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The Factors Governing the Coordination Number in the Anion Derivatives of Carbonic Anhydrase

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The electronic and ^1H NMR spectra of cobalt(II)-substituted carbonic anhydrase in the presence of NCS^-, N_3^- and NCO^- on one side and CH_3COO^-, ClO_4^- and NO_3^- on the other are presented here and discussed in the light of the available X-ray data on two kinds of inhibitors. The aim of this article is to focus on the factors which determine the coordination number of the inhibitor derivatives. It is proposed that the hydrophobic or the hydrophilic character of the inhibitors provide an important contribution to the energy balance between four and five coordination.

Key Words: *carbonic anhydrase, ^1H NMR spectroscopy, electronic spectroscopy, anion binding to metalloenzymes, cobalt-substitution in metalloenzymes*

INTRODUCTION

Carbonic anhydrase (CA hereafter) is a zinc enzyme that catalyzes a simple but fundamental reaction, the hydration of carbon dioxide[1-3]:

$$CO_2 + H_2O \rightleftarrows HCO_3^- + H^+$$

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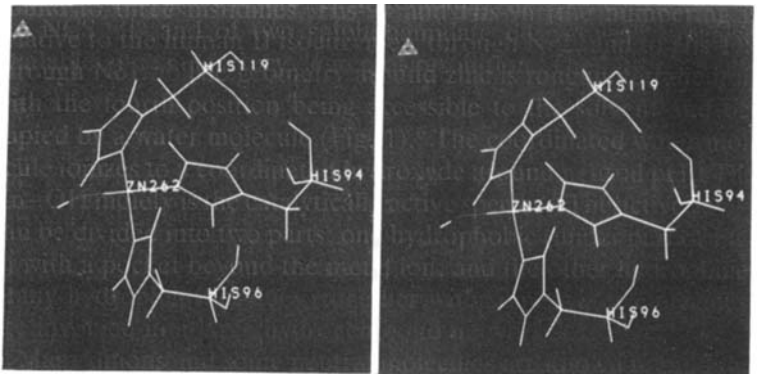


FIGURE 1 Stereo view of the donor groups in carbonic anhydrase. (See color centerpiece.)

The enzyme has a MW of 30,000 and is constituted by a single polypeptide chain. It contains an essential zinc ion; the zinc is bound to three histidines, His-94 and His-96 (the numbering is relative to the human II isoenzyme), through Nε2, and to His-119 through Nδ1.[4,5] The geometry around zinc is roughly tetrahedral, with the fourth position being accessible to the solvent and occupied by a water molecule (Fig. 1).[6] The coordinated water molecule ionizes to a coordinated hydroxyde around neutral pH.[7] The Zn–OH moiety is the catalytically active species.[8] The active cavity can be divided into two parts, one hydrophobic (upper part of Fig. 2) with a pocket beyond the metal ion, and the other hydrophilic. Many hydrophilic residues, together with some water molecules, are involved in a large hydrogen-bond network.

Many anions and some neutral molecules are known to bind the zinc ion and to inhibit the enzyme.[7,9,10] They may displace the coordinated water molecule so that the metal ion remains tetracoordinated, or add to the N_3O donor set giving rise to a five-coordinated adduct.[11]

The X-ray structure of the human II isoenzyme has been recently refined at 2.0 Å of resolution.[6] This isoenzyme has a high homology with the bovine isoenzyme (80% homology).[12–15] The structure of the NCS^- [16] and of two sulphonamidate derivatives[16] are also available at a satisfactory degree of resolution. The latter are tet-

