Spectroscopic Studies of Nickel(II) Carbonic Anhydrase and its Adducts with Inorganic Anions

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Nickel(II) carbonic anhydrase, NiBCA II, and its adducts with nitrate, acetate, cyanate and azide, have been investigated through ^{1}H NMR and electronic absorption spectroscopies. From the pH dependence of the molar absorbance the acidity constants of NiBCA were determined. The anions bind to the metal ion forming 1 : 1 adducts, and the corresponding affinity constants have been determined. The ^{1}H NMR spectra of NiBCA and its adducts have been recorded, and the signals corresponding to the *meta*-like protons of the co-ordinated histidines followed by ^{1}H NMR titration. The T_1 values of these signals were measured and resonance assignments made based on nuclear Overhauser enhancement experiments. The co-ordination geometry of the metal ion in NiBCA and its adducts is discussed on the basis of the temperature dependence of the isotropic shifts, molar absorbance, and longitudinal relaxation times.

The zinc-containing metalloenzyme carbonic anhydrase (CA) (M = 30 000) is a much studied enzyme which catalyses the simple, reversible reaction $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$ as well as the hydrolysis of esters and hydration of aldehydes.[1–5] The X-ray structure has recently been refined at a resolution of 2 Å for the human isoenzymes HCA I and HCA II.[6–11] Although a high-resolution crystal structure is not available for bovine carbonic anhydrase II (BCA II) due to its high homology with the corresponding human isoenzyme HCA II (80%), a very similar structure is expected.[12–14] In all cases the catalytic zinc ion is co-ordinated to three imidazole groups from His-94, -96 and -119 (the numbering is relative to the CA II isoenzyme) and to one or two solvent molecules.

There is now convincing evidence that deprotonation of co-ordinated water provides the active form of the enzyme.[2,15–17] Presently, it is agreed that the enzymatic properties of carbonic anhydrase depend on more than one acidic group,[1,17–19] as do the spectroscopic properties of cobalt-substituted CA (CoCA).[16,20,21] The pH dependence of the kinetic parameters in the absence of anions have been analysed on the basis of the ionization of two interacting acidic groups.[17,22] Furthermore, Bertini *et al.*[23] have studied the acid–base properties of cobalt-substituted BCA II and HCA I (CoBCA II and CoHCA I) and four acidic microconstants were obtained describing two interacting acidic groups as in the cited kinetic study. One acidic group is the metal-co-ordinated water molecule, and His-64 (or His-200 in HCA I) has been proposed as the second ionizable group.[2,22,23]

There are two main groups of inhibitors of CA, sulfonamides and monovalent anions.[18,21] Even many anions that do not normally bind to metal ions inhibit, for example NO_3^-, SO_4^{2-} and ClO_4^-.[18,21,24–26] Spectroscopic studies of CA have indicated that inhibitor molecules can bind to the metal ion either by replacing the co-ordinating water molecule, so that the metal ion remains four-co-ordinated, or by expanding the co-ordination number giving rise to a five-co-ordinated adduct.[16,18,27–29] In many cases an equilibrium between the two species takes place.[16,18,28,29] The X-ray structures of the NCS^- and of two sulfonamidate derivatives have been determined to a satisfactory degree of resolution,[30] and the factors governing the co-ordination number in the anion derivatives of CA have recently been analysed.[3,29]

Nickel(II) has been used as a probe to monitor the structure and reactivity of several zinc enzymes such as carboxypeptidase A (CPA), liver alcohol dehydrogenase, LADH, or carbonic anhydrase.[31–33] Whereas the nickel derivatives of CPA and LADH practically retain completely the enzymatic activity,[34,35] it has been reported that nickel-substituted carbonic anhydrase (NCA) has weak CO_2 hydration and esterase activities.[18,36,37] Studies on NiCA are very scarce and the co-ordination geometry of nickel(II) is not known. This derivative has been studied by electronic spectroscopy[37–39] and its water ^{1}H NMR dispersion spectrum investigated.[33,40] On the basis of the energies and relative intensity of the d–d bands a six-co-ordinate stereochemistry had been suggested.[37,38] However, the intensity of the d–d transitions are pH dependent and from a more detailed study at high pH a five-co-ordinated nickel(II) geometry has been proposed.[39] Recently, magnetic susceptibility measurements at neutral pH have been performed and the results obtained were consistent with a distorted octahedral co-ordination.[41]

Octahedral nickel(II) complexes have an orbitally non-degenerate ground state $^3A_{2g}$, and since the first excited level is at relatively high energy the electronic relaxation mechanism will not be very efficient.[33,42] As a consequence, their ^{1}H NMR spectra will display ill defined relatively broad signals with short T_1 values. In addition, the magnetic anisotropy is expected to be quite small, unless sizeable anisotropy is apparent in large zero-field splitting. Therefore the dipolar term, which originates from magnetic anisotropy, will usually be negligible and the isotropic shifts are largely contact in origin. On the other hand, the high-spin four- or five-co-ordinated nickel(II) complexes possess low-lying spin-triplet excited states which allow an efficient electronic relaxation and, due to the existence of sizeable anisotropy, a dipolar contribution to isotropic shifts is expected.[33,42,43] The ^{1}H NMR spectra of such complexes show well resolved relatively narrow signals with reasonably long relaxation times.[44–46]

