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NMR STUDIES OF THE TWO BINDING SITES OF ACETATE IONS TO MANGANESE(II) CARBONIC ANHYDRASE

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SUMMARY

The interaction of acetate ions with Mn(II) bovine carbonic anhydrase as was studied by NMR, revealed the close proximity between two anion molecules and the metal ion at the active center of the enzyme. However, only one acetate molecule is responsible for the inhibition of the enzymic activity. The two binding sites could also be distinguished by their ability to bind sulfonamide inhibitors. The distances between the acetate methyl groups and the Mn^{2+} was calculated using the longitudinal and transverse relaxation rates for the bound ligands, and were found to be 4.3 ± 0.3 and 4.8 ± 0.3 Å for the acetate ions bound to the inhibitory and noninhibitory sites, respectively. These distances are consistent with a model in which the carboxylic groups of the acetate anion are directly coordinated to the metal. However, it is likely that at least the noninhibitory acetate, which is also untitratable by sulfonamide, is bound to another site.

INTRODUCTION

In a previous work we have characterized two different anion-binding sites on the Zn-containing enzyme, carbonic anhydrase by NMR and kinetic measurements [1]. Only one anion is linked to the inhibition of the esterase activity of the enzyme, and it is generally accepted that this anion is directly bound to the metal ion [2]. The kinetic properties of some inhibitory anions were also characterized [3].

On the other hand, not much is known yet about the "noninhibitory anionic binding site" [1]. Recent EPR studies of the Co(II) carbonic anhydrase indicated cloae proximity between two CN^- molecules and the metal [4–6]. It was argued by Grell and Bray [6] that the binding of the second CN^-, which induces a low-spin Co enzyme form, is accompanied by an increase in the coordination number of the Co^{2+}. However, while a sulfonamide inhibitor could displace the two CN^- bound to the Co enzyme [5], we could not observe such a titration of acetate ion from the "noninhibitory binding site" in the Zn enzyme by a sulfonamide inhibitor [1]. Moreover, the metal–anion distance in the human carbonic anhydrase was found by X-ray measurements at a resolution of 2 Å [7], to be greater than that expected for direct binding. It seems that the problems of the number of anions bound in the active site crevice of carbonic anhydrase, their geometry and replacement by other enzyme inhibitors, are not yet

settled. Therefore, we further investigated the anion binding to bovine carbonic anhydrase by the NMR method, by using its Mn derivative.

The NMR technique using paramagnetic probes has the advantage that in certain cases, one can calculate direct distances between a paramagnetic center at the active site of a protein, and proton groups of a small molecule attached to this protein [8–10]. In the present paper the distances between the Mn^{2+} and the methyl groups of the two acetate ions located at the active site region were estimated, as well as the kinetic parameters for the anion–enzyme complexes.

MATERIALS AND METHODS

Bovine carbonic anhydrase B, prepared and purified by the method of Lindskog [11] was obtained from Seravac. The preparations and properties of the Mn-substituted enzyme were described in a previous publication [10]. Prior to the addition of Mn^{2+}, the various batches of apoenzyme had 3–7% of their activity, due to residual Zn enzyme.

The enzyme activity was monitored as esterase activity against *p*-nitrophenylacetate. In the activity measurements of the Mn(II) enzyme, the solutions contained $0.7 \cdot 10^{-5}$ M Mn(II) carbonic anhydrase and $2 \cdot 10^{-6}$ M *p*-toluenesulfonamide inhibitor. This concentration of sulfonamide was sufficient to saturate traces of Zn(II) carbonic anhydrase [12], while the Mn(II) enzyme molecules remained free from inhibitor binding, owing to the larger dissociation constant of $3.5 \cdot 10^{-5}$ M of the *p*-toluenesulfonamide–Mn(II) carbonic anhydrase complex [10].

Samples were prepared for NMR measurements by dialysing the Mn enzyme against four changes of a 10-volume excess of ^2H_2O solutions containing $5 \cdot 10^{-4}$ M $MnCl_2$ in 0.01 Tris–sulfate buffer at the appropriate pH value. Blank solutions of Mn(II) were prepared from the last dialysis solution.