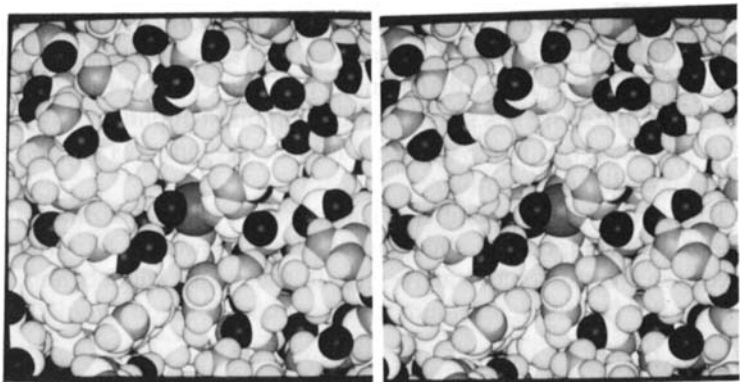


FIGURE 2 The active cavity of human carbonic anhydrase II viewed as CPK model (Ref. 6). The zinc ion is the green sphere at the bottom of the cavity (center of the figure). The color codes are: C = white, H = cyan, N = blue, O = red, S = yellow. Among the zinc ligands, the His-94 ring and the OH group are visible at the right- and left-hand sides of the zinc ion, respectively, while the His-96 ring is below the zinc ion, partially covered by the ring of His-64. (See color centerpiece.)

racoordinated, with the inhibitor NH^- moiety occupying the OH^- (H_2O) position. This binding site was called A.[16,17] The NCS^- adduct is five coordinated with a water molecule bound in a binding site of the hydrophilic part of the cavity, called C, while the exogenous ligand is located in the hydrophobic pocket at a site called B. The structural characterization has also shown that the residues coordinated at the metal site and those nearby essentially maintain the same position upon inhibitor binding. These structural studies confirm the coordination arrangements proposed on the basis of spectroscopic investigation[7-11] and of EXAFS studies.[18]

In the light of this recent information, we would like to reexamine the electronic and ^1H NMR spectra of the derivatives of cobalt(II)-substituted carbonic anhydrase with NCO^-, NCS^-, N_3^- on one side, and acetate, nitrate, perchlorate on the other in order to understand what are the factors determining the coordination geometry. The choice of the cobalt(II)-substituted carbonic anhydrase is due to its suitable spectroscopic properties,[19,20] the enzymatic activity being comparable with that of the native enzyme.[21] The electronic spectra of all the compounds but the perchlorate had already been reported,[11,22] whereas the ^1H NMR spectra have

been recorded again at higher magnetic field in order to relate the signals from one derivative to another and to have a closer look at the signals close to the diamagnetic position. The protons giving rise to the latter signals sense, through dipolar coupling, the magnetic anisotropy of the system and may be exploited as indicative of the coordination number.

These NMR studies and electronic spectra characterization together with computer graphics studies have allowed us to perform a deeper analysis of the active site in the many adducts and to discuss the factors determining the final coordination geometry.

RESULTS AND DISCUSSION

Electronic Spectra

The electronic spectra in the range 10,000–25,000 cm^{-1} of the adducts of cobalt(II)-substituted CA with the inhibitors under investigation at pH 6.2–6.4 are reported in Fig. 3 together with those of the native derivative.[23–27] We should note here that the various anions give rise to a large variety of spectral intensities. Acetate, nitrate and, to a smaller extent, perchlorate induce a decrease in the molar absorption coefficient. A small absorption at 14,000 cm^{-1} is evident, which was proposed to be diagnostic of five coordination.[7,11] Azide does not appreciably change the intensity of the spectra with respect to the uninhibited enzyme; cyanate, on the other hand, induces a dramatic increase in the molar absorbance together with a decrease in the transition energies. These data are in agreement with those already reported for acetate,[23] nitrate[24] and pseudohalides.[11,25,26] As already suggested, acetate and nitrate are considered five coordinated and cyanate tetrahedral.[7,10,11] The other anions give rise to an equilibrium between four and five coordination.[7,28]

^{1}H NMR Titration

The 200 MHz ^{1}H NMR spectra of the completely formed adducts with the anions are reported in Fig. 4; the shifts and T_1 values are collected in Table I. When available, the literature data agree with the present findings. All of the spectra show three or four signals