In order to understand the binding properties of anions and to shed light on the structural features of nickel-substituted BCA (NiBCA) and its adducts, we have determined the affinity constants of nitrate, acetate, cyanate and azide, registered their ^{1}H NMR spectra and measured the ^{1}H longitudinal relaxation times, T_1, of the co-ordinated histidines. In addition, we have

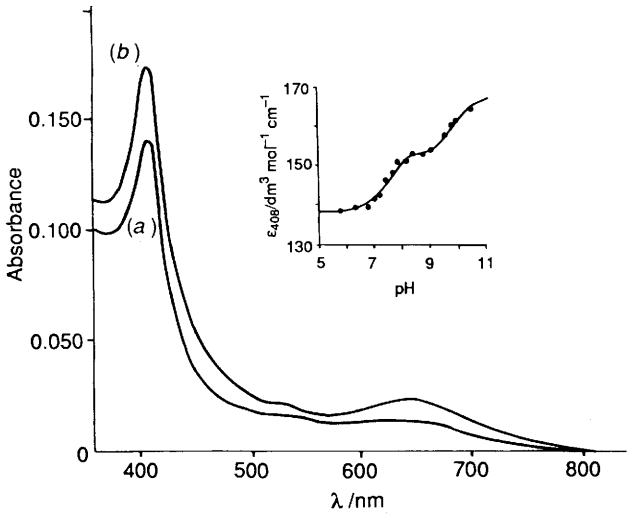


Fig. 1 Electronic spectra in the visible region of nickel-substituted carbonic anhydrase (0.3 × 10^{-3} mol dm^{-3}) at pH 6.2 (*a*) and 10 (*b*) and 20 °C. The insert shows the pH dependence of the molar absorbance at 408 nm. The solid line is calculated assuming pK_a 7.4 and 9.6

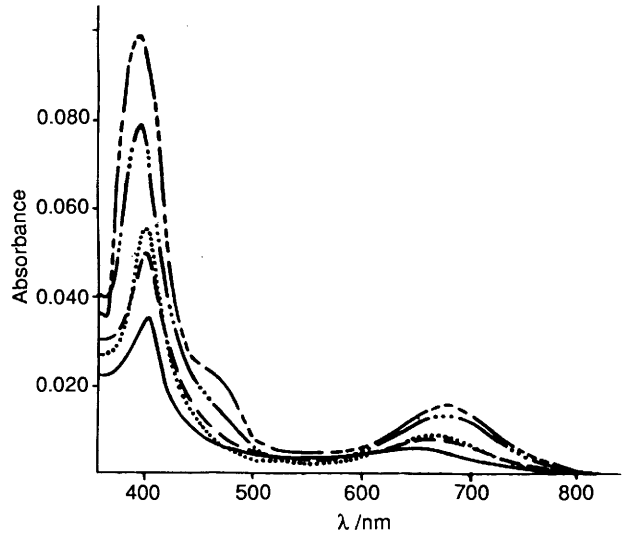


Fig. 2 Electronic absorption spectra of some anion adducts of nickel carbonic anhydrase at pH 6.2: (——) NiBCA; (– – –) NiBCA(NO_3); (· · ·) NiBCA(O_2CCH_3); (— · —), NiBCA(NCO); (— — —) NiBCA(N_3). Solution conditions: 0.3 × 10^{-3} mol dm^{-3} NiBCA, 0.05 mol dm^{-3} mes, 20 °C

performed nuclear Overhauser enhancement (NOE) experiments and analysed the temperature dependence of the chemical shifts.

Experimental

Bovine carbonic anhydrase II was purchased from Sigma and purified through chromatography on DEAE cellulose.[47] Apocarbonic anhydrase was prepared as previously reported[48] and its concentration determined by spectrophotometric titrations with a cobalt(II) sulphate solution[25] as well as from the absorbance at 280 nm (ε = 5.6 × 10^4 dm^3 mol^{-1} cm^{-1}). The two values were always within 10%. The nickel(II) enzyme was obtained by addition of nickel(II) sulphate to a solution of the apoenzyme. In order to avoid possible binding to extra sites in the enzyme, Ni^{II} was added in slightly less than the stoichiometric amount. The formation of NiBCA II could be monitored by electronic and ^{1}H NMR spectroscopies. The salts $NiSO_4$·$6H_2O$, KNO_3, NaO_2CCH_3, NaNCO and NaN_3 and all the other chemicals were Merck analytical grade reagents. The 99.7% D_2O was obtained from Fluka.