NMR spectra were recorded on a Varian HA-100 spectrometer. The probe temperature was measured by the peak separation of dry methanol and was 30 ± 1 °C unless otherwise stated. Hexamethyldisiloxane was used as an external lock. Values of 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. T_1 values of acetate ions were measured directly by a 180–90 ° pulse sequence using the method for measuring relaxation times in a two-line NMR spectra system [13].

The net relaxation rates, $1/T_{1p}$ and $1/T_{2p}$ are the differences between the relaxation rates of the acetate ions in the enzyme solution and those in the blank solutions.

RESULTS

Acetate binding to Mn(II) carbonic anhydrase

The single NMR line of the methyl group of the acetate ion is appreciably broadened in a solution of Mn(II) carbonic anhydrase. The broadening is about 250-fold larger than that observed for acetate ion bound to the diamagnetic Zn enzyme at 30 °C, pH 8.0, [1] and more than 150-fold than for the acetate–Co(II) enzyme complex [3]. The apparent affinity of inhibitiors to an enzyme, studed by NMR, can be obtained by plotting $1/\Delta\nu_p$, the bound ligand vs the ligand concentration [12, 14]. Such plots for five different temperatures for the acetate–Mn(II) carbonic anhydrase com-

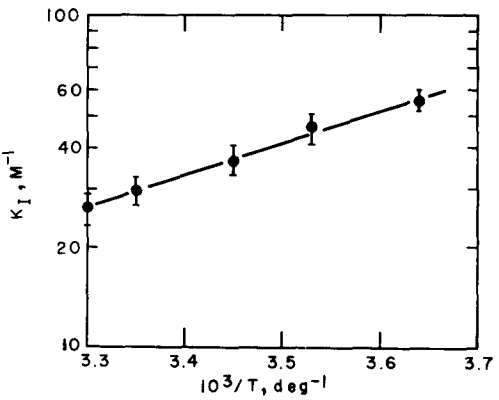

Fig. 1. Arrhenius plot of the binding constant of acetate to Mn(II) carbonic anhydrase as determined by the NMR method. The binding constant value at each temperature was obtained by plotting $1/\Delta\nu_p$ versus acetate concentration [1]. The solution contained $6.25 \cdot 10^{-5}$ M Mn(II) enzyme, $2 \cdot 10^{-4}$ M $MnCl_2$ and various concentrations of sodium acetate, in 0.1 M Tris–sulfate buffer, pH 7.5.

plex are drawn and the association constants are summarized in an Arrhenius plot (Fig. 1). The enthalpy of the binding reaction is $\Delta H = -4.4$ kcal/mole.

The inhibition constant of Mn(II) carbonic anhydrase esterase activity by acetate ion was found to be 0.8 ± 0.2 M^{-1} at 25 °C, pH 7.5 (Fig. 2), as compared to the value of 29 ± 2 M^{-1} for the binding constant, which was determined by the NMR method, under the same conditions. The large difference clearly indicates binding of acetate to two distinct sites for which the stronger one is noninhibitory. This result is very similar to that obtained for the binding of acetate to the Zn-containing enzyme [1].