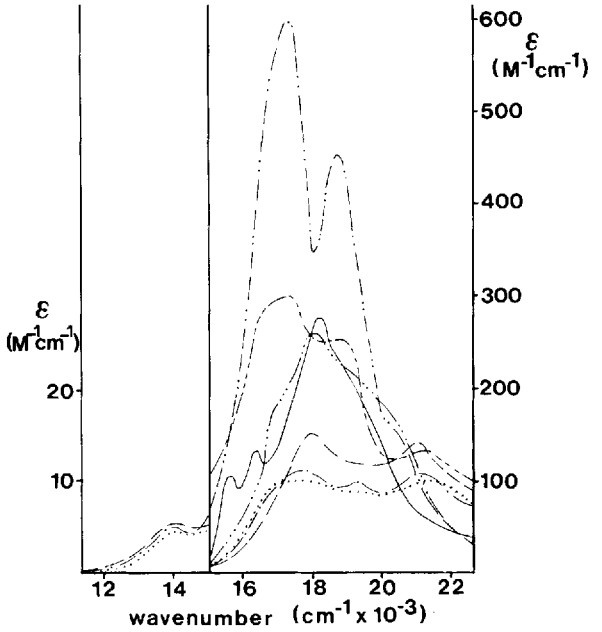


FIGURE 3 Electronic spectra of unbuffered solutions of some anion adducts of cobalt(II)-substituted CA at pH 6.2–6.4. (— native, ... + NCS^-, – · – + CH_3COO^-, – – – + NO_3^-, – · · · – + ClO_4^-, – – – – + N_3^-, – – · · · – – + NCO^-).

far downfield and, in some cases, some broad and ill-defined signals. The three or four resolved signals account for the four protons that are in a meta-like position with respect to the metal ion (see Scheme I). Three of them are exchangeable and are assigned to the ring NH protons of the three coordinated histidines, while the non-exchangeable one is assigned to the Hδ2 of His-119. The ring NH of the same histidine is assigned on the basis of the field dependence of the linewidth[29] and NOE experiments.[30]

The signals of all the derivatives but cyanate could be followed through titration because the free–bound inhibitor exchange is fast or quasi-fast on the NMR time scale.[31] In some derivatives the signal of the ring NH of His-119, which experiences a large variation in the shift upon anion binding, becomes broad and then

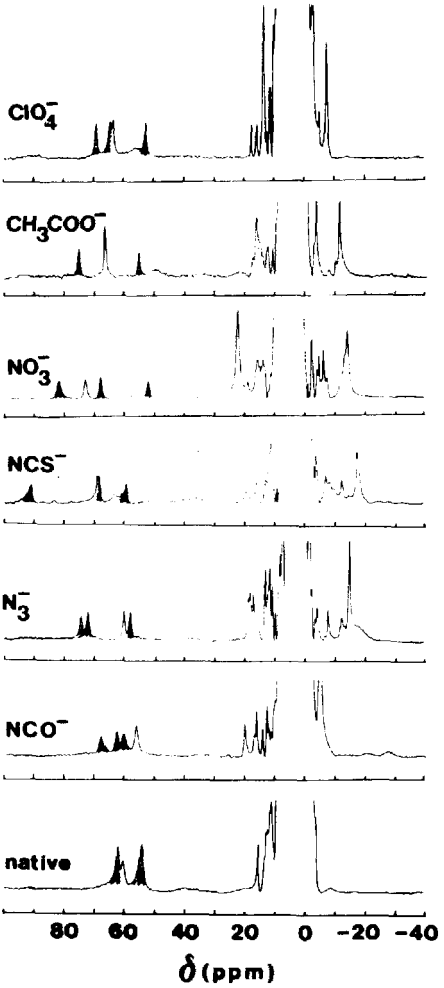


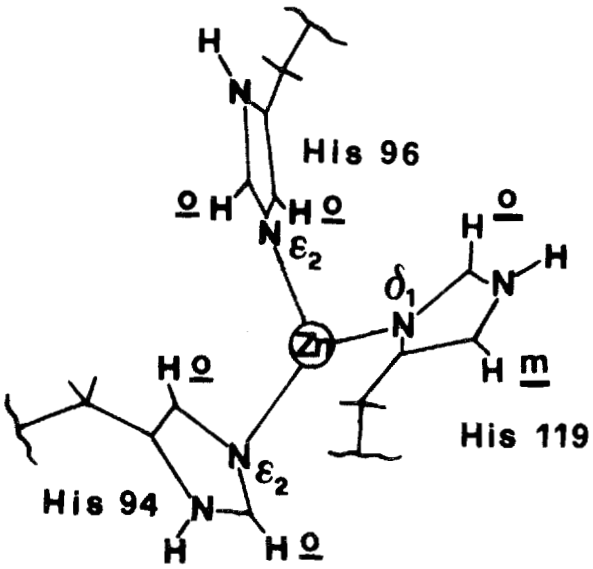
FIGURE 4 ^{1}H 200 MHz NMR spectra at 300 K of some anion adducts of cobalt(II)-substituted CA. The samples were in HEPES (2[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid) buffer at pH 6.2–6.4. The dashed signals disappear when the spectra are recorded in D_2O.

