kinetic properties. Most of the spectral properties are also the same.

The concentrations of native and modified enzymes were determined from their absorbance at 280 nm (ε = 47,000 M⁻¹ cm⁻¹). The extinction coefficient was calculated from the absorbance of a 1 mg/ml solution at 280 nm of 1.63 (27), and a molecular weight of 28,850 (23).

Esterase activity was assayed with p-nitrophenyl acetate as described (6), except that 70 µl of 50 mM substrate in dimethylsulfoxide was used.

Iodoacetate, bromoacetate, and p-nitrophenyl acetate were purified as previously described (6). Bis-Tris was purchased from Sigma Chemical Co., St. Louis, Mo.

Results for the modified enzymes definitely show more than one ionization. The theoretical curves are shown for a simple two-group case of two independent ionizing groups in the enzyme which influence the binding of I⁻. The ionization of the two groups on the enzyme are illustrated in Equation 1, with the most acidic form arbitrarily assigned to be neutral, and the dissociated protons omitted.

\[ \text{HY-E-XH} \rightarrow K_1 \text{HY-E-X} + \text{H}^+ \]

\[ \text{HY-E-X} \rightarrow K_2 \text{HY-E-X}^- \]

\[ \text{HY-E-X}^- \rightarrow K_3 \text{HY-E-X}^{-2} \]

\[ \text{HY-E-X}^{-2} \rightarrow K_4 \text{HY-E-X}^{-3} \]

\[ K_1, K_2, K_3, \text{and } K_4 \text{ are acid dissociation constants, and it is assumed that } K_1 = K_4 \text{ and } K_2 = K_3 \text{ and that } pK_a > pK_p. \text{ The binding of iodide to } \text{HY-E-XH} \text{ is assumed to be strongest; it is weaker to } \text{Y-E-XH}, \text{ and not significant to the other two forms. Although } pK_a \text{ and } pK_p \text{ cannot be assigned with great accuracy, the data closely fit the theoretical lines generated when } pK_2 = 6.1 \text{ and } pK_4 = 8.2 \text{ for the Cam-enzyme and } 6.1 \text{ and } 9.0 \text{ for the Cm-enzyme.} \]

The pH dependence of Cl⁻ inhibition of native enzyme was investigated at ionic strengths of 0.075 and 0.2. The minimum \( K^* \) at low pH was 2 mM at an ionic strength of 0.075 and 3 mM at an ionic strength of 0.2. The pH dependence was similar to that for iodide with an apparent \( pK_a \) near 6.8 for the case where a single \( pK_a \) is assumed.

Evidence that the primary binding site is the same for Cl⁻, Br⁻, and I⁻ is shown in Table I. The degree of inhibition of esterase activity at pH 6.5 by Cl⁻ + Br⁻, Cl⁻ + I⁻, or Br⁻ + I⁻ agrees with that calculated for the case where the binding of one inhibitor is strictly competitive with the other.

**Binding of Halide Ions to Co**++-enzymes**—The pH dependence of the \( K^* \) of iodide for the Co**+-enzymes are shown in Fig. 2. A surprising result is that the group with a \( pK_a \) of 6.1 has absolutely no effect on the binding of iodide to the Co**+ form of the Cam- or Cm-enzyme. Additional results show that the difference between Cm-enzyme and CmCo**+-enzyme is due to
the substitution of Co²⁺ for Zn²⁺ and not to the procedure of removing the metal. Cm-enzyme was dialyzed against o-phenanthroline at pH 5.5 for 20 days, and the apoenzyme separated from the metalloenzyme by affinity chromatography using the same conditions as described under "Materials and Methods" for purification of Cm-enzyme. Since the metal dissociates slowly from Cm- or Cam-enzyme, most (80%) of the enzyme was recovered as CmZn²⁺-enzyme, and this enzyme showed the same complex pH dependence of $K_i$ for iodide ion as the untreated Cm-enzyme. Cobalt was added to half of the apoenzyme and zinc was added to the other half. The Zn²⁺-enzyme showed the complex pH dependence and the Co²⁺-enzyme did not.