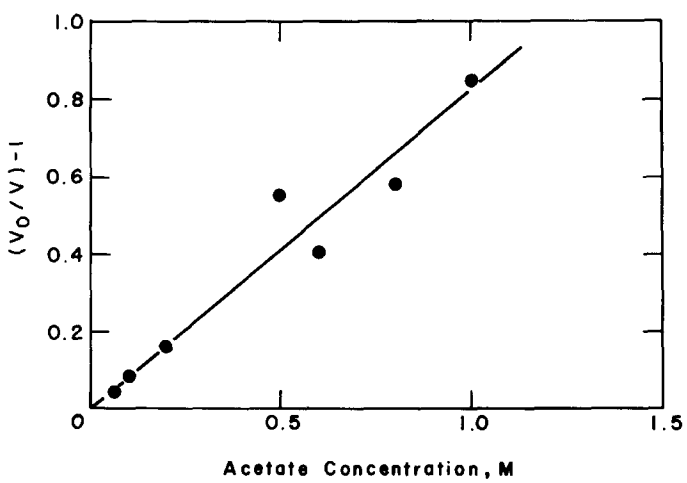

Fig. 2. Determination of the inhibition constant of the esterase activity of Mn(II) carbonic anhydrase by acetate ions, at 25 °C. The solution contained $7 \cdot 10^{-6}$ M Mn(II) enzyme, $2 \cdot 10^{-4}$ M $MnCl_2$, $2 \cdot 10^{-6}$ M *p*-toluenesulfonamide (see text) in 0.01 M Tris–sulfate buffer, pH 7.5.

NMR titrations

Acetate ions are weakly bound to the inhibitory site of Zn(II) carbonic anhydrase and their line broadening is very small in the diamagnetic system [1]. Hence, we could not follow the acetate binding to the inhibitory anionic site of the native enzyme by the NMR method [1]. However, in the paramagnetic system the considerable relaxation effect enabled us to raise the acetate concentration above 0.75 M and still observe an appreciable line broadening. In this case we could titrate the two anionic binding sites. In Fig. 3 we presented the displacement of acetate ions from their complex with Mn(II) enzyme by N_3^- as well as by the sulfonamide inhibitor *p*-toluenesulfonamide. A narrowing of about 20% in the acetate linewidth is observed at a 1:1 sulfonamide to enzyme ratio. On the other hand, the titration of the acetate linewidth by N_3^- exhibits two sigmoidal regions. The lower N_3^- concentration titration curve has a very similar behavior to the sulfonamide titration profile, except that higher

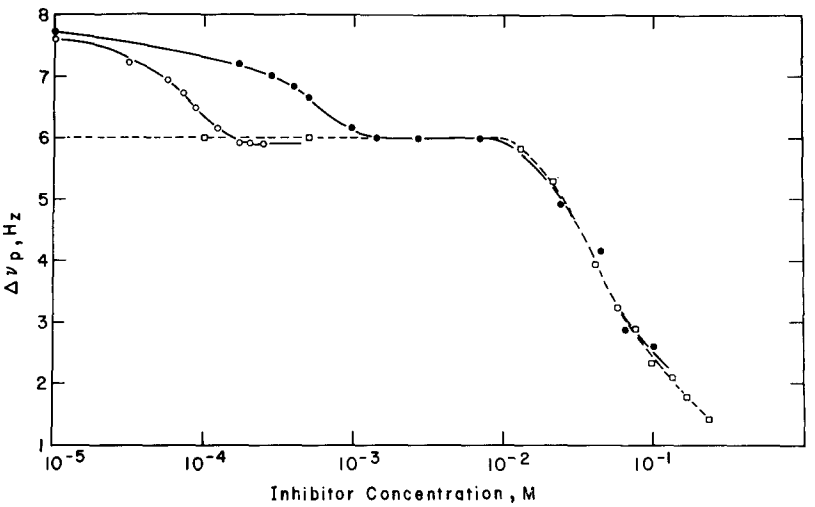

Fig. 3. Titration of 0.5 M acetate bound to $2.07 \cdot 10^{-4}$ M Mn(II) carbonic anhydrase by: ○—○, *p*-toluenesulfonamide; ●—●, N_3^-; □—□, N_3^- to solution of enzyme containing $6.5 \cdot 10^{-4}$ M *p*-toluenesulfonamide. All the ^2H_2O solutions contained $2 \cdot 10^{-4}$ M $MnCl_2$ in 0.01 M Tris–sulfate buffer, pH 7.5.