TABLE I

200 MHz ^{1}H NMR shifts and T_1 values[a] for some anion derivatives of bovine Co(II)-substituted carbonic anhydrase

Signal	Native	+ CH_3COO^-	+ NO_3^-	+ ClO_4^-	+ NCS^-	+ NCO	+ N_3^-
NH-His	62.1 (7.3)	66.0 (20.3)	66.2 (17)	69.0 (9.5)	68.7 (18)	62.1 (3.1)	57.8 (6.2)
m-His 119	60.3		70.1 (12)	63.4 (5.7)	69.0	55.5 (3.6)	59.9 (7.8)
NH-His 119	54.2 (8.1)	75.0 (19.5)	78.7 (12)	64.3 (5.4)	91.2 (14)	59.8 (2.5)	74.1 (5.5)
NH-His	54.2	55.0 (22.5)	51.1 (27)	52.4 (14.6)	59.5 (25)	67.3 (2.2)	71.7 (5.4)
downfield CH_3 group	<6	15.7 (24)	20.1 (19)	13.3 (10)	11.4 (−)	<10	<10
upfield CH_3 group	≃ −3	−11.8 (40)	−13.1 (28)	−7.7 (20)	−17.3 (44)	−5.6 (7)	−14.9 (17)

[a] Only the meta-like signals for the histidine rings are reported.



SCHEME I

narrow again during the titration. In the case of cyanate, the free–bound exchange rate is slow on the NMR time scale, thus preventing the possibility of following the proton signals. In this case a titration with cyanate on the nitrate adduct was performed in order to decrease the apparent affinity constant and to relate the signals of the former adduct with those of the latter, which in turn are related to those of the native derivative.

Analysis of the limit spectra shows a large variety in the behavior of the paramagnetically shifted signals upon inhibitor binding. The signal assigned to the ring NH of His-119 in all the adducts but the cyanate derivative experiences the largest variation in shift and, except in the perchlorate adduct, is the most downfield shifted signal. The meta-like proton of the same histidine, on the other hand, does not seem to follow the same pattern as the NH signal, in some cases remaining almost unaffected by anion binding. In Fig. 5 the pattern of the shift values for the four meta-like signals for the various inhibitors is reported.

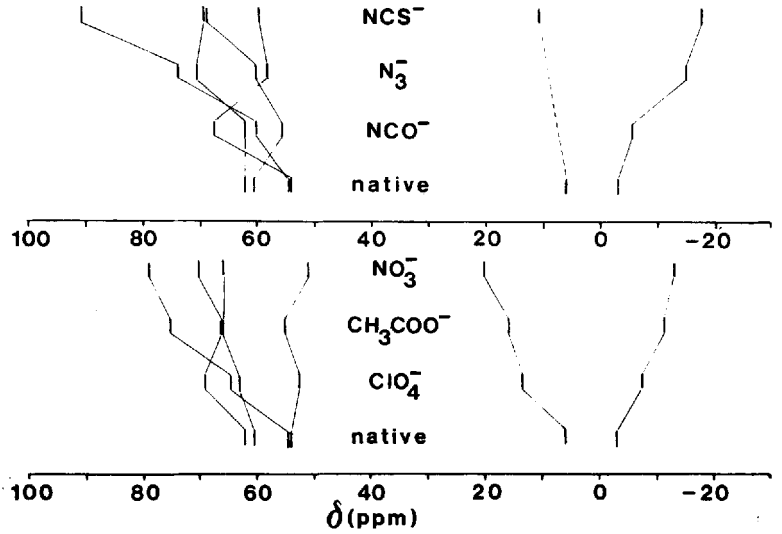


FIGURE 5 Pattern of the shift values for the hyperfine shifted signals in the anion adducts of cobalt(II)-substituted carbonic anhydrase, reported in Fig. 4.

