Characterization of Cobalt(II) Bovine Carbonic Anhydrase and of Its Derivatives

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Abstract: Cobalt bovine carbonic anhydrase and a number of inhibitor derivatives have been investigated by electronic spectroscopy, in the range 8000–25 000 cm^{-1}, and by ^1H longitudinal relaxation studies of water protons. The electronic spectra have distinguished between four- and five-coordinate derivatives. The two forms of the enzyme are essentially pseudotetrahedral. ^1H T_1 values have been interpreted as being due to the presence of exchangeable protons of water bound to the metal ion at every pH. Some inhibitors replace water, whereas others bind to a fifth donor position.

X-ray data at 2.0 Å of resolution have shown that the zinc(II) ion in carbonic anhydrase is bound to three histidyl nitrogens in an arrangement which has been considered typical of a distorted geometry.[1] Although the problem is still quite controversial,[2-5] a fourth group has been guessed to be a water molecule.[6-8]

The cobalt analogue, which substantially retains the enzymatic activity of the native enzyme,[9,10] shows an electronic spectrum in the visible region which is strongly pH dependent.[3] The equilibrium between an acidic and a basic form of the enzyme can be represented as:

$$EH^+ \rightleftharpoons E + H^+ \qquad (1)$$

with a pK_a of 6.6.[3]

The electronic spectra of both the acidic and basic forms are typical of a low symmetry chromophore, and susceptibility measurements indicate that the cobalt(II) ion is high spin.[11] These data could be consistent with either a tetracoordinate pseudotetrahedral or five-coordinate structure, since planar complexes are expected to be low spin and six-coordinate complexes are expected to have quite a lower molar absorbance of the absorption bands.[12-16] Any further statement on the geometry of the chromophore was mostly based on the authors' personal feelings rather than on experimental evidence.

Relaxivity data on water protons in solutions containing cobalt carbonic anhydrase were consistent with the hypothesis that the low pH form of the enzyme contained a slowly exchanging water molecule or no water at all.[4,17]

The recent findings that high-spin five-coordinate cobalt(II) complexes are relatively common[18-21] and the full understanding of the electronic properties of these complexes[20-22] led us to reinvestigate the electronic spectra of the cobalt carbonic anhydrase and of its inhibitor derivatives extended down to the near-infrared region. The conclusion of such research induced us to repeat the ^1H relaxivity measurements in water solutions containing the cobalt enzyme derivative,[23] prepared under different experimental conditions.

In order to evaluate the extent of the interaction between the enzyme and the inhibitors, the apparent affinity constants have been determined when not available in the literature. The relationship between the latter constant, K_{app}, and the actual affinity constant, K_I, is:

$$K_{app} = K_I \left(1 + \frac{K_a}{[H^+]}\right)^{-1} \qquad (2)$$

However, when the pH is lowered the protein part of the enzyme becomes more and more protonated in such a way that there are other effects besides that relative to eq 1. As a matter of fact, binding of anions is more and more favored even with respect to eq 2 when the pH is lowered.[24]

Experimental Section

Bovine carbonic anhydrase (EC 4.2.1.1) was purchased as a lyophilized powder from Sigma Chemical Co. All reagents used were of analytical grade. Native enzyme concentrations were determined from the absorbance at 280 nm, using a molar absorbance of 5.7×10^4 M^{-1} cm^{-1},[25] and enzymatic activities were assayed using *p*-nitrophenyl acetate as substrate, at 25 °C.[26] Carbonic anhydrase B was obtained by chromatography on DEAE-cellulose and apocarbonic anhydrase was prepared according to the usual procedure, as previously described.[9] The cobalt derivative was obtained by dialyzing apoenzyme solutions against twice-distilled water to remove 1,10-phenanthroline and acetate buffer, then against unbuffered solutions of 10^{-3} M cobalt sulfate (pH 7–8), and finally against freshly distilled deionized water, until the isoionic pH of 5.5 was reached.[27] The pH was monitored on each single sample of enzyme using a glass microelectrode. Cobalt(II) bovine carbonic anhydrase concentrations were determined by measuring the absorbance at both 280 and 550 nm (ϵ_{550} = 250 M^{-1} cm^{-1} at pH 6.0). Concentrations obtained from the measurements at the two wavelengths were the same within 10% error, indicating that the protein was almost entirely in the cobalt enzyme form.