concentrations of N_3^- are needed in order to reduce the acetate linewidth by the same factor. This is understandable on the basis of the stronger binding of the sulfonamide to the enzyme. Further addition of N_3^- caused complete acetate displacement. Furthermore, upon gradual addition of N_3^- to the Mn(II) enzyme–acetate system, which was saturated with an excess of *p*-toluenesulfonamide, we could titrate the residual acetate linewidth, and the residual titration curve overlapped with the curve obtained in the absence of sulfonamide (Fig. 3). Thus, by the NMR titration curves, the two anionic binding sites of Mn(II) carbonic anhydrase, can be clearly distinguished. The first site, which will be shown later to be the inhibitory anion binding site, can be titrated by both sulfonamide and N_3^- inhibitors. The second site seems to bind only anion ligands and may be designated as a noninhibitory binding site.

Fig. 4 shows the determination of the relative affinities of various inhibitors to

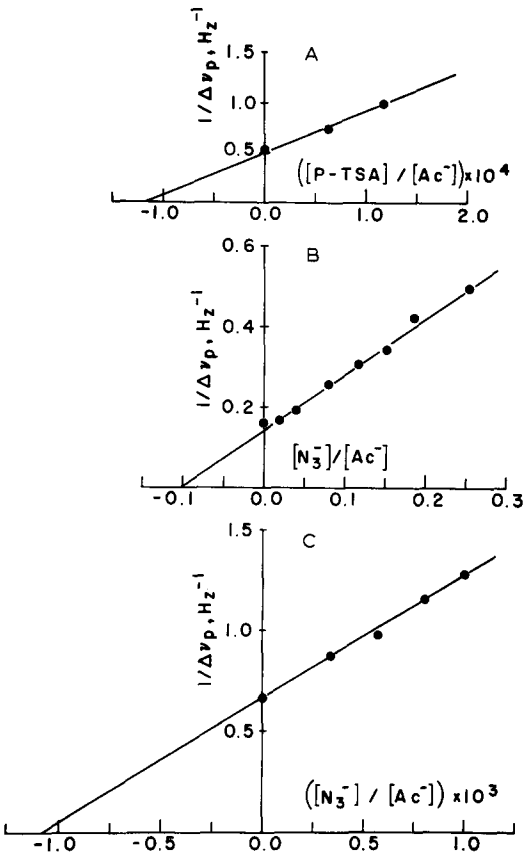

Fig. 4. Determination of the affinity of some inhibitors for the two anionic binding sites, taken from Fig. 3: (A) Titration of the inhibitory site by *p*-toluenesulfonamide (P-TSA). (B) Titration of the noninhibitory site by N_3^-. (C) Titration of the inhibitory site by N_3^-.

the two different anionic binding sites, as plots of the reciprocal acetate broadening against the inhibitor to acetate ratio. As was noted in the previous paper [1], the *x*-axis negative intercept is equal to $K_{I_2}^{-1}(K_{I_1} + [I_1]^{-1})$, where, in the present case, I_1 denotes the acetate ion and I_2 the other inhibitor. Using the association constants 0.8 M^{-1} and 29 M^{-1} for acetate binding to the inhibitory and noninhibitory sites, respectively (at 30 °C), we could calculate the binding constants of the inhibitors to these sites. They are summarised in Table I.

It can be seen from the table that the binding constants of *p*-toluenesulfonamide and N_3^- to the first site are in close agreement with those obtained previously by the titration of sulfacetamide bound to Mn(II) carbonic anhydrase, and by inhibition measurements [10]. Thus, the designation of this site as an inhibitory binding site is justified.

Temperature dependence of relaxation and T_1/T_2 ratio

Fig. 5 shows the temperature dependence of the transverse relaxation rate, $1/fT_{2p}$, for acetate ions at a concentration of 0.05 M. *f*, the fraction of inhibitor bound