In Fig. 6 the expanded 25/−20 ppm region of the spectra of all the derivatives is reported. Nitrate, acetate and perchlorate adducts show a downfield shifted signal of intensity 3, whose shift ranges from 21 to 13 ppm, respectively. In the NCS^- derivative it experiences a smaller shift (11 ppm), while the NCO^- and N_3^- adducts do not show this signal above 10 ppm outside the diamagnetic envelope. In addition, all the adducts show a three proton intensity signal shifted upfield. These two methyl signals and the signals in between presumably experience pseudocontact shifts, i.e., are close to the metal ion but do not belong to groups directly coordinated to the metal ion. Inspection of the crystal structure shows that among several candidates there are the methyl groups of Thr-199, Thr-200, Val-143 and Val-121.

Pseudocontact shifts are due to the anisotropy of the magnetic susceptibility tensor.[31] It has already been discussed that five coordinated complexes have larger orbital contribution to the magnetic susceptibility and therefore larger anisotropy of the magnetic

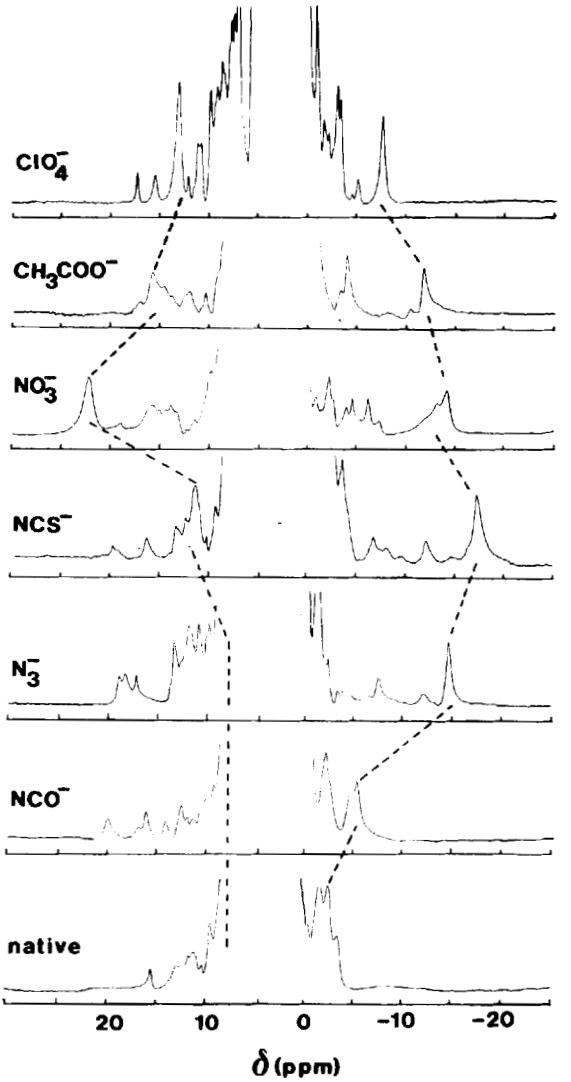


FIGURE 6 Expanded "quasi-diamagnetic region" of the ^{1}H 200 MHz NMR spectra of some anion derivatives of cobalt(II)-substituted CA. Experimental conditions are the same as in Fig. 4.

susceptibility tensor ($\Delta\chi$)[27,31] than pseudotetrahedral complexes do. The different patterns for the two sets of inhibitors could indicate that a different orientation of the magnetic susceptibility tensor (χ) could occur, whereas the extent of the shifts, i.e., the spreading of the signals in the near diamagnetic region, depends on the share of five coordination.

The T_1 values of protons of histidine rings bound to a paramagnetic metal ion may be strongly dominated by ligand centered contributions.[32] This makes the analysis of the T_1 values and the estimation of the electronic correlation times, τ_s, quite difficult. However as already discussed,[7,10,27] the longer the T_1's, the larger the share of five coordination. Therefore we have three criteria to decide the coordination number: (i) the intensity of the electronic spectra; (ii) the absolute magnitude of the pseudocontact shifts experienced by the protons of residues not directly coordinated to the metal ion; (iii) the T_1 values.

From the three criteria it appears that thiocyanate, acetate and nitrate are on one extreme, in the sense that they provide five coordinated derivatives. On the other side the cyanate adduct is tetrahedral, whereas perchlorate and azide adducts, and possibly the native enzyme, display an intermediate behavior.

