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INTERACTION OF BOVINE CARBONIC ANHYDRASE WITH ACETATE IONS

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SUMMARY

The binding of acetate ions to bovine carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) was investigated by NMR and by inhibition measurements. Two acetate ions were found to interact with the protein but only one is linked with the inhibition of the esterase activity of the enzyme. The NMR spectrum of the anion is sensitive to the stronger acetate binding, which is to a noninhibitory binding site. This acetate ion could be titrated with monovalent anions as N_3^-, but not with *p*-toluene-sulfonamide. The noninhibitory acetate ion performs an isotropic random motion faster than that of the enzyme. The correlation time for the tumbling of the methyl group was calculated from the T_1/T_2 ratio, as well as from the NMR line-broadening ratio at two NMR frequencies and was found to be $(2.3 \cdot 10^{-9}\ 0.8) \cdot 10^{-9}$ s. The binding of the two ligands decreases with pH and is probably dependent upon two different groups on the enzyme with pK_a values of 7.5 and 7.4 for the binding of the inhibitory and noninhibitory acetate ions, respectively.

INTRODUCTION

The interactions of anions with the Zn metallo-enzyme carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) has been extensively studied (see Lindskog et al. [1] and references therein) since the original discovery of their inhibitory power by Meldrum and Roughton [2]. Inhibition was found to be noncompetitive for the CO_2 hydration reaction [3], as well as for the esterase activity of the enzyme [4–6]. It appears that the anions are bound to a form of the enzyme which predominates at low pH values [3, 6–10]. Recently, magnetic resonance methods have been applied to investigate the carbonic anhydrase-anions system. Taylor et al. [11] determined the rates of association and dissociation of several carboxylate ligands with the Co enzyme, using ^1H and ^{19}F NMR spectroscopy. Ward [9, 10] has examined the interactions of Cl^- with carbonic anhydrase by NMR and on the basis of his results he proposed direct anion binding to the metal coordination site. This is in agreement with the previous results, showing Co spectral changes upon binding of an anion to Co(II) carbonic anhydrase [7, 12–14].

Although the stoichiometry of specific anion binding was believed to be 1:1,

Verpoorte et al. [5] found that both human carbonic anhydrase and its apoenzyme bind approx. 6 Cl^- in 0.1 M KCl. More recently studies of the Co- and Cu-substituted enzymes by EPR [15–17] indicated a 2:1 CN^- complex with carbonic anhydrase. The formation of the 2:1 complex could be also followed spectrophotometrically [17]. In the present work we have examined, using high-resolution NMR spectroscopy and inhibition measurements, the acetate-ion binding to bovine carbonic anhydrase as an example of anion–carbonic anhydrase interaction.

MATERIALS AND METHODS

Bovine carbonic anhydrase B, prepared and purified by the method of Lindskog [18], was obtained from Seravac. To prevent possible contaminations of paramagnetic impurities, all metal ions were removed by prolonged dialysis against 1,10-phenanthroline, and the Zn^{2+} was restored by further dialysis as has been described previously [19]. A precipitate of denatured material was centrifuged off. Solutions of Cu(II) carbonic anhydrase were prepared by dialysing the apoenzyme against a 250-fold volume excess of $1 \cdot 10^{-4}$ M $CuSO_4$. The excess of Cu^{2+} was removed by further dialysing the Cu enzyme against three changes of 0.1 M Tris–sulfate buffer, pH 7.4. After this treatment the Cu enzyme, which was completely inactive, contained 1.0 Cu^{2+}/enzyme molecule. (Shinar, H., unpublished result.)

Enzyme concentrations were determined spectrophotometrically at 280 nm using the molar extinction coefficient $\varepsilon_{280\ nm} = 5.7 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ and a molecular weight of 30 000 [20].

Enzymatic activity was determined by measuring the esterase activity toward the hydrolysis of *p*-nitrophenylacetate by following the absorbance at 400 nm [21].

Samples were prepared for NMR measurements by dialysing the enzyme against four changes of a 10-fold volume excess of solution of ^2H_2O containing 0.01 M Tris–sulfate or phosphate buffers; pH values were recorded as the uncorrected glass-electrode reading.