Spectrophotometric Measurements. Optical spectra were recorded on a Cary 17 spectrophotometer, on the 0–0.1 absorbance range, using 10^{-3}–10^{-4} M solutions of cobalt enzyme. The pH dependence of the d–d spectrum of cobalt(II) carbonic anhydrase was followed between pH 5.5 and 10.0 by adding increasing amounts of NaOH to the unbuffered enzyme at the isoionic pH. The electronic spectra of the various adducts were recorded on samples obtained by mixing unbuffered solutions of enzyme and inhibitor at the same pH. The pH was monitored in situ after every addition using a microelectrode. The affinity constants of the inhibitors were determined by following the spectral changes in the visible region under addition of increasing amounts of ligand.

Relaxivity Measurements. Proton T_1 measurements in water solutions were carried out on a Varian CFT 20 spectrometer, operating at 15 °C. The cobalt enzyme concentration varied from 2 to 5×10^{-3} M. The pH was varied from 5.5 to 9.0 by addition of NaOH. Inhibitors were added until no further variations in both electronic spectra and relaxivity data were observed. The pH of each single sample was monitored using a glass microelectrode. The assembly for the NMR experiments consisted of two coaxial tubes: the inner tube contained the enzyme solutions, whereas the outer one contained hexadeuterioacetone and Me_4Si (1%). In such a way, the intensity of the ^1H signal was conveniently reduced and the presence of Me_4Si allowed checking the absence of chemical shift for the water protons. The chemical shift of the acetone signal with respect to the transmitter offset was found completely insensitive to the presence of the paramagnetic species at the used enzyme concentrations. T_1 measurements were performed using the inversion recovery method.

Results and Discussion

Electronic Spectra. The electronic spectra of the acidic and basic forms of the enzyme as well as of the adducts with a large number of inhibitors have been measured. Some typical spectra

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Table I. Affinity Constants and Spectral Data for Some Cobalt(II) Bovine Carbonic Anhydrase Derivatives