Analysis of the Factors Determining the Coordination Number

In order to proceed with the analysis of the data it may be convenient to treat separately N_3^-, NCS^- and NCO^- on one side and NO_3^-, ClO_4^-, and acetate on the other.

The structure of the NCS^- derivative is reported in Fig. 7.[16] The sulphur atom is reported to interact with the residues Val-143, Leu-198 and Trp-209, i.e., with the hydrophobic pocket of the active cavity. Such interactions contribute to the stability of the fifth (or B) coordination site. The coordinated water molecule has changed its position, though it still interacts with the OH group of Thr-199. In agreement with this structure, the intensity of the electronic spectra is low and the energy of the transitions is spread over a large energy range. A weak band is observed at about 14,000 cm^{-1}, which is assigned to the high energy $F \rightarrow F$ transition. Five coordinated cobalt(II) complexes have low lying excited energy levels which induce large zero field splitting, large magnetic anisotropy and short electronic relaxation times. The last property

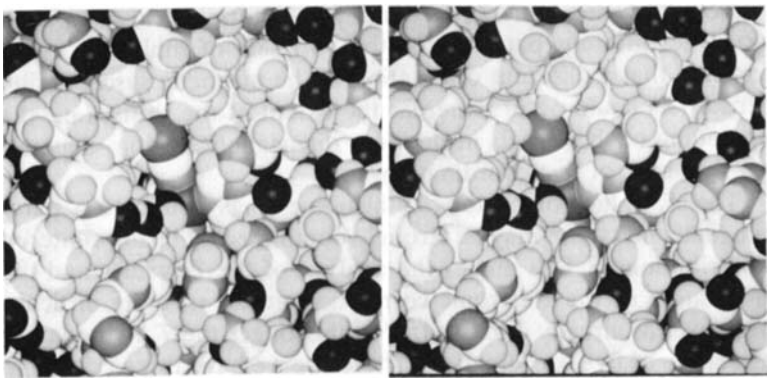


FIGURE 7 Structure of the NCS^- adduct of human carbonic anhydrase II viewed as a CPK model (Ref. 16). The NCS^- ion occupies a binding site (B site) that is more buried than the binding site of the OH^- ion in the native form (A site). The water molecule in the NCS^- adduct occupies the C site, which is pointing more towards the entrance of the cavity. (See color centerpiece.)

is reflected in the longest proton T_1 values, because they are inversely proportional to the electronic relaxation times.[31] The large magnetic anisotropy gives rise to pseudocontact contributions to the isotropic shifts and spreads the NMR signals. This is easily seen from Figs. 4 and 6. The NCS^- derivative shows the histidine signals far apart from each other, and the signals in the $25/-20$ ppm region numerous and well spread. The assignment of the latter signals is a hard task, but we refer for the present discussion to the upfield signal of intensity 3 present in all the derivatives and the downfield signal, again of intensity 3, present in the NCS^-, NO_3^-, CH_3COO^-, and ClO_4^- derivatives (see Table I).

Cyanate has just the opposite behavior: small spreading of the NMR signals, short T_1 values and intense electronic spectrum. We propose that NCO^- substitutes water in the A site. From computer graphics analysis, it appears reasonable that the terminal oxygen atom interacts through H bonds with the NH of Thr-199, the NH of Thr-200 and the OH of Thr-199 (Fig. 8). The difference in behavior between NCS^- and NCO^- is possibly determined by the different hydrophobic or hydrophilic properties of the terminal atom. It is now reasonable to accept that the terminal nitrogen of N_3^- is intermediate between oxygen and sulphur and that the

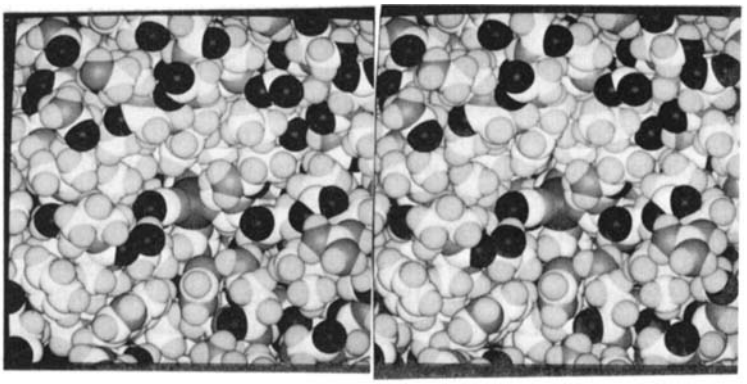


FIGURE 8 Computer graphics representation (as CPK model) of the proposed binding mode of the NCO^- anion to human carbonic anhydrase II. (See color centerpiece.)

resulting adduct gives rise to a four–five coordination equilibrium, though shifted towards tetracoordination. It is also possible that the native enzyme shares some fraction of pentacoordination.