The pH dependence of iodide inhibition of Co²⁺-enzyme shows a maximum at pH 6.7 (Fig. 2). The data were fitted with a curve with one group ($pK = 7.2$) which must be in its acidic form and a second group ($pK = 6.2$) which must be in its basic form for binding to occur. The esterase activity falls off rapidly below pH 6. Since the metal dissociates from the nonalkylated enzyme much more rapidly than from the Cam- or Cm-enzymes, the possibility that the metal may be dissociating from the Co²⁺-enzyme was investigated. It was found that neither the activity nor the inhibition was affected by addition of 20 μM excess Co²⁺ at pH 5.5.

The effects of halide ions on the spectra of Co²⁺-enzyme and its carboxamidomethyl and carboxymethyl derivatives are shown in Fig. 3. The spectra of the native Co²⁺-enzyme with Cl⁻ and Br⁻ are very similar, but the spectrum with I⁻ shows more resolved peaks in the 600 to 650 nm region and an additional large band below 350 nm. The spectra of the modified Co²⁺-enzymes with Cl⁻ and I⁻ are similar to those for anions with the native Co²⁺-enzyme, but the modified enzymes have much smaller peaks in the 500 to 650 nm region and a pronounced shoulder in the spectra with I⁻ is evident near 340 nm. The basic similarity of the spectra of the three Co²⁺-enzymes with I⁻ is more evident at lower pH values (Fig. 4).

The changes in the spectra as the pH value increases are also quite similar. It seems reasonable to conclude that the site and general manner of binding of I⁻ is the same for the three Co²⁺-enzymes.

Some of the dissociation constants of these ions with the different Co²⁺-enzymes were determined by spectral changes. In Table II, they are compared with $K_i$ values determined using the esterase activity of Zn²⁺- and Co²⁺-enzymes. Their similarity implies that the spectral changes are due to the binding of the halides in their inhibitor site.

The spectral titrations with Cl⁻ and Br⁻ follow a simple curve, indicating that binding in a single site is responsible for the observed spectral changes. But when all three enzymes are titrated with I⁻ at pH 6.1, secondary changes in the spectra were noted as the concentration of I⁻ exceeded saturating levels of the primary site. The general shape of the spectra did not change, but the height of the peaks increased. For example, as the concentration of I⁻ with Co²⁺-enzyme was increased from about 10 $K_{diss}$ to 100 $K_{diss}$ the absorbance at 628 nm increased approximately 20%. At wavelengths where the absorbance of the Co²⁺-enzyme-I⁻ complex was less than for free Co²⁺-enzyme, the absorbance first decreased at low concentrations of I⁻ and then increased at higher concentrations. Small corrections for the secondary effects were made by linear extrapolation toward zero concentration for calculations of dissociation constants of I⁻ with the Co²⁺-enzymes. Secondary effects were not observed in the titrations with I⁻ at pH 8.2.
Fig. 4. pH-dependent changes in the spectra of Co⁺⁺-enzymes associated with iodide ions. The buffer was 10 to 20 mM bis-Tris sulfate plus 100 mM NaI and enough Na₂SO₄ to give an ionic strength of 0.14 for (A) and 0.20 for (B) and (C). The spectrum of each solution was determined at low pH (solid lines) and the solutions were titrated to high pH (dashed lines) with 1 M Tris. A, Co⁺⁺-enzyme at pH 5.7 and 7.4; B, CamCo⁺⁺-enzyme at pH 5.3 and 6.8; C, CmCo⁺⁺-enzyme at pH 5.7 and 8.0.