inhibitor	log K	band position ($cm^{-1} \times 10^{-3}$) and molar absorptivity ($M^{-1}\ cm^{-1}$)
pure enzyme at pH 5.9		8.2 (47), 10.5 (13) sh, 15.6 (100), 16.2 (135), 18.1 (250), 19.2 (180)
pure enzyme at pH 8.0		8.2 (80), 11.0 (18), 15.6 (260), 16.2 (280), 18.1 (380), 19.2 (280)
acetate	2.1[a] pH 7.5	8.3 (7), 10.2 (6), 14.1 (9), 18.0 (110), 19.4 (80) sh, 21.2 (100)
acetazolamide	6.8,[a,b] pH 8.0	7.9 (65), 9.2 (65), 11.0 (45), 16.8 (500) sh, 17.4 (530), 18.2 (220), 19.3 (390)
aniline	1.2[a] pH 7.5	8.9 (100), 10.5 (55), 17.4 (470) sh, 17.9 (580), 18.9 (460)
azide	3.6,[a] pH 7.5	8.6 (24), 10.3 (31), 10.6 (30), 15.6 (65) sh, 17.6 (250), 18.4 (210), 21.2 (110)
benzoate	1.8,[a] pH 7.5	8.5 (15), 10.6 (6), 13.2 (4), 16.9 (160) sh, 18.0 (220), 19.7 (170) sh, 20.8 (110) sh
bromide	2.1[c]	8.5 (3), 10.5 (7), 14.0 (10) sh, 16.8 (200), 18.2 (200), 18.7 (90) sh, 20.3 (170)
chloride	1.7[c]	8.0 (14), 10.6 (14), 13.9 (4) sh, 16.9 (270), 18.1 (220), 20.2 (160)
cyanate	5.1[c]	8.0 (60), 10.7 (75), 17.4 (600), 19.1 (450), 20.4 (180) sh
cyanide	>5,[a,d] pH 8.0	7.8 (100) sh, 8.7 (120), 10.2 (110) sh, 17.2 (700), 18.4 (500) sh
fluoride	1.6[c]	7.8 (15), 8.4 (15), 9.0 (22), 9.7 (19) sh, 10.7 (16), 16.9 (170) sh, 18.2 (270), 19.2 (210) sh
formate	4.1,[a] pH 6.0	10.2 (18), 11.0 (11) sh, 13.6 (15), 17.8 (130), 18.2 (120) sh, 18.7 (110) sh, 22.0 (55) sh
hydrogen carbonate	1.0,[a] pH 8.2	7.8 (19), 8.5 (21), 8.8 (17), 11.0 (9), 15.6 (85), 16.3 (105), 18.2 (220), 19.4 (170) sh
hydrogen sulfite	>5,[a] pH 6.5	10.2 (4), 13.6 (8) sh, 18.2 (110), 20.0 (90) sh
hydrosulfide	5.8,[a,e] pH 8.2	7.8 (25), 10.2 (30) sh, 10.8 (32), 16.3 (600), 18.4 (400)
iodide	3.0[c]	10.5 (1), 13.2 (9), 15.9 (80) sh, 16.7 (120) sh, 18.1 (170), 18.9 (180), 20.1 (190)
nitrate	3.5[c]	8.6 (16), 10.2 (9), 14.2 (8), 18.0 (130), 21.1 (100)
oxalate	2.5,[a] pH 7.5	14.5 (14), 17.7 (165), 19.3 (110) sh, 21.6 (100)
2,4-pentanedione	1.7[a] pH 6.0	9.2 (9), 11.0 (7), 13.7 (12) sh, 18.2 (130), 20.7 (90) sh
thiocyanate	3.8,[a] pH 8.1	10.2 (4), 10.6 (4) sh, 14.5 (9), 17.5 (100), 18.9 (90) sh, 21.5 (100)

[a] Apparent affinity constant (K_{app} of eq 2). [b] See ref 10. [c] Actual affinity constant (K_1 of eq 2) as reported in ref 38. [d] The value for the zinc bovine carbonic anhydrase is 5.5 at pH 8.0 (see ref 29). [e] See ref 50.

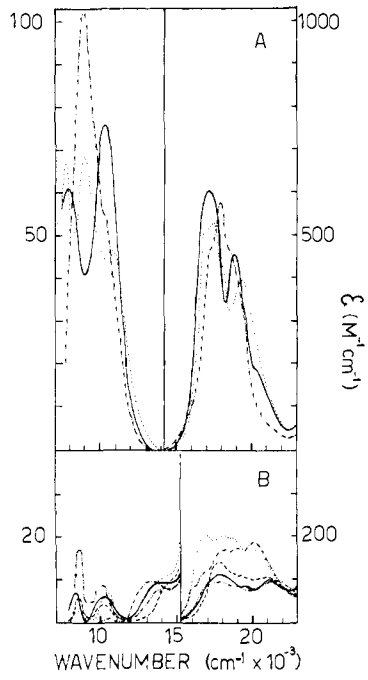


Figure 1. Limit electronic spectra of cobalt(II) bovine carbonic anhydrase with the following inhibitors: (A) aniline at pH 6.0 (·-·-), acetazolamide at pH 8.0 (···), cyanate at pH 5.9 (—); (B) bromide (···); iodide (·-·-); acetate (—); nitrate (- - -), and thiocyanate (·····) at pH 5.9.

are reported in Figure 1, whereas the absorption maxima and molar absorbances are reported in Table I. The affinity constants of Table I determined during the present research are obtained as described in ref 28. All of them experience pH dependence of the type displayed in eq 2, except for the affinity constants of ligands as CN^-, SH^-, and acetazolamide, whose pH dependence is also affected by the acid-base equilibria displayed by the binding group.[29,30] The absorption maxima and molar absorbances are referred to the enzyme-inhibitor species, as calculated from the computer program and checked experimentally in ligand excess; in any case the electronic spectra of the adducts are pH independent.