In the case of nitrate, acetate, and perchlorate, low intensity of the electronic spectra, large spreading of the NMR signals and long T_1 values suggest that the three derivatives are largely five coordinated, though the percentage of tetracoordinated species increases in the case of perchlorate. It would be tempting to suggest that these anions bind in a bidentate fashion in the A site. Owing to the similarity of NO_3^- with the natural substrate HCO_3^-, this point is particularly meaningful. However, the terminal O atoms would not be in a position to form any H-bond with neighbor groups.

The ^1H NMR spectra of the nitrate derivative are similar to those of the thiocyanate derivative, the minor differences being possibly ascribed to the difference between N and O as donor atoms. Even the signals at about 20 and -13, 16 and -12, and 13 and -7 ppm in the nitrate, acetate and perchlorate adducts, respectively, are due to the same protons as shown from titration, and compare well with 11 and -17 ppm observed in the case of tiocyanate. The two pairs of signals are at the extremes of the near-diamagnetic portion of the spectrum. This shows that the four derivatives that experience five coordination are quite similar, since

a different type of coordination would be expected to give rise to severe scrambling of signals. Water ^1H Nuclear Magnetic Relaxation Dispersion (NMRD) has shown that water is present in the coordination sphere together with nitrate.[33] Therefore we propose that NO_3^-, CH_3COO^- and, to a lesser extent, ClO_4^- sit in the hydrophobic pocket B and water remains coordinated but moves to the C site. The charge is delocalized on the oxygen atoms of these anions, and they may well contribute to hydrophobic interactions as in sulphonamides one oxygen atom of SO_2 moiety does.[16]

It therefore appears that the preference of one coordination geometry with respect to another depends on a number of factors. The strength of the coordination bond favors tetracoordination as in small complex compounds. Indeed, CN^- and SH^- form tetracoordinated adducts.[11,33] The ability to form hydrogen bonds with Thr-199 or Thr-200 again favors tetracoordination. OH^-, SH^-, CN^-, the NH group of the sulfonamidato moiety and NCO^- give rise to H-bonds with the above residues and tetrahedral adducts. If these anions are largely hydrated in solution they liberate the water molecules of their solvation shells, providing an entropic contribution to strong binding. Anions like NCS^-, I^-, and CH_3COO^- bind in the B site providing hydrophobic contacts; their solvation energy is smaller than for some of the previously discussed anions. A water molecule still remains coordinated to the metal and interacting with Thr-199. Apparently, NO_3^- and, to a lesser extent, ClO_4^- belong to the latter class of compounds. Several other anions give rise to equilibria between the two species, indicating that the energy barrier is small and the interconversion kinetic fast.

About the Catalytic Mechanism

It is now accepted that HCO_3^- binds the metal ion giving rise to an equilibrium between tetra and penta coordinated species. CO_2 is attracted inside the cavity by the hydrophobic pocket and under these circumstances could be activated by interacting, at the B site, with the zinc ion. Ab initio[34] and molecular dynamics[35] calculations show that a weak interaction between the terminal oxygen of CO_2 and the zinc ion may well take place. The OH group in the A site would be in the right position to perform the nucleophilic attack, providing a transition state with a bidentate bicarbonate moiety. At this stage the system may evolve towards two new species in