Table II

Binding of halide ions to native and modified carbonic anhydrase B

The buffers were bis-Tris sulfate at pH 6.1 and Tris sulfate at pH 8.2. The ionic strength was maintained at about 0.2 by addition of Na₂SO₄. The primary numbers are Kₛ values determined using the esterase assay. The numbers in parentheses are dissociation constants determined from spectral titrations of the Co⁺⁺-enzymes with halide ions.

| Enzyme                  | Cl⁻ | Br⁻ | I⁻ | I⁻ |
|-------------------------|-----|-----|----|----|
|                         | pH 6.1 | pH 6.1 | pH 6.1 | pH 8.2 |
| Carbonic anhydrase B    | 3.8 | 0.13 | 2.9 |
| Co⁺⁺-carbonic anhydrase B | (21) | (10) | 1.7 (1.8) | 7.5 (7.0) |
| Cam-carbonic anhydrase B | 0.6 | 4.3 |
| CamCo⁺⁺-carbonic anhydrase B | (25) | 0.4 (0.3) | 0.8 |
| Cm-carbonic anhydrase B  | 35  | 2.9  | 11  |
| CmCo⁺⁺-carbonic anhydrase B | 130 | 2.4 (2.0) | 2.7 |

**pH-dependent Spectral Changes**—The pH-dependent changes in the spectra of the three Co⁺⁺-enzymes in the presence (Fig. 4) and absence (Fig. 5) of I⁻ were determined in the region of pH 5.5 to 8 (Fig. 6). Special care was taken while titrating the enzymes to low pH, since addition of strong acids causes denaturation and precipitation of the enzyme. The pH was decreased to about 5.5 by addition of bis-Tris sulfate buffer at pH 5.1 to the unbuffered enzyme solution. Even so, a solution of CamCo⁺⁺-enzyme with 100 mM I⁻ developed some turbidity. The solution was quickly filtered, scanned, and titrated to higher pH. Since this laboratory previously reported that the CamCo⁺⁺-enzyme did not show a secondary transition in its spectrum at low pH (22), the present results were checked with three different preparations of enzyme. The reason it was not observed before may be due to slightly different methods of preparation of the CamCo⁺⁺-enzyme. The procedure used for these studies was more gentle, since the dialysis time against o-phenanthroline at pH 5.5 was much shorter. The alkaline spectrum of Co⁺⁺-enzyme is given in Fig. 4A to show that the spectral changes at low pH are small compared to the changes which occur in the major pH-dependent spectral transition. The alkaline spectra for Cam- and CmCo⁺⁺-enzymes are similar to that of the Co⁺⁺-enzyme (6, 22). Since these spectral changes were small compared to the major transitions which occur at higher pH, the pH dependence of the minor transitions was followed at wavelengths near an isosbestic point for the major transition (Fig. 6). The data were fitted with theoretical titration curves with pKₛ values of 6.2 for native Co⁺⁺-enzymes and 6.0 for the modified enzymes. The magnitude of the changes were similar in all six cases but the direction of the changes in the presence of I⁻ was opposite from those in its absence. Since the same pKₛ is obtained in the presence or absence of I⁻, it is likely that the same pH-dependent ionization is responsible for the observed spectral changes in the Co⁺⁺-enzyme and the Co⁺⁺-enzyme-I⁻ complex.

**Discussion**

The purpose of this study was to investigate how the ionization of charged groups in human erythrocyte carbonic anhydrase B influences properties of the active site. The pH dependence of inhibition by halide ions was studied for the
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![Figure 6: pH dependence of the spectra of Co²⁺-enzymes in the presence and absence of I⁻.](http://www.jbc.org/)

From the data of the pH dependence of inhibition of native carbonic anhydrase B by anions it is difficult to make any definite conclusions about the pK of the ionizing group or groups which control the binding. Assuming that a single ionizing group controls the binding of iodide and chloride, its apparent pKₐ is about 6.8. This agrees with previous results for inhibition by iodoacetate (7) and bromoacetate (6). However, Ward studied the pH dependence of chloride ion binding using "Cl NMR techniques and calculated a pKₐ of 8.2 (11). This value was calculated using a literature value of 51 mM for the Kᵣ of chloride. By replotting Ward's data using the Kᵣ, one of the pKₐ values is the same as the major one affecting activity and the visible spectrum. The ambiguities and uncertainties in the determination of the number and pK values of groups influencing anion binding to this enzyme serves to re-emphasize the fact that it is difficult to assign reliable pK values from one set of data on a pH-dependent property, since an undetected secondary group may affect the curve in subtle ways if its pK is near that of the primary group which controls binding.