The most significant observation about the electronic spectra is the large variation in the molar absorbance from the aniline to the acetate derivative. Such a drastic change cannot reasonably be accounted for by exchanging in the chromophore an oxygen of the acetate with a nitrogen of the aniline. Actually, the spectra of the acetate and benzoate derivatives were assigned to a five-coordinate chromophore on account of the low molar absorbance and of the presence of a transition at 13 000 cm^{-1}.[28,31] The absorptions below 14 000 cm^{-1} have the shape of F-F transitions and, as shown in Table II, pseudotetrahedral complexes do not absorb at that high an energy. Therefore, based on similarity with other five-coordinate model compounds both the presence of a band at 12 000-14 000 cm^{-1} and the low intensity of the absorptions are taken as diagnostic of five-coordinate species. Indeed, the spectra of Table I can be roughly divided into three groups: (i) weak intensity spectra with the characteristic absorption between 12 000 and 14 000 cm^{-1} (acetate, nitrate, iodide, etc.); (ii) intense spectra with molar absorbance above 300 $M^{-1}\ cm^{-1}$ in the visible region (aniline, sulfonamides, cyanate, etc.); (iii) spectra with molar absorbance in the visible region between 200 and 300 $M^{-1}\ cm^{-1}$, some of which shows an absorption in the range 12 000-14 000 cm^{-1}. Whereas the compounds of the second class are definitely tetrahedral as previously suggested[11,31-33] and those of the first class are substantially five coordinate, the derivatives of the third class deserve more comment. The temperature dependence of the spectra, although in a very limited excursus, has been of some help.[34] The chloride derivative (ϵ_{max} in the visible region = 270 $M^{-1}\ cm^{-1}$; Figure 2) shows a temperature-dependent spectrum especially with respect to the shoulder at 13 900 cm^{-1}; in particular, it decreases with increasing temperature. This is consistent with an equilibrium between four- and five-coordinate species, the former species being favored by higher temperatures for entropic reasons.[35] The spectrum in the visible region decreases in intensity only slightly because both tetrahedral and five-coordinated species absorb in the same region. It may be interesting to note that five-coordinate species absorb more at higher energies (22 000 cm^{-1}).[12,13,22,36,37] The spectrum of the acetate which belongs to the first class is not temperature dependent in the range 4-35 °C, indicating that a single five-coordinate species is present.

In conclusion, the absorption at 13 000-14 000 cm^{-1} indicates the presence of five-coordinate species; however, since these species have low molar absorbances, they may be present

Table II. Spectral Band Energies ($cm^{-1} \times 10^{-3}$) and Molar Absorptivity ($M^{-1}\ cm^{-1}$) of Four- and Five-Coordinate Cobalt(II) Complexes