NMR spectra were recorded on a Varian HA-100 spectrometer. Chemical shifts were measured relative to 0.02 M acetone which also served as a linewidth reference with typical linewidths ranging between 0.25 and 0.3 Hz. The sample temperature was 30 °C unless otherwise stated. Values of $1/T_2$ were obtained from the spectral linewidth using the expression $1/T_2 = \pi \Delta\nu$, where $\Delta\nu$ is the full linewidth at half-maximum peak height. The net broadening $\Delta\nu_p$ of acetate due to binding to the enzyme was obtained by subtracting the linewidth of the acetate ions in the buffer solutions. T_1 values were obtained using the progressive saturation method. H_1 was calibrated as previously described [22].

RESULTS

Inhibition of the esterase activity by acetate ions

Acetate inhibition of the rate of *p*-nitrophenylacetate hydrolysis by bovine carbonic anhydrase B was measured as a function of pH at 25 °C. The association constants of the inhibitor K_I were determined from a plot of $1/v$, the reciprocal hydrolysis rate, against total acetate concentration. The pH dependence of the inhibition constant is given in Fig. 1. The pH profile of the affinity of acetate for bovine

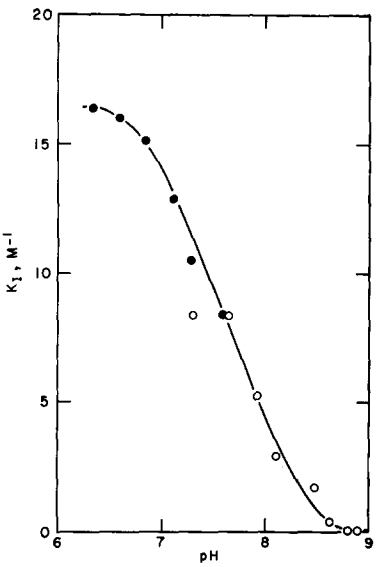


Fig. 1. Acetate inhibition constant K_I, as a function of pH at 25 °C. ○, Tris–sulfate buffer, 0.1 M; ●, phosphate buffer, 0.1 M.

carbonic anhydrase is similar to that obtained for other anions. The anion binding site has an apparent pK of 7.5.

NMR measurements of the binding of acetate ions to the enzyme

The acetate ion has a sharp, single peak which is 221.6 Hz downfield from external hexamethyldisiloxane at 30 °C. Besides the line broadening, a very small downfield shift of about 0.2 Hz was observed for the acetate signal upon addition of bovine carbonic anhydrase; 0.02 M acetone was used as an internal reference. As this shift was independent of the acetate concentration, it was considered to be due to a very slight binding of acetone [6] or of acetate to a different site on the enzyme and was subsequently ignored. The binding constant of small molecules to macromolecules can be obtained from the concentration dependence of the NMR line broadening [19, 23]. In the absence of differences in the chemical shift:

$$\Delta\nu_p = \frac{f}{\pi(T_{2M} + \tau_M)} \tag{1}$$

where f is the fraction of inhibitor molecules bound to the enzyme:

$$f = [E_0]/(K_I^{-1} + [I]) \tag{2}$$

T_{2M} is the transverse relaxation time of the bound inhibitor, τ_M is the exchange lifetime of the ligand, and E_0 is the total concentration of bovine carbonic anhydrase used in the experiment.

Fig. 2 shows $1/\Delta\nu_p$ versus the acetate concentration at five different temperatures. The negative x-axis intercept is equal to the dissociation equilibrium constant,

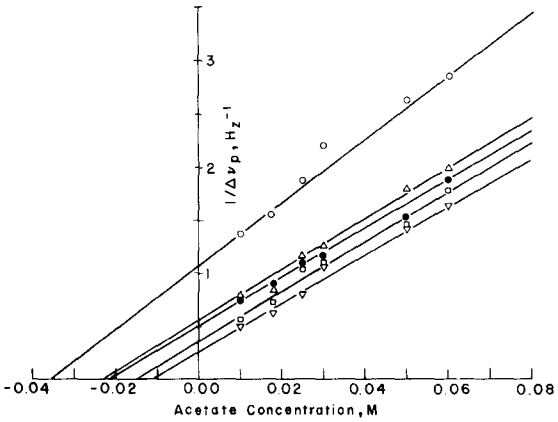
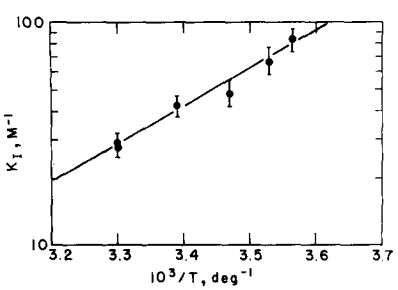