Optical spectra were recorded on a UV/VIS/NIR Perkin-Elmer Lambda 9 spectrophotometer, using microcells with an optical path length of 10 mm and the NiBCA concentrations were approximately (0.2–0.4) × 10^{-3} mol dm^{-3}. The microcells were thermostatted with a Haake F3-K cryostat. Samples for spectrophotometric measurements were prepared by adding to a buffered solution of NiBCA (0.05 mol dm^{-3} 2-morphilino-ethanesulphonic acid (mes), pH 6.2) variable amounts of buffered solutions of the inhibitors containing the same concentration of NiBCA in order to avoid dilution effects of the enzyme. The pH values of all solutions were measured on a Crison digit-501 pH meter provided with an Ingold combined microelectrode.

The samples for NMR measurements were concentrated to (3–8) × 10^{-3} mol dm^{-3} protein by ultrafiltration at 2–4 °C using a Centricon microconcentrator (Amicon) with a molecular weight cut-off of 10 000. The ^{1}H NMR spectra were recorded on a Bruker AC-200 MHz spectrometer at 20 °C (0.05 mol dm^{-3}, mes, pH 6.2) using the modified driven equilibrium Fourier transform (MODEFT) pulse sequence[49] to suppress signals from water and bulk diamagnetic protein. Spectra typically consisted of ≈16 000 scans with 8K data points and a spectral width of 50 kHz. The ^{1}H NOE experiments were performed as already reported.[50] The SUPERWEFT[51] multipulse sequence was used, 180°–τ–90°–acquisition + delay, with τ values of about 84 ms and 83 ms recycle time. The signals were selectively saturated by using a selective decoupling pulse, whose power was predetermined (lesser than 0.1 W), kept on for 19/20 of the τ value. Difference spectra were collected by applying the decoupler frequency on and off alternately following the scheme, ω, ω + Δω, ω, ω − Δω, where ω is the frequency of the irradiated signal and Δω is the offset for the off-resonance irradiation. The value of Δω depends on the linewidth of the irradiated signal and the proximity of other signals of interest, typically values of 100–400 Hz were used. For each final difference spectrum 80–90 blocks were collected, 8192 scans each. Difference spectra were analysed by area. Chemical shifts were measured from the H_2O or HDO signals and referenced to $SiMe_4$ assumed at −4.8 ppm from the water signal. The spin–lattice relaxation times ^{1}H T_1 of the isotropically shifted signals were determined by measuring the intensity of the signals I_τ as a function of the time (τ) between subsequent pulses of the MODEFT sequence. The data were best fitted using equation (1)[49] with a non-linear two parameter best-fitting program to obtain I_0 and T_1 values. The estimated error is about 5%.

$$I_\tau = I_0(1-2\,e^{-\tau/T_1} + e^{-2\tau/T_1}) \quad (1)$$

Results

Electronic Spectra.—The electronic spectrum in the range 360–1000 nm of an aqueous solution of 0.3 × 10^{-3} mol dm^{-3} Nickel(II)-substituted bovine carbonic anhydrase II (NiBCA II) at pH 6.2 (0.05 mol dm^{-3} mes) and 293 K are shown in Fig. 1. There are three absorption bands at 408 (50), 634 (20) and ≈1000 nm ($\varepsilon \approx 10$ dm^3 mol^{-1} cm^{-1}) in agreement with reported values.[38,39] This spectrum is pH sensitive and the intensity of the band at 408 nm increases when the pH is increased (Fig. 1). The plot of the molar absorbance at this wavelength against pH is indicative of two acid–base equilibria (Fig. 1, insert). From the analysis of the pH-dependent spectral variation two pK_a values of about 7.4 and 9.6 were estimated.

When nitrate or acetate are added to a solution of NiBCA (pH 6.2) the visible spectrum of the nickel enzyme is slightly modified (Fig. 2). The intensity of the bands increases and, in addition, the band at 634 nm is displaced to 663 nm (see Table 1). The absorption data can be fitted by a simple equilibrium of the type NiBCA + X $\rightleftharpoons$ NiBCA(X) (X = NO_3^- or $CH_3CO_2CCH_3^-$) and the values of the affinity constants obtained were K_{app} = (5 ± 1) × 10^3 and (3 ± 0.5) × 10^3 dm^3 mol^{-1} (pH 6.2, 293 K) for the nitrate and acetate adducts respectively.