	donor set	F-F transitions	F-P transitions
four coordinate			
$Co(Ph_3P)_2Br_2$[a,b]	P_2Br_2	6.2 (110), 7.7 (95), 10.2 (80)	13.1 (610), 14.9 (970), 15.6 sh
$Co(Me_4en)Br_2$[c]	N_2Br_2	5.9 sh, 6.9 (73), 9.6 (27.3)	15.2 (270), 16.7 (310), 17.5 sh
$Co(PA)_2$[d,e]	N_4	8.3 (28), 9.7 (36)	16.0 (58), 17.1 (129), 19.6 (600), 20.8 (629)
Co(MOB)(en)Net_2[f]	N_2Cl_2	6.0 (34), 7.5 (417), 10.3 (20)	15.4 (41), 15.7 (425), 17.15 (425)
$Co(Me_4en)(NCS)_2$[c]	N_4	7.4 sh, 8.7 (132), 11.2 (68)	16.1 (1050), 17.8 sh
five coordinate			
$Co(Me_5dien)Cl_2$[g]	N_3Cl_2	8.7 sh, 10.6 (19)	16.1 (106), 18.8 (112)
Co(MAB)Cl_2[h]	N_3Cl_2	9.5 (23), 11.7 sh	15.8 (187), 16.7 sh, 17.8 sh
$Co(Me_6tren)Cl_2$[i]	N_4Cl	5.8 (32), 12.6 (30)	15.5-16.1 (87), 20.2 (118)
$Co(Me_6tren)(NCS)_2$[j]	N_5	5.8 (50), 14.8 sh	16.5 (230), 21.0 sh
$Co(py(cy)_2)Cl_2$[k]	N_3Cl_2	5.3 (12), 8.3 (10), 11.3 (7)	16.5 (57), 17. (52), 23.8 sh
Co(dpca)Br_2[l]	N_3Br_2	5.5 (12), 7.2 (18), 10.0 (20), 12.5 (14)	15.3 (162), 16.6 (214), 18.0 (64), 19.4 sh
$Co(dpca)(NCS)_2$[l]	N_5	5.8 (24), 13.4 (23)	16.3 (181), 16.8 (80), 18.3 (75), 19.3 (77), 20.5 sh
$Co(Et_4dien)Cl_2$[m]	N_3Cl_2	11.2 (18)	15.4 (58), 19.0 (60)
Co(tren)H_2O^{2+}[n]	N_4O	13.8 (12)	17.4 (42), 20.8 (46)

[a] F. A. Cotton, O. D. Faut, D. M. L. Goodgame, and R. H. Holm, *J. Am. Chem. Soc.*, **83**, 1780 (1961). [b] Assignment verified through single-crystal linearly polarized spectra on the chloride derivative; C. Simo and S. L. Holt, Jr., *Inorg. Chem.*, **7**, 2655 (1968). [c] L. Sacconi, I. Bertini, and F. Mani, *Inorg. Chem.*, **6**, 262 (1967); Me_4en = *N,N,N',N'*-tetramethylethylenediamine. [d] R. H. Holm, A. Chakravorty, and L. J. Theriot, *Inorg. Chem.*, **5**, 625 (1966); PA = *N-tert*-butylpyridine-2-carbaldimino. [e] I. Bertini, D. Gatteschi, and A. Scozzafava, *Inorg. Chim. Acta*, **13**, 145 (1975). [f] L. Sacconi and I. Bertini, *Inorg. Chem.*, **7**, 1178 (1968); MOB(en)NEt_2 = *N,N*-diethyl-*N'*-(3-methoxybenzylidene)ethylenediamine. [g] M. Ciampolini and G. P. Speroni, *Inorg. Chem.*, **5**, 45 (1966); Me_5dien = bis(2-dimethylaminoethyl)methylamine. [h] L. Sacconi, I. Bertini, and R. Morassi, *Inorg. Chem.*, **6**, 1548 (1967); MAB = *N,N*-diethyl-*N'*-(3-methylaminobenzylidene)ethylenediamine. [i] M. Ciampolini and N. Nardi, *Inorg. Chem.*, **5**, 41 (1966); Me_6tren = tris(2-dimethylaminoethyl)amine. [j] I. Bertini, M. Ciampolini, and D. Gatteschi, *Inorg. Chem.*, **12**, 693 (1973). [k] L. Sacconi, R. Morassi, and S. Midollini, *J. Chem. Soc. A*, 1510 (1968); $Py(Cy)_2$ = *N,N'*-dicyclohexyl-2,6-diacetylpyridinebisimine. [l] D. P. Madden and S. M. Nelson, *J. Chem. Soc. A*, 2342 (1968); dpca = bis[2-(2'-pyridyl)ethyl]amine. [m] Z. Dori and H. B. Gray, *J. Am. Chem. Soc.*, **88**, 1394 (1966); Et_4dien = 1,1,7,7-tetraethyldiethylenetriamine. [n] P. Paoletti, M. Ciampolini, and L. Sacconi, *J. Chem. Soc. A*, 3589 (1963); tren = tris(2-aminoethyl)amine.