Although the data showing that more than one ionizing group influences the active site, two chemically modified forms of the enzyme were used. From the pH dependence of esterase activity and the visible spectra of the Co²⁺-enzymes, it was observed that the pKₐ of the primary group affecting these properties was shifted from 7.3 for the native enzyme to about 8.3 for the Cm-enzyme and 9.2 for the Cm-enzyme (6, 22). Hence, in these derivatives the ionization of the primary group is shifted enough to make a secondary group with a lower pK more evident. This is clearly demonstrated by the results in Figs. 1 and 2. The major pH-dependent transition can now be seen to have the same pH as observed for esterase activity and the visible spectra. The pKₐ for the secondary group is about 6.1 in both modified enzymes. The pKₐ values of 6.4 estimated previously for native enzyme (22), and the pK of 6.1 determined here for the modified enzymes are within experimental uncertainty, so it is likely that the pK of this group is affected very little by alkylation of histidine 200 with either a neutral or negatively charged group.

The most surprising finding is that the substitution of cobalt for zinc causes such marked changes in the pH dependence of anion binding at low pH. The finding that the secondary group (pKₐ = 6.1) does not influence the Kᵣ for iodide with CmCo²⁺- or CmCo⁺-enzyme is in accord with the results showing that the binding of iodide does not shift the pKₐ of the spectral transition. However, the case with the native enzyme is not so simple, since there are pH-dependent transitions with pKₐ values near 6.1 which promote the binding of I⁻ to Zn²⁺-enzyme, prevent I⁻ binding to Co²⁺-enzyme, and produce spectral changes in the Co²⁺-enzyme and the Co⁺-enzyme-I⁻ complex. If the same ionization which prevents binding of I⁻ to Co⁺-enzyme at low pH was responsible for the spectral transition, then the spectral transition would have a higher pKₐ in the presence of I⁻. Such is not the case, so another ionization is implicated. It is unlikely that the loss in ability to bind I⁻ at low pH is due to the binding of a proton to a nearby basic group, so it may be due to a small pH-dependent conformational change which shows up in the native Co²⁺-enzyme but not in the alkylated Co⁺-enzymes.

The identities of the ionizing groups involved in enzymic functions are not known. The ionizing group of primary importance has a pKₐ of 7.3 in the native enzyme and is thought to be a zinc-bound water molecule (2, 28, 29), although alternatives involving histidyl side chains have been proposed (18, 30, 31). The group with a pK near 6.1 could be due to histidines 64, 67, or 200 (26), all of which are in the active site region. Histidine 200 is the one modified in the Cm- and Cam-enzyme (7, 23, 24). In the native enzyme, this histidine has a pKₐ of 5.6 in the presence of reversibly bound bromoacetate (6) and 5.8 with iodoacetate (7). Its pKₐ in the absence of inhibitor could be higher and it would not be expected to change much upon alkylation (21). Therefore, any of these histidyl residues are possible candidates for affecting anion binding.

An important question is whether the secondary group has...
any other functional significance. The pH dependence of CO₂-hydrating activity of enzyme B does not follow a simple titration curve (1), and this may be due to a positive or negative influence by this secondary group. Perhaps the effects of the secondary group will show up more distinctly in studies using dehydrating activity, since this has a maximum at low pH. Some dehydration studies have been done (3), but, due to experimental limitations, these were not extended to low enough pH to see these effects. The involvement of a second ionizing group has also been postulated to account for hydrogen isotope effects on the kinetics of CO₂ hydration and HCO₃⁻ dehydration by human carbonic anhydrase C (32). Possible roles of the secondary groups may be to enhance binding of anions, to influence the water structure in the active site, and to function in concert with the primary group which is responsible for enzymic activity.

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