TABLE I

AFFINITY OF SULFONAMIDE AND N_3^- TO THE TWO ANIONIC BINDING SITES OF Mn(II) CARBONIC ANHYDRASE

Type of binding site	Ligand	Negative x-axis intercept*	K_{I_2} (M^{-1})**	K_{I_2} (M^{-1})***
Inhibitory	p-toluenesulfonamide	$1.1 \cdot 10^{-4}$	$2.6 \cdot 10^{4}$	$2.9 \cdot 10^{4}$
Inhibitory	N_3^-	$1.04 \cdot 10^{-4}$	$2.8 \cdot 10^{3}$	$3.3 \cdot 10^{3}$
Noninhibitory	N_3^-	$1.0 \cdot 10^{-1}$	$2.8 \cdot 10^{2}$	—

* For experimental conditions, see Fig. 4.
** Calculated from the negative x-axis intercept values given in the previous column.
*** Taken from Lanir and Navon [10].

to the Mn enzyme was calculated using the value of the binding constants given in Fig. 1. At the acetate concentration used in this experiment, the binding of acetate to the inhibitory anionic binding site can be neglected. The relaxation rates show a positive temperature dependence (Fig. 5) which may indicate one of the following two alternative relaxation mechanisms: (a) The ligand exchange is slow and the relaxation is controlled by the rate of exchange of the ligand between the bulk phase and the paramagnetic environment. (b) The fast exchange condition is valid, and the correlation time is dominated by the electronic spin relaxation of the Mn^{2+}. One can distin-

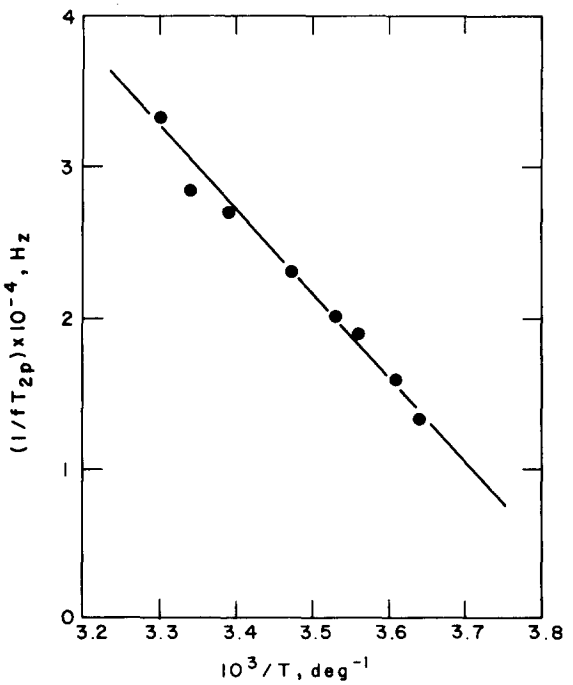

Fig. 5. Temperature dependence of the relaxation rate of acetate ions bound to Mn(II) carbonic anhydrase. The ^2H_2O solution contained $4.58 \cdot 10^{-5}$ M Mn(II) enzyme, 0.05 M sodium acetate and $2 \cdot 10^{-4}$ M $MnCl_2$ in $5 \cdot 10^{-3}$ M Tris–sulfate buffer, pH 8.0.

guish between these two possibilities by measuring the T_{1p}/T_{2p} ratio. While the slow exchange limit predicts a T_{1p}/T_{2p} ratio equal to unity, in the fast exchange region the T_{1p}/T_{2p} ratio is strongly dependent on the correlation time. The experimental T_{1p}/T_{2p} values at 100 MHz are given in Table II.

TABLE II

VARIOUS PARAMETERS OBTAINED FOR THE TWO ANIONIC BINDING SITES

Parameter	Type of binding site	
	Inhibitory	Noninhibitory
T_{1p}/T_{2p}	1.6	2.35
fT_{2p} (s)	$2.17 \cdot 10^{-5}$	$2.04 \cdot 10^{-5}$
T_{2M} (s)*	$0.20 \cdot 10^{-5}$	$0.41 \cdot 10^{-5}$
r (Å)**	4.3	4.8
τ_M (s)*	$1.97 \cdot 10^{-5}$	$1.63 \cdot 10^{-5}$
K_I (M^{-1})	0.8	29
k_{off} (s^{-1})	$5.08 \cdot 10^4$	$6.14 \cdot 10^4$
k_{on} ($M^{-1} \cdot s^{-1}$)	$4.06 \cdot 10^4$	$1.78 \cdot 10^6$

* τ_M and T_{2M} values were derived using Eqns 6 and 7, and a correlation time of $\tau_c = 5 \cdot 10^{-9}$ s.
** The distances between the Mn^{2+} and the methyl groups of the bound acetate ions in Mn(II) carbonic anhydrase.