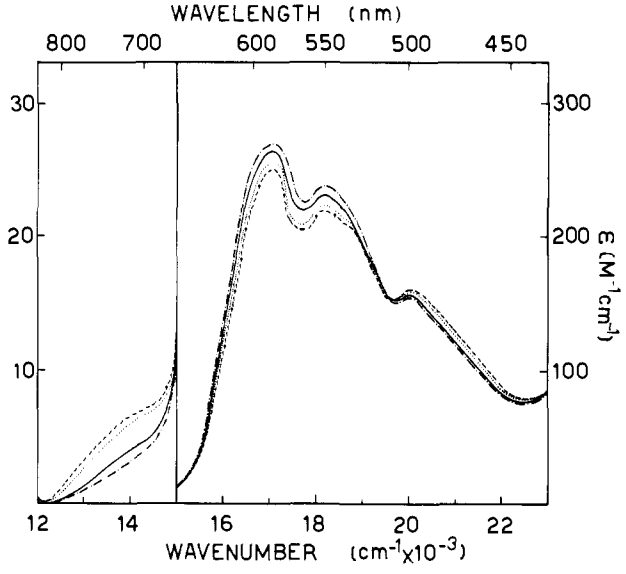


Figure 2. Temperature dependence of the electronic spectrum of the chloride cobalt(II) bovine carbonic anhydrase (pH 5.9): 5 °C (- - -), 17 °C (···), 26 °C (—), and 35 °C (·-·-).

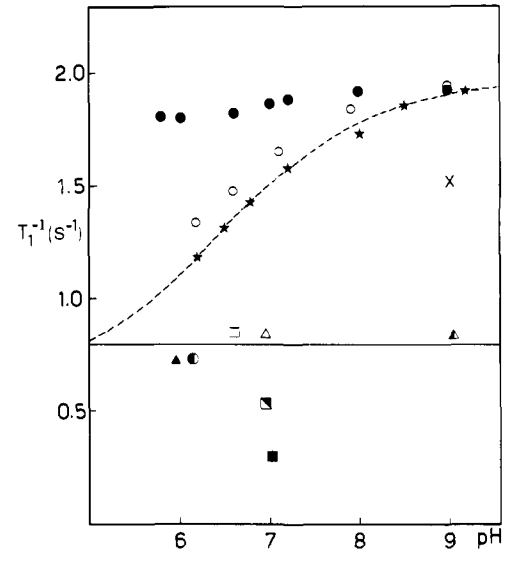


Figure 3. T_1^{-1} values for water protons of a solution of 2.4×10^{-3} M cobalt(II) bovine carbonic anhydrase as a function of pH: (●) pure enzyme; (○) plus 10^{-1} M Tris-sulfate; (★) plus 10^{-1} M sulfate. Residual values obtained after addition of the following inhibitors: (—) *p*-toluenesulfonamide; (□) cyanate; (△) acetate; (▲) thiocyanate; (◪) oxalate; (X) hydrosulfide. The inhibitors were added in such an amount as to fully develop the electronic spectrum of the inhibited enzyme: (■) T_1^{-1} value of H_2O; (◐) T_1^{-1} value for a 2.4×10^{-3} M solution of $Co(H_2O)_6^{2+}$; (▲) T_1^{-1} value for a 2.4×10^{-3} M solution of Co(tren)H_2O^{2+}.

in small amounts even in the absence of such a diagnostic absorption when the intensity of the absorption in the visible region is intermediate between that of class i and class ii derivatives. In the case of the pure enzyme, the alkaline form has a spectrum typical of class ii whereas the acidic form belongs to class iii.

The halogens show a decrease in the population of five-coordinate species in the order ($I^- > Br^- > Cl^- > F^-$); also, the apparent affinity constants follow the same pattern[38] which is contrary to the affinity of halogens toward 3d metal ions.[39] However, the affinity constants refer to different amounts of the two species in solution and therefore cannot meaningfully be compared.