Fig. 2. Concentration dependence of the reciprocal net line broadening of the methyl group of acetate ion bound to bovine carbonic anhydrase B, at various temperatures. ○, 30; △, 22; ●, 15; □, 10.5; ▽, 5.5 °C, Tris–sulfate buffer in ^2H_2O, pH 7.53.

k_I^{-1}. A logarithmic plot of the measured equilibrium constants is given in Fig. 3. The apparent enthalpy for the acetate binding reaction is −2.3 kcal/mole. It can be seen from Fig. 3 that the binding constants found using the NMR method are about an order of magnitude greater than those obtained from inhibition measurements. This means that NMR is sensitive to the binding of acetate ions to a site different from the binding site involved in the inhibition of enzymatic activity.

The pH dependence of the broadening due to bound acetate ions at 6 °C is given in Fig. 4. Assuming a 1:1 binding of ligand to enzyme at an acetate concentration of 0.037 M as used in this experiment, and assuming $1/T_{2M}$ as being independent



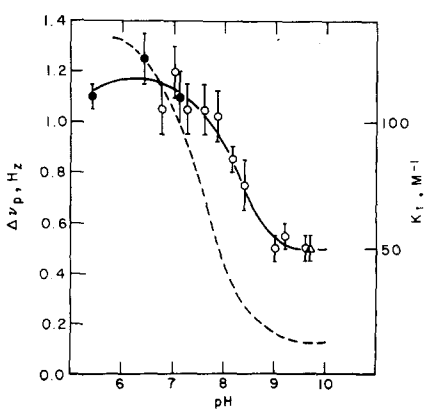


Fig. 3. Arrhenius plot of the noninhibitory acetate binding to bovine carbonic anhydrase B. Experimental conditions as in Fig. 2.

Fig. 4. pH dependence of: (———) Acetate line broadening upon binding to bovine carbonic anhydrase at 6 °C. (— — —) Binding constant of acetate ion to the enzyme. The binding constant was calculated from values at each point as described in the text. Bovine carbonic anhydrase B, $9.35 \cdot 10^{-4}$ M; acetate concentration, 0.037 M. ●, citrate–phosphate buffer; ○, Tris–sulfate buffer; △, glycine–NaOH buffer.

of pH, we could then calculate the pH dependence of the noninhibitory acetate binding constant by using Eqns 1 and 2 and the value of K_I at 6 °C taken from Fig. 3. Such a plot is given by the dashed line in Fig. 4. An apparent pK of 7.4 is obtained for the noninhibitory binding site.

One of the possible complications in the field of NMR relaxation enhancement by diamagnetic macromolecules is the possible presence of paramagnetic impurities. It was found that the negative enzyme preparation contained about 0.24 Cu atoms per Zn atom (18,19). There is a danger that a very small contamination of Cu^{2+} would remain even after prolonged dialysis against 1,10-phenanthroline and would influence the NMR results. Although no Cu could be detected after this treatment, by atomic absorption, some control experiments were conducted with the Cu enzyme, analogous to those carried out with the Zn enzyme.

We examined the pH dependence of the broadening of 0.034 M acetate ions, bound to $0.9 \cdot 10^{-4}$ M Cu(II) carbonic anhydrase. The net line broadening was found to be 3.1 ± 0.2 Hz independent of the pH, in the range of 6.3–8.8. This is in sharp contrast to the results obtained in Fig. 4 for the Zn enzyme, indicating that no Cu impurity is affecting our results.

NMR titrations

The displacement by sulfonamides of anions specifically bound close to the active site of carbonic anhydrase has been shown by various investigators using different methods [9, 25]. *p*-Toluenesulfonamide is a highly potent inhibitor of bovine carbonic annydrase B with a binding constant of $2.1 \cdot 10^6$ M^{-1} [19]. However, addition of this inhibitor up to concentrations of 0.01 M did not affect the observed acetate