Table 1 Absorption maxima and molar absorbance of nickel-substituted carbonic anhydrase and its adducts at pH 6.2 and 20 °C

	λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1})		K_{app}/
	λ_{max} (ε)	λ_{max} (ε)	dm^3 mol^{-1}
NiBCA (pH 6.2)	408 (50)	634 (20)	
(pH 10)	406 (90)	634 (24)	
NiBCA(NO_3)	406 (100)	660 (27)	5×10^3
NiBCA (O_2CCH_3)	406 (120)	664 (30)	3×10^3
NiBCA(NCO)	406 (190)	670 (44)	2×10^4
NiBCA(N_3)	406 (260)	680 (53)	1.5×10^5

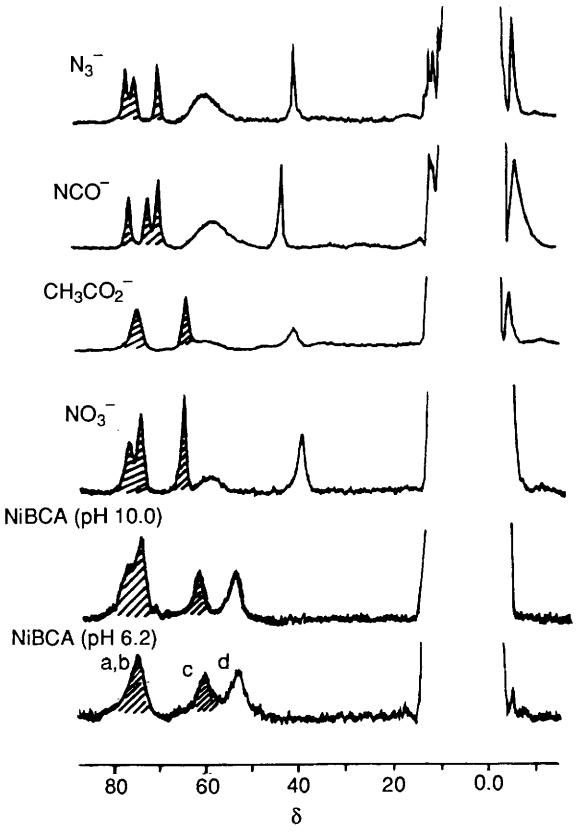
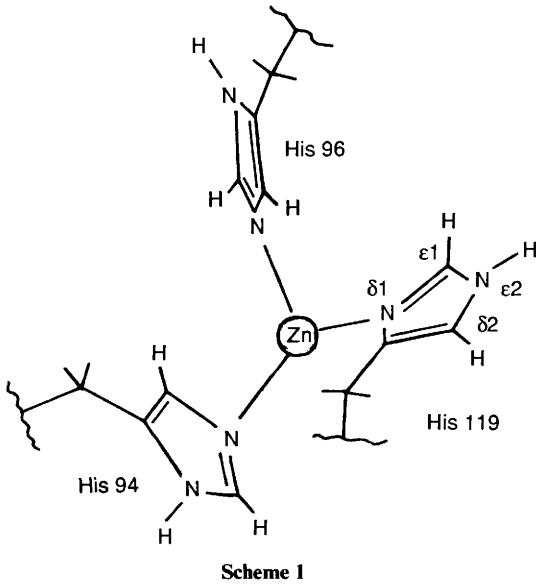


Fig. 3 Proton NMR spectra of nickel substituted carbonic anhydrase and its adducts with anions. Protein concentration: (3–4) $\times 10^{-3}$ mol dm^{-3}; other conditions as in Fig. 2



Scheme 1

Upon addition of cyanate or azide the visible spectrum of NiBCA undergoes more marked changes. The spectra of the formed 1:1 adducts display two main absorption bands at 406 and 670–680 nm as well as a shoulder at about 455–460 nm (Fig. 2), and the intensity of these bands are higher than those of NiBCA or its nitrate and acetate adducts (Table 1). From the absorption data we have calculated the apparent affinity constants for the complexes as $K_{app} = (2 \pm 0.2) \times 10^4$ and $(1.5 \pm 0.2) \times 10^5$ dm^3 mol^{-1} for cyanate and azide respectively (pH 6.2, 293 K).

^{1}H *NMR Spectra.*—The ^{1}H NMR spectrum of an aqueous solution of $\approx 5 \times 10^{-3}$ mol dm^{-3} NiBCA II (pH 6.2, 293 K) shows, in the downfield region, three resolved paramagnetically shifted signals at δ 75.2 (a, b), 60.8 (c) and 53.7 (d), the first being of intensity two (Fig. 3). In addition, broad and ill defined signals are observed at the range δ 50–65. The three resolved signals account for the four protons that are in a *meta*-like position with respect to the metal ion, and provide evidence that the three histidines are still co-ordinated to the metal ion, as in the native enzyme (Scheme 1). Thus, when the spectrum of NiBCA II is recorded in D_2O, the signals at δ 75.2 (a, b) and 60.8 (c) disappear completely whereas that at 53.7 (d) remains unchanged. This behaviour is in accord with the presence of three exchangeable NH protons corresponding to the three co-ordinated histidines, whereas the non-exchangeable signal is assigned to the H(δ2) of His-119 (Scheme 1).