Relaxivity Data. Proton relaxation measurements on water solutions containing cobalt carbonic anhydrase show large effects caused by the paramagnetic center[17,23] (Figure 3). The T_1^{-1} values are much larger than when the solutions contain the diamagnetic native enzyme and are substantially independent of pH, provided no anionic species is introduced in the solution.[23] Previous measurements were carried out in the presence of Tris-sulfate buffer and a drop in the T_1^{-1} values at acidic pHs was observed.[17] Actually, the same results obtained in the presence of Tris-sulfate buffer are obtained when

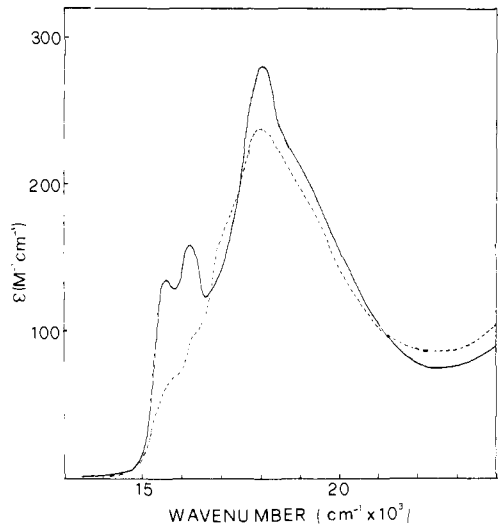


Figure 4. Visible absorption spectrum of salt-free cobalt(II) bovine carbonic anhydrase (—) and in the presence of 10^{-1} M sodium sulfate (- - -). The pH of both samples is 6.5.

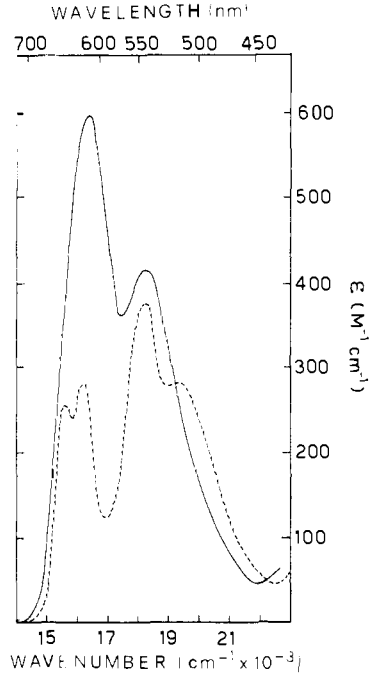


Figure 5. The visible absorption spectra of cobalt(II) bovine carbonic anhydrase at pH 9.0 (- - -) and of its adduct with HS^- (—).

sodium sulfate is added to the solution containing cobalt enzyme.[23] Therefore, the sulfate ion is responsible for the dropping of T_1^{-1} at low pH values. Indeed, the electronic spectra are also affected by the presence of the sulfate ion in the sense that the absorptions typical of the alkaline form disappear by adding sulfate to the solution (Figure 4). The effect of the sulfate ion may be that of a weak inhibitor[40] and/or reflect a change of the ionic strength which may affect the apparent K_a of the acid-base equilibrium of the enzyme. The present data, however, do not allow discrimination between the two possibilities. Since the catalytic process actually takes place in the presence of salts both in vivo and in vitro, the influence of sulfate and of the other ions present in the usual buffers on the exchangeable protons is a primary point, and deserves further investigation.[41]

The present results are interpreted as due to a group with exchangeable protons bound to the metal at every pH in the range 5.6-9.0. Addition of *p*-toluenesulfonamide, oxalate, thiocyanate, etc. (Figure 3), in such amounts (10^{-2}-5×10^{-1} M) as to fully develop the electronic spectra of the inhibited enzyme, titrates T_1^{-1} enhancement down to a value which was defined as residual relaxation[17] and which is probably due to exchangeable protons not far from the paramagnetic center.