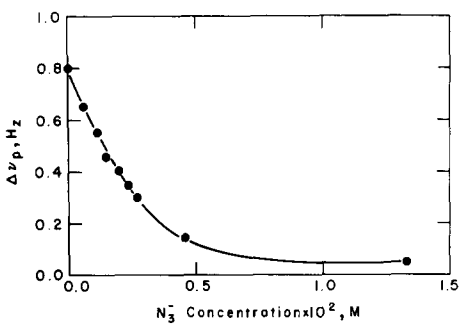


Fig. 5. The net line broadening of acetate ion bound to bovine carbonic anhydrase B upon titration with N_3^-. Enzyme concentration of $0.98 \cdot 10^{-3}$ M, and acetate 0.033 M, were kept constant throughout the experiment. Tris–sulfate buffer in ^2H_2O, pH 7.0. 30 °C.

line-broadening by the enzyme, at an acetate concentration of 0.037 M. This is further evidence that the binding of acetate at that concentration is to a site, different from the inhibitory site which is occupied by the sulfonamide inhibitor.

On the other hand, this bound acetate anion can be titrated by N_3^- ions. The narrowing of the linewidth of the acetate methyl group upon gradual addition of N_3^- is given in Fig. 5. For a simple competition between acetate and N_3^-, which are de-

noted by I_1 and I_2, respectively, the following equation applies [19] provided that $[I_1] \gg [EI_1]$ and $[I_2] \gg [EI_2]$:

$$\Delta\nu_p^{-1} = \frac{\pi(T_{2M} + \tau_M)[I_1]}{[E_0]} \left(1 + \frac{1}{K_{I_1}[I_1]} + \frac{K_{I_2}[I_2]}{K_{I_1}[I_1]}\right) \quad (3)$$

I_1 and E_0 were kept constant during the titration. The plot of the reciprocal of the net broadening of the acetate ion, $\Delta\nu_p^{-1}$, versus N_3^- to acetate ratio gave us a straight line (see Fig. 6). Its negative x-axis intercept is equal to:

$$K_{I_2}^{-1}\left(K_{I_1} + \frac{1}{[I_1]}\right) = 0.055$$

using K_{I_1} of 31 M^{-1} obtained from Fig. 3, the association constant of N_3^- to the noninhibitory binding site of carbonic anhydrase was calculated to be $1.2 \cdot 10^3$ M^{-1}.

When we titrated acetate ions bound to Cu(II) carbonic anhydrase a plot analogous to Fig. 6 gave a negative x-axis intercept of 0.005 ± 0.001, which is an order

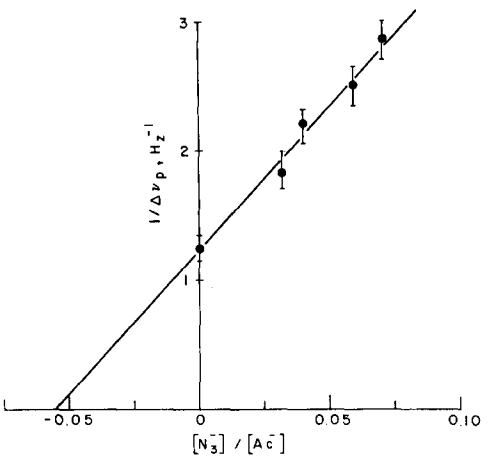


Fig. 6. N_3^- to acetate ion ratio dependence of the reciprocal net line broadening of acetate bound to bovine carbonic anhydrase B. Enzyme concentration $0.98 \cdot 10^{-3}$ M, Tris–sulfate buffer in ^2H_2O, pH 7.02. 30 °C.

of magnitude smaller than that obtained for the Zn enzyme, thus providing further evidence that no Cu^{2+} is present in the active site of the enzyme in our preparation.

Mechanism of relaxation

In many cases it is possible to determine whether the relaxation times are governed by the exchange lifetime or by the relaxation of the bound ligand, following the influence of temperature on the transverse relaxation rate of its protons [19, 24]. $(T_{2M} + \tau_M)^{-1}$ values for bound acetate from Eqn 1, are plotted versus temperature in Fig. 7. The temperature dependence suggests that the relaxation rate is in the fast exchange limit and is governed by T_{2M}.

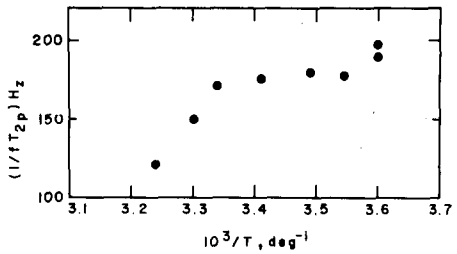


Fig. 7. The temperature dependence of the relaxation rate of acetate ion bound to bovine carbonic anhydrase. Enzyme concentration $1.34 \cdot 10^{-3}$ M. Acetate concentration 0.04 M. Tris–sulfate buffer in ^2H_2O, pH 7.5.