Separation of T_{2M} and τ_M and calculation of distances

Since no chemical shift difference between the free and the bound acetate ions was observed, the following expressions for the relaxation times are valid:

$$fT_{ip} = T_{iM} + \tau_M \qquad i = 1,2 \tag{1}$$

$$\frac{T_{1p}}{T_{2p}} = \frac{T_{1M} + \tau_M}{T_{2M} + \tau_M} \tag{2}$$

where T_{1M} and T_{2M} are the NMR relaxation times of the bound acetate ions, and τ_M is the residence time of the acetate ion in the complex. For the Mn-containing enzyme in which $\omega_s^2 \tau_c^2 \gg 1$ and when the transfer hyperfine interaction can be neglected, the Solomon–Bloembergen equations for T_{1M} and T_{2M} simplify to:

$$\frac{1}{T_{1M}} = \frac{2}{15} \frac{S(S+1)\gamma_I^2 g^2 \beta^2}{r^6} \left(\frac{3\tau_c}{1 + \omega_I^2 \tau_c^2} \right) \tag{3}$$

$$\frac{1}{T_{2M}} = \frac{1}{15} \frac{S(S+1)\gamma_I^2 g^2 \beta^2}{r^6} \left(4\tau_c + \frac{3\tau_c}{1 + \omega_I^2 \tau_c^2} \right) \tag{4}$$

where S is the electron-spin quantum number; γ_I is the nuclear magnetogyric ratio; r, the Mn^{2+}–proton internuclear distance; g, the electronic g factor; β, the Bohr magneton; ω_I, the Larmor angular procession frequency for the nuclear spin; and τ_c, the correlation time, which is a function of T_{1M}/T_{2M} alone.

$$\frac{T_{1M}}{T_{2M}} = \frac{7}{6} + \frac{2}{3} \omega_I^2 \tau_c^2 \tag{5}$$

In a previous work [10] we have shown the possibility for separating T_{2M} and τ_M from their combined experimental value in a system where the signals of at least two groups can be observed in the molecule bound to the Mn enzyme. The same method cannot be applied for the acetate ion which obviously has only one NMR signal. However, the separation of T_{2M} and τ_M from the experimental $(T_{2M} + \tau_M)$ can be accomplished using the ratio T_{1p}/T_{2p} if the correlation time is given.

By rearranging Eqn 2, the following convenient expressions for T_{2M} and τ_M can be obtained:

$$T_{2M} = (T_{2M} + \tau_M)\frac{(T_{1p}/T_{2p}) - 1}{(T_{1M}/T_{2M}) - 1} \tag{6}$$

$$\tau_M = (T_{2M} + \tau_M)\frac{(T_{1M}/T_{2M}) - (T_{1p}/T_{2p})}{(T_{1M}/T_{2M}) - 1} \tag{7}$$

The ratio T_{1M}/T_{2M} is calculated from the correlation time according to Eqn 5. We adopt for this calculation the correlation time of the Mn(II) carbonic anhydrase–sulfacetamide complex, which is very similar to the present system. At 30 °C the correlation time was $\tau_c = 5 \cdot 10^{-9}$ s [10], which yields $T_{1M}/T_{2M} = 7.73$. The values of τ_M and T_{2M} calculated using Eqn 6 and 7 are given in Table II. Substituting $\tau_c = 5 \cdot 10^{-9}$ s and T_{2M} values in Eqn 4, the distances from the metal ion in Mn(II) carbonic anhydrase to the methyl group of the two bound acetate ions could be evaluated.