In the fast exchange limit, T_1, corrected for the molar fraction, corresponds to T_{1M}, the longitudinal relaxation time due to the paramagnetic center. An important factor determining the value of T_{1M} is the electronic correlation time, τ_c, which, in turn, is determined by which is shorter, the electronic spin-lattice relaxation time, τ_e, or the rotational time, τ_r, according to the relations:[42,43]

$$\frac{1}{T_{1M}} = \frac{2}{15}\frac{S(S+1)\gamma_I^2 g^2 \beta^2}{r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right) + \text{contact term} \quad (3)$$

and

$$\frac{1}{\tau_c} = \frac{1}{\tau_e} + \frac{1}{\tau_r} \quad (4)$$

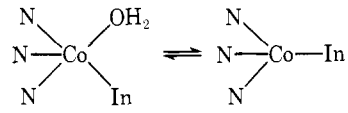
The contact term is usually believed to be less important than the other contribution which is dipolar in origin.[44,45] Cobalt(II) complexes show τ_c values with magnitudes of 10^{-11}-5×10^{-13} s, which are far shorter than the rotational times even for the hexaaquo ion.[17,46,47] Six-coordinated octahedral compounds have the shortest τ_c[46] and therefore give rise to relatively small T_1^{-1} enhancements. Indeed, proton NMR signals of such compounds are very sharp.[46,47] Accordingly, the T_1^{-1} values of 10^{-3} M solutions of hexaaquo cobalt(II) are small. They correspond to the residual relaxivity of the inhibited enzyme at the same concentration. In the enzyme derivative, on the contrary, T_1^{-1} is quite large in agreement with a substantially tetrahedral configuration of the enzyme at both high and low pH values. Inhibitors may replace the group with exchangeable protons or bind to a fifth site. In both cases, T_1^{-1} values drop to the residual relaxation, in the former case because the group has been removed, and in the second case probably because five-coordinate complexes have electronic relaxation times close to the values of octahedral compounds. Indeed, the ESR line widths for five- and six-coordinate adducts have been found substantially equal.[49] Furthermore, the relaxivity of the five-coordinated species $Co(tren)H_2O^{2+}$ (tren = tris(2-aminoethyl)amine) is close to that of the hexaaquo ion and to the residual relaxivity due to the inhibited enzyme.

The hydrosulfide derivative is of some interest since the SH group is similar to the OH group which is a possible coordinated group in the alkaline form of the enzyme. The electronic spectra of the two derivatives are similar[50] (Figure 5) and the proton T_1 values are also similar if allowance is made for the different Co-H distances.[51] This might be considered in support of the hydroxide being coordinated to the enzyme at high pH values. However, calculations through eq 3, in agreement also with those previously performed at alkaline pH,[17] are consistent with an H_2O being bound to the metal. Therefore, owing to the substantial pH independence of T_1^{-1} values, water would not be involved in the acid-base equilibrium, as already suggested by several authors.[4,54-56]

Conclusions

The present data are consistent with the hypothesis that exchangeable proton(s) are bound to at least one donor group at every pH in salt-free solutions. The electronic data are consistent with two tetrahedral species in equilibrium. Anionic inhibitors bind the metal in the acidic species although the pH dependence of the apparent affinity constants might in some cases be consistent with a model according to which the in-

hibitor binds the alkaline form of the enzyme.[6,30] The inhibited species experience the following equilibrium:



The position of the equilibrium depends on factors which are not yet understood. A quite peculiar result is that the NCO^- derivative is purely tetrahedral whereas the NCS^- derivative is probably fully five coordinate. The spectrophotometric titration of the enzyme with inhibitors indicates that both NCO^- and NCS^- bind in a 1:1 ratio. Therefore, a fine balance based on hydrogen bonding and other interactions with that part of the protein close to the metal must control the relative distribution of the two species in equilibrium.

Note Added in Proof. Discussions with Dr. K. K. Kannan of the University of Uppsala drew our attention to the possibility that sulfonamides might have a second donor group pointing at the metal but located at a long distance (K. K. Kannan, I. Vaara, B. Notstrand, S. Lövgren, A. Borell, K. Fridborg, and M. Petef, "Proceedings of the Symposium on Drug Action at the Molecular Level", G. C. K. Roberts, Ed., Macmillan, New York, N. Y., 1976, p 73). In our opinion, such a group would hardly affect the eletronic spectra.

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