Two other measurements, the frequency dependence of the relaxation rate at 60 and 100 MHz, and the T_1/T_2 ratio at 100 MHz gave us additional information about the relaxation mechanism of the bound ligand. The determination of the T_1/T_2 ratio in three different solutions is summarized in Table I. The rotational correlation time was

TABLE I

DETERMINATION OF T_{1p}/T_{2p} RATIO AT 100 MHz

The solutions contained 0.01 MTris–sulfate buffer, pH 7.5, in ^2H_2O, 30 °C. The rotational correlation time was calculated as described in ref. 26.

Enzyme concentration (M)	Acetate concentration (M)	$1/T_{1p}$ (s^{-1})	$1/T_{2p}$ (s^{-1})	T_{1p}/T_{2p}	τ_c (s)
$1.32 \cdot 10^{-3}$	0.04	0.74	2.82	3.82	$2.7 \cdot 10^{-9}$
$1.31 \cdot 10^{-3}$	0.05	0.73	1.91	2.62	$1.9 \cdot 10^{-9}$
$1.30 \cdot 10^{-3}$	0.05	0.60	1.88	3.14	$2.4 \cdot 10^{-9}$

calculated as discussed previously [26]. The average value $T_1/T_2 = 3.1$ confirms the prevalence of the fast exchange limit. A ratio of 1.3 for the relaxation rates at 60 and 100 MHz [26] indicates a dipolar relaxation, rather than chemical shift mechanism. The rotational correlation time obtained from these two experiments is $(2.3 \cdot \pm 0.8) \cdot 10^{-9}$ s, which is shorter than the tumbling correlation time of the enzyme molecule of about $1.5 \cdot 10^{-8}$ s. It was concluded [26] that the methyl group of the acetate performs a fast isotropic random motion rather than just rotating around its symmetry axis. Furthermore, about half of the relaxation rate was assigned to intramolecular dipolar interaction while the other half probably arises from intermolecular dipolar interactions with the protein protons.

DISCUSSION

Carbonic anhydrase is inhibited by most monovalent anions. Kernohan [3] found that the inhibition can be described as resulting from the binding of one anion per active site. However, Verpoorte et al. [5] found that in 0.1 M KCl at the isoionic pH both apo- and native human carbonic anhydrase bind approx. 6 Cl^-. Moreover,

they found that at 0.005 M KCl one Cl^- is bound to the native enzyme, whereas the inhibition constant of the enzyme by Cl^- is 0.05 M. It is evident, therefore, that Cl^- is also bound to noninhibitory binding sites at low concentrations. Taylor et al. [15, 16] and Grell and Bray [17] found a 2:1 CN^-–Co(II) enzyme complex in millimolar concentrations of CN^-. The two CN^- are bound very close to the metal ion. It appears that the NMR technique is also sensitive to this secondary anion binding, which in the case of acetate ion is stronger than the ligand binding to the activity-linked binding site. The two binding sites of acetate were shown to be very close to the metal ion in the Mn-substituted enzyme [27].

The noninhibitory bound acetate was shown in the present work to be untitratable with the *p*-toluenesulfonamide inhibitor. However, other monovalent anions can titrate the acetate, suggesting a second specific anionic binding site. On the other hand, Taylor and Coleman [16] claimed that ethoxazolamide, another sulfonamide potent inhibitor, can titrate the two CN^- at the active site of the Co enzyme, as judged from the disappearance of the EPR signal characteristic of the 2:1 complex. However, the 1:1 anion or sulfonamide complexes of the Co enzyme were shown to be of a high-spin type [28] and hence their ESR signals were very broad and undetectable at room temperatures, while the 2:1 CN^-–Co(II) enzyme complex is low spin, and has characteristic ESR absorption features. It is possible that the titration of the CN^- bound to the inhibitory anionic binding site restores the Co complex to its high-spin state, although one anion molecule is still bound to the noninhibitory site in addition to the sulfonamide group. Other possibilities are that the result reflects differences between the coordination of the Zn and the Co carbonic anhydrases, or between the behavior of the CN^- and acetate anions.