When the pH is increased some improvement in the resolution of the ^{1}H NMR spectrum of NiBCA II is attained (Fig. 3). The linewidth of the *meta*-like protons is slightly decreased and, as indicated in Table 2, the positions of these signals are changed. The signal of double intensity (a, b) is split and while the position of signal b remains essentially unchanged, signal a is shifted by about 2 ppm downfield. The temperature dependence of the ^{1}H NMR spectrum at pH 10 shows that signal a is observed only at temperatures < 15 °C, and this behaviour is indicative of fast exchange between the NH proton of histidine and solvent protons.

The ^{1}H NMR spectra for the nitrate, acetate, cyanate and azide adducts of NiBCA are shown in Fig. 3, and the shifts and T_1 values are reported in Table 2. At first sight all adducts display a similar ^{1}H NMR pattern, and the more remarkable features are the smaller linewidth and higher spread of signals when compared with NiBCA II. The spectrum of the nitrate adduct displays four resolved signals at δ 77.4, 74.7, 65.4 and 40.2 as well as a broad signal centred at ≈ 59.5. The first three are exchangeable and are assigned to the NH protons of the three co-ordinated histidines, while the non-exchangeable signal at δ 40.2 is assigned to the H(δ2) of His-119. The broad signal corresponds to *ortho*-like protons of these histidines.

In order to correlate the proton signals of the nitrate adduct with those of NiBCA II we performed a ^{1}H NMR titration (Fig. 4). On addition of nitrate signal a, b is split and whereas signal a moves a few ppm downfield b remains at practically the same position. In addition signal c moves downfield and d upfield. So the signals at δ 77.4, 74.7, 65.4 and 40.2 of the nitrate adduct are related to signals a, b, c and d, respectively, of NiBCA. The changes in shifts of the *m*-H signals as a function of the nitrate concentration are reported in Fig. 4. The affinity constant estimated from this plot, $(6 \pm 1) \times 10^3$ dm^3 mol^{-1}, is consistent with the value obtained from spectrophotometric titration.

Similarly, we have performed ^{1}H NMR titrations for the other complexes of NiBCA II. However, due to the poor quality of the spectrum of NiBCA, to simplify the process of spectra recording, the titrations have been performed by adding the corresponding anion to the nitrate complex. The obtained pattern of shifts for the four *meta*-like signals is shown in Fig. 5.

Analysis of the limiting spectra shows a similar behaviour of the paramagnetically shifted signals upon inhibitor binding. Signal d, corresponding to H(δ2) of His-119, for all the adducts experiences the largest variation in shift and is the most upfield

Table 2 Proton NMR (200 MHz) chemical shifts (δ) and T_1/ms (in parentheses) values for nickel-substituted carbonic anhydrase and its adducts at pH 6.2 and 20 °C

	a(NH)	b(NH)	c(NH-119)	e	d(CH-119)	
NiBCA (pH 6.2)	75.2	75.2	60.8		53.7 (<1)	14.3
(pH 10)	76.1	74.5	62.7		54.5	14.2
NiBCA(NO_3)	77.4 (2.9)	74.7 (4.0)	65.4 (4.7)	59.5	40.2 (6.5)	13.6
NiBCA(O_2CCH_3)	76.0 (2.0)	76.0 (2.0)	64.8 (3.1)	61.2	41.9 (7.1)	13.7
NiBCA(NCO)	72.9 (2.9)	76.9 (1.8)	70.5 (3.2)	59.0	44.1 (8.7)	13.3
NiBCA(N_3)	75.4 (2.9)	77.2 (2.5)	70.1 (3.0)	60.4	41.5 (10)	13.9

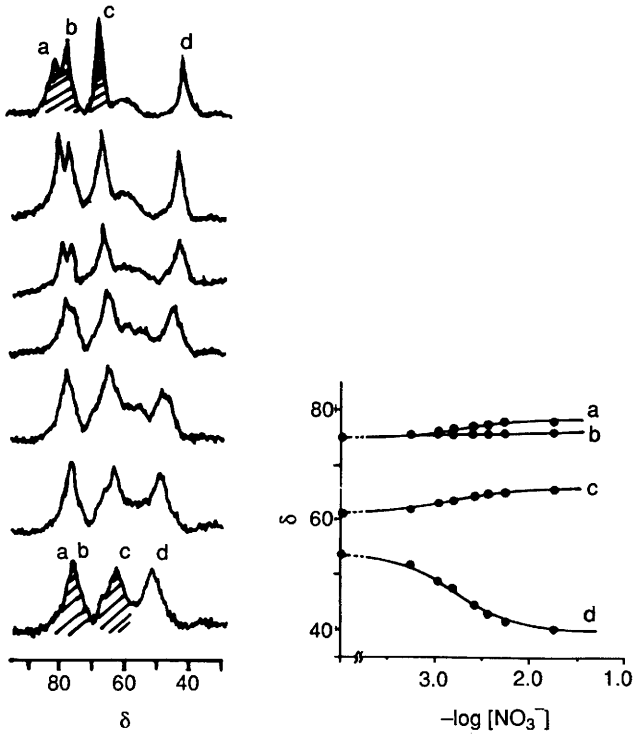


Fig. 4 Proton NMR titration of NiBCA with nitrate. Variations of chemical shifts of signals a–d *vs.* nitrate concentration together with the best fitting curve. Protein concentration 4 × 10^{-3} mol dm^{-3}; other conditions as in Fig. 2

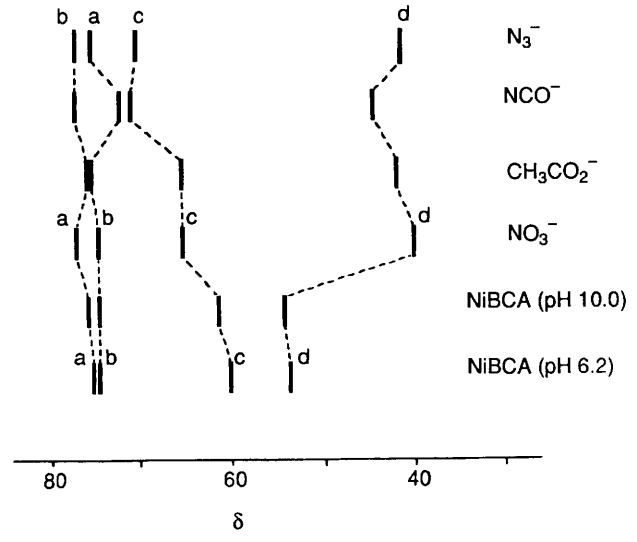


Fig. 5 Pattern of the shift values for the *meta*-like proton signals of nickel carbonic anhyrase and its adducts

shifted *meta*-like signal. However, signal c assigned to the NH of the same histidine (see below) does not follow the same pattern as that of the CH signal and is shifted downfield. On the other hand, signals a and b only move a few ppm when the respective complexes are formed, and for the azide and cyanate adducts their positions are interchanged.

Assignments based on ^{1}H *NOE Experiments.*—NOE measurements on large paramagnetic biomolecules are made difficult due to the short nuclear relaxation times which decreases the NOE compared to that obtained with diamagnetic systems.[50,52] However, the NOE, when observed, provides a powerful tool for resonance assignment and for obtaining structural information. Although other methods can be used, NOE studies are becoming the preferred assignment method.[52,53] The NOE, η_{ij}, for a proton i represents the fractional change in intensity of signal i upon saturation of the resonance of another proton j, in the same molecular species. It is dependent on the time t of irradiation of signal j, and is given by equation (2) where $\sigma_{ij} = -h^2\gamma^4\tau_c/10\ r_{ij}^6$ is the cross-

$$\eta_{ij} = \frac{\sigma_{ij}}{\rho_i}(1 - e^{-t\rho_i}) \quad (2)$$

relaxation rate between protons i and j, ρ_i is the selective spin–lattice relaxation rate of proton i, τ_c is the reorientation time of the vector connecting H_i and H_j and r_{ij} is the interproton distance. For long irradiation times, $t \gg \rho^{-1}$, the steady state is reached, and the NOE is maximal [equation (3)].

$$\eta_{ij} = \frac{\sigma_{ij}}{\rho_i} = -\frac{h^2\gamma^4\tau_r}{10\ \rho_i r_{ij}^6} \quad (3)$$

As in Scheme 1, His-119 is the only histidine co-ordinated through N(δ1). So, the NH proton of His-119 has one vicinal *meta*-proton, whereas the NH proton of the other histidines have no vicinal *meta*-proton. Therefore, saturation of the *m*-CH signal will provide only one NOE among the paramagnetic signals. In the case of N(δ2) binding no NOE is expected due to the very short relaxation times of the vicinal *ortho*-protons (T_1 < 1 ms). So it is possible to distinguish between the two types of co-ordination and should permit unambiguous identification of His-119.

The difference spectra obtained upon saturation of the *meta*-protons of the azide derivative are reported in Fig.6. Saturation of signal d only induces an NOE in peak c. The c–d correlation was checked and confirmed by saturating signal c; this experiment gave a NOE on signal d. Similarly, saturation of signals a and b was performed and no NOE was detected for any isotropically shifted signal. On the basis of equation (3) it is possible to calculate interproton distances. By taking $\tau_r \approx 1.4 \times 10^{-8}$ s as calculate by the Stokes–Einstein equation,[33] we determined the interproton distance for the c–d pair of protons as 2.2 ± 0.2 Å, consistent with the hypothesis of vicinal protons in histidine rings. The fact that c is connected through a NOE to signal d unambiguously assigns c as HN(ε2) of His-119.

It should be noted that in the difference spectra of Fig. 6 several NOE are observed in the +15 to −5 ppm region. However, their assignments are beyond the scope of this report.

Temperature Dependence of the Shifts.—Variable-temperature ^{1}H NMR spectra of NiBCA (pH 6.2 and 10), and its

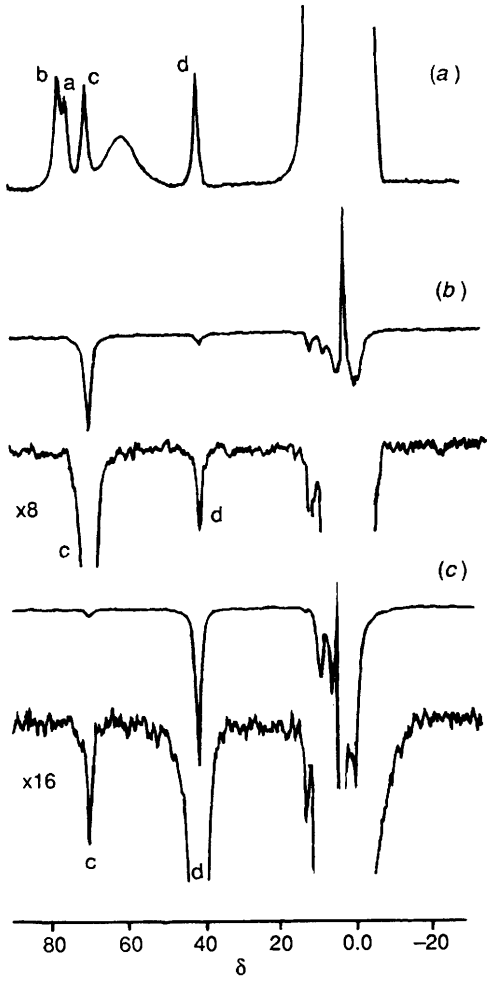


Fig. 6 200 MHz Proton NMR spectra of the azide adduct of nickel carbonic anhydrase in water at 20 °C: (*a*) reference spectrum; (*b*) and (*c*) difference spectra obtained from a steady-state NOE experiment by saturating peaks c and d respectively. Solution conditions 8.3 × 10^{-3} mol dm^{-3} NiBCA, 90 × 10^{-3} mol dm^{-3} azide, pH 6.2 (0.05 mol dm^{-3} mes)

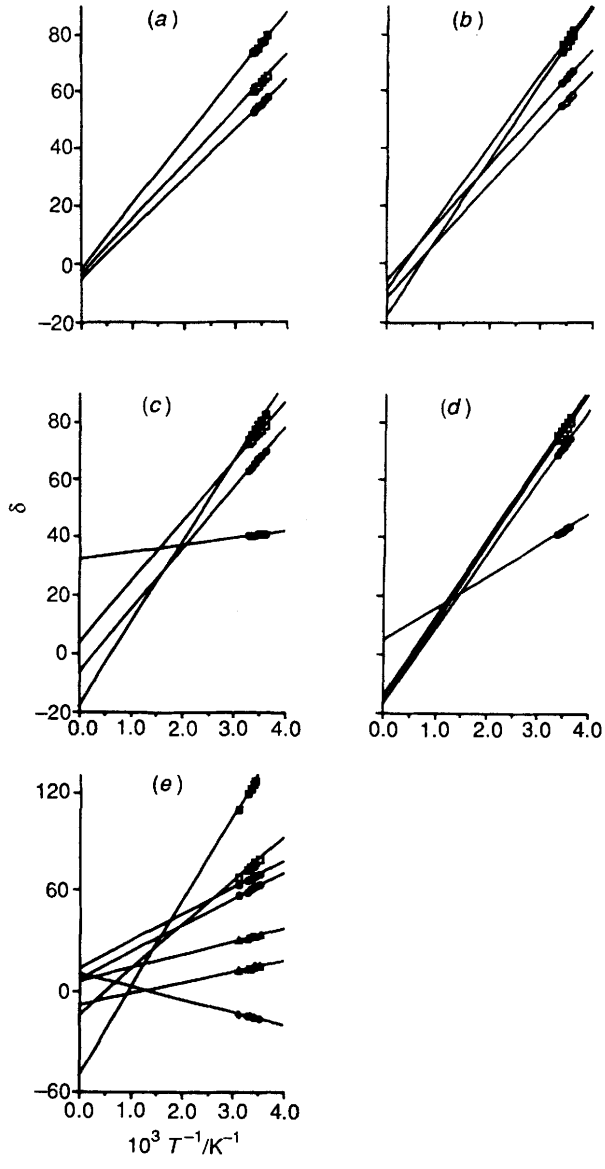


Fig. 7 Temperature dependence of the isotropic shifts for NiBCA adducts at pH 6:2: (*a*) NiBCA; (*b*) NiBCA (pH 10); (*c*) NiBCA(NO_3); (*d*) NiBCA(N_3); (*e*) Nickel-azurin (data from ref. 44)

nitrate and azide adducts at pH 6.2, were registered from 4 to 30 °C. In Fig. 7 the observed isotropic shifts of the *meta*-like protons are plotted *vs.* T^{-1}. For comparison, the temperature dependence of the isotropic shifts for the nickel derivative of azurin, a copper protein in which the co-ordination of the metal ion is tetrahedral, taken from ref. 44, is also plotted. For NiBCA the chemical shifts of all *meta*-like proton signals are temperature dependent. At pH 6.2 the intercept values at infinite temperature were negative but close to zero, whereas at pH 10 all signals displayed higher slope values and showed more negative intercepts.

In the case of the nitrate complex, signals a–c are very temperature dependent, whereas d is practically independent of temperature (Fig. 7). Only the shift of signal b obeys an equation of the type $\delta = \alpha T^{-1}$. The intercept values for the two other proton signals (a, c), at infinite temperature, were non-zero. For the azide adduct all *meta*-like proton signals are temperature dependent and only signal d obeys the equation $\delta = \alpha T^{-1}$. Signals a–c show more negative intercepts than those of the corresponding signals of the nitrate complex. These results are indicative of some dipolar contribution to the isotropic shifts in NiBCA at pH 10 as well as in the nitrate and azide complexes, and it is more important in the latter complex.

Discussion

The visible spectrum of nickel(II)-substituted carbonic anhydrase is pH dependent as is that of the cobalt(II) derivative. From the pH dependence of the molar absorbance we have estimated the acidity constants of NiBCA II as $pK_{a1} = 7.4$ and $pK_{a2} = 9.6$. The pK_{a1} value is quite similar to that reported for CoCA and can be assigned to the ionization of His-64.[23] The acidic group responsible for pK_{a2} may be associated with the metal-co-ordinated water molecule.[54]

The analysis of the position and molar absorbance of the absorption bands of NiBCA at neutral pH is consistent with a pseudo-octahedral co-ordination around the metal ion. However, at alkaline pH the molar absorbance is significantly higher and although the positions of the main absorptions are consistent with six-co-ordination, from an analysis of the CD spectrum a five-co-ordinated geometry has been suggested.[39]

At neutral pH, NiBCA forms 1:1 complexes with nitrate, acetate, cyanate and azide. The visible spectra of the nitrate and acetate adducts are similar to that of the alkaline form of NiBCA and only minor differences in the molar absorbance and position of the absorption bands were observed. However, the spectra of the cyanate and azide adducts are quite different. In particular, a new band at 460 nm is present and the molar absorbance is practically double of those in the nitrate and acetate adducts. The analysis of the spectra is consistent with a five-co-ordinated geometry for the metal ion.

The ^1H NMR spectra of the acidic and alkaline forms of NiBCA are similar but certain differences in chemical shifts and linewidths of the signals were observed, suggesting that the metal binding environment is changed. At high pH some improvement in the quality of the spectra is obtained. These spectra show three exchangeable signals isotropically shifted, in the downfield region, reflecting that three histidines are co-ordinated to the metal ion as in the native enzyme. Upon anion binding to the NiBCA, its ^1H NMR spectrum is dramatically changed and the adducts formed exhibit good-quality spectra. All display similar ^1H NMR patterns and the linewidths of the signals are smaller than those for NiBCA. Furthermore, the spread of the signals is greater, indicating larger magnetic anisotropy and therefore larger dipolar shifts in the adducts. Their *meta*-proton signals have been correlated, by ^1H NMR titrations, with those of NiBCA II. Since the longitudinal relaxation times of the *meta*-protons, T_1, are longer for the adducts compared with those for NiBCA, NOE experiments have been successfully performed on the azide adduct. On the basis of these measurements we have unambiguously identified the signals corresponding to the *meta*-protons of histidine-119.

The temperature dependence of the isotropic shifts as well as the T_1 values can provide additional information about the co-ordination geometry of the nickel ion in NiBCA II and its adducts. The observed isotropic shifts of paramagnetic complexes may be of contact or dipolar origin or a combination of both.[33,42] Whereas the contact term displays a reciprocal temperature dependence, the dipolar term is known to exhibit a T^{-2} dependence. However, due to the small temperature range usually investigated, the shifts are typically inversely proportional to temperature. The usual procedure for verifying a non-T^{-1} dependence of the shifts is to extrapolate the plot to infinite temperature. A non-zero value of the intercept indicates a sizeable dipolar contribution to the isotropic shifts.[33] As seen in Fig. 7, the acidic form of NiBCA displays intercepts practically in the diamagnetic region, indicating that the isotropic shifts are essentially contact in origin as expected for six-co-ordinated nickel(II) complexes. However, in the case of the azide adduct some dipolar contribution to the isotropic shifts is detected, although less than for the nickel derivative of azurin where the metal ion displays