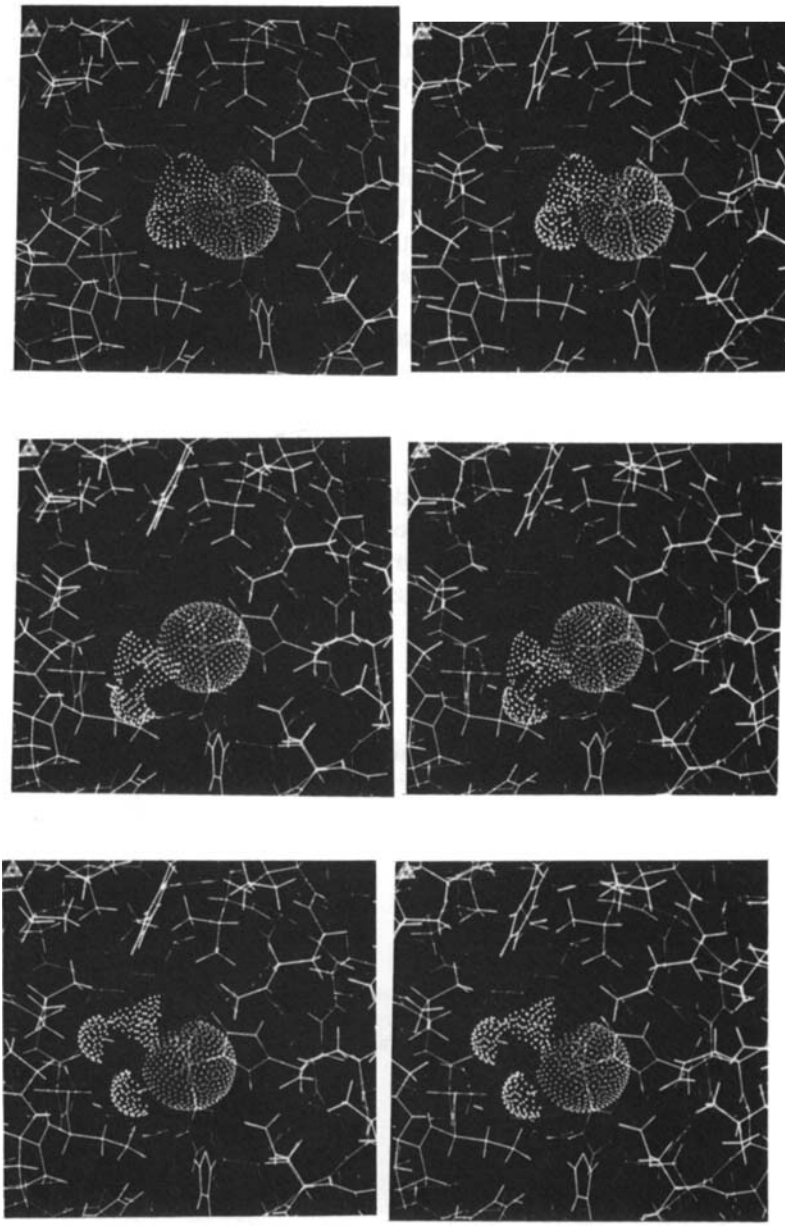


FIGURE 9 The possible binding schemes of HCO_3^- to carbonic anhydrase. (See color centerpiece.)

equilibrium between themselves (Fig. 9). A specific binding site for CO_2 is not necessary. An increased concentration of CO_2 in the hydrophobic part of the cavity could also account for the fast attack performed by Zn–OH. ^{13}C data on the CO_2–HCO_3^- system in the presence of copper(II)-substituted CA are consistent with both models.[36] It is possible that HCO_3^- in the four coordinated adduct interacts with Thr-200. The five coordinated form is probably necessary to allow the bicarbonate anion to leave the coordination sphere.[7] In this way the acidic form of the enzyme is again formed.

Some researchers have suggested from ^{13}C NMR studies on HCO_3^- interacting with manganese(II)-substituted CA that the anion binds in a bidentate fashion because the distance between the metal ion and the resonating nucleus is found to be short.[37,38] The matter is subtle since, once HCO_3^- is coordinated in a monodentate fashion, unpaired spin delocalization on the ligand occurs which may simulate a shorter carbon–metal ion distance. However, the possibility of some share of bidentate behavior of HCO_3^- cannot be ruled out at this stage.

EXPERIMENTAL SECTION

The samples were prepared as already reported;[11,28] the electronic spectra were collected on a Cary 17D; the ^1H NMR spectra were recorded on a Bruker MSL 200 using the super-WEFT pulse sequence.[39]

Acknowledgments

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