From the pH variation of the inhibition constant of bovine carbonic anhydrase B by Cl^-, Pocker and Stone [6] have obtained a $pK_H = 7.5$ for the ionizing group which is probably controlling the Cl^- binding. Ward [10], on the other hand, using the NMR broadening of Cl^- estimated a pK_H of 6.2 for this group. Our value of $pK_H = 7.5$ for the acetate affinity for the enzyme (Fig. 1) is in a good agreement with Pocker's data for Cl^-.

The pH dependence of the anionic inhibition constant is well established for a variety of anions. The NMR method is sensitive to noninhibitory anion binding as well. The affinity of acetate ions for the noninhibitory site also depends on the ionization state of a titratable group and the binding is stronger at low pH values. However, while the binding of acetate to the inhibitory site, as was measured by the inhibition of activity (Fig. 1), was practically zero above pH 9.0, there is still an appreciable line broadening of acetate at basic pH values, and the binding constant is reduced only by a factor of 8.7 compared to that of the acidic region.

A pH-dependent binding of inhibitors to carbonic anhydrase is conventionally represented by the following scheme:

$$\begin{array}{ccc} EH + I & \overset{K_H}{\rightleftarrows} & E + H^+ + I \\ \uparrow\downarrow K_A & & \uparrow\downarrow K_B \\ EHI & \overset{K'_H}{\rightleftarrows} & EI + H^+ \end{array} \qquad (4)$$

Where K_A and K_B are the association constants of the inhibitor to the acidic and the basic forms of the enzyme, respectively. K_H' and K_H are the ionization constants of

the enzyme with and without bound inhibitor. Assuming the same linewidth from the EHI and EI enzyme–inhibitor complexes the observed line broadening is given by

$$\Delta\nu_p = \frac{[E_0]}{\pi(T_{2M} + \tau_M)} \frac{K_B + K_A \dfrac{[H^+]}{K_H}}{1 + \dfrac{[H^+]}{K_H}(1 + K_A[I]) + K_B[I]} \tag{5}$$

The apparent binding constant, K_I, which can be obtained at each pH value, is derived from Eqn 2 by plotting $1/\Delta\nu_p$ versus [I]. Hence:

$$K_I = \frac{K_B + K_A \dfrac{[H^+]}{K_H}}{1 + \dfrac{[H^+]}{K_H}} \tag{6}$$

using Eqn 4, an expression for the activity in the presence of inhibitors was given by Lindskog [7]. From this expression the apparent binding constant measured by inhibition of the activity can be obtained and is identical to Eqn 6.

It is interesting to note that both NMR broadening and activity pH profiles do not directly give the pK of the ionizable group in the enzyme. However, in a plot of the association constant, K_I versus pH, K_I approaches K_A and K_B in the acidic and basic pH limits, respectively, and the midpoint $K_I = 1/2(K_A + K_B)$ occurs at pH = pK_H. Certainly, it can be seen that the pK = 8.2 obtained from the plot of $\Delta\nu_p$ versus pH differs by 0.8 pH unit from pK_H = 7.4 which is directly observed from the K_I plot (Fig. 4).

Since the pH profiles of the acetate binding sites were found to be rather similar, it is interesting to discuss whether or not the same ionizing group is involved in the two binding sites. In the case of one common ionizing group, the first acetate ion which is preferentially bound to the acidic form of the enzyme, will shift the K_H to $K_H' = K_H\, K_B/K_A$. Since K_A/K_B = 8.7, the second inhibitor site will depend on pK_H' which is shifted from pK_H by about one pH unit and one expects to find a pK_H of 8.4 for the inhibition of the activity by acetate. But as this pK was found to be 7.5 it seems that these two sites involve separate ionizable groups with similar pK values.

Even for separate ionizable groups which are not too far apart, binding of acetate to one site may shift the pK of the adjacent group. It seems reasonable in the present case that binding of acetate to the noninhibitory site shifts the pK of the inhibitory site from a value below 7 to our experimental pK of 7.5. Therefore, a pK below 7 is not unexpected in the absence of bound anions.

It has been pointed out that the pH dependence of the anion binding can be correlated with the pK of the activity pH profile. There are large variations in the reported pK of the activity of bovine carbonic anhydrase which are all in the range of 6.35–7.5. It is possible that these variations are due to anions binding near the catalytic center which shifts the pK of activity according to the anion and the buffer concentrations used in the experiment.

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