The values $k_{off} = \tau_M^{-1}$ for the acetate ions–Mn(II) enzyme complexes were obtained from the calculated exchange lifetime. Combining τ_M and the equilibrium constants, the kinetic rate constants, k_{on}, for the enzyme–anion binding reactions could be derived, using the expression $K_I = k_{on}/k_{off}$. All the parameters are summarized in Table II. It may be noted that the value of k_{off} is similar for the two acetate binding sites in the Mn(II) enzyme, and is also similar to those obtained for sulfacetamide–Mn(II) enzyme ($k_{off} = 7.4 \cdot 10^4$ s^{-1} at 30 °C [10]) and for carboxylate anion–Co(II) carbonic anhydrase complexes [3]. On the other hand, k_{on} varies considerably for the different inhibitors and metallo-carbonic anhydrases.

DISCUSSION

Investigation employing magnetic resonance methods [1, 4–6] confirmed the binding of two anion molecules close to the metal ion in the Zn(II) and Co(II) carbonic anhydrases. In the present communication, these works have been extended to the Mn-substituted enzyme, in which direct distances between the methyl groups of bound acetate ions and the paramagnetic Mn^{2+} could be calculated. The Mn enzyme, like the native enzyme, was also found to bind two acetate molecules, only one of which gave rise to inhibition of the enzymatic activity and could be titrated with the sulfonamide inhibitor. The distance between the methyl group of this acetate ion and the Mn^{2+} was calculated from the relaxation rate of the bound anion, and was found to be 4.3 Å (Table II). This distance is consistent with a model in which the carboxylic group of the acetate anion is directly bound to the metal ion. Direct coordination of the inhibitor anions with the single Zn atom in the native enzyme has been suggested before by Ward using ^{35}Cl NMR relaxation measurements [15, 16]. It should be noted that the broadening effects of halide ions due to binding to proteins were also ob-

tained in cases where no metal ion was present [17, 18]. Such a broadening is expected for the binding of Cl^- to any positive center on the protein, e.g. protonated histidine residues, because of the long rotational correlation time for the quadrupolar interaction. However, comparison of the magnitude of this effect to that obtained by Ward for Cl^- bound to carbonic anhydrase indicated a larger effect by an order-of-magnitude in the latter case, which probably originated from the metal-ion field gradient.

Our NMR results for the inhibitory anionic site conform to other indirect proofs for metal ion–anion coordination which includes visible and infrared spectroscopy [19, 20] and solvent relaxation measurements [21]. By the latter technique, Fabry et al. [21] found that anions could displace a water molecule which apparently exists within the coordination sphere of the metal in Co carbonic anhydrase. On the other hand, this single water molecule could not be titrated by anion ligands in the Mn-substituted enzyme [22]. It seems that in that case the anion molecule possibly increases the number of metal ligands to five, without affecting the interaction of the Mn with the water protons. Similar effects were previously reported for F^- bound to Mn carboxypeptidase A [14]. Also, it was found that NMR parameters of water molecules bound to Mn–EDTA complexes are similar to those in the $Mn(H_2O)_6^{2+}$ in spite of the increased coordination number of 7 in the EDTA complex [23, 24].

The two anionic binding sites located in the active-site region may explain the number of positive densities, higher than the solvent densities, in the active center of the enzyme, observed by X-ray difference maps between the anion derivative and the native enzyme.

The second "noninhibitory anion binding site" was found in the present work to be slightly further apart from the metal ion. Although the distance obtained, $r = 4.8$ Å, is still within the limits allowed for direct metal carboxylate coordination of the acetate ion, the fact that it does not inhibit the enzyme activity and cannot be replaced by sulfonamide inhibitors suggests that this acetate ion is bound to a different group. A possible candidate is a protonated histidine group, analogous to the binding of $Au(CN)_2^-$ to the human carbonic anhydrase C [7]. Such a binding site for the noninhibitory acetate ion is also compatible with the marked pH dependence of the acetate binding to this site, which was observed for the Zn carbonic anhydrase B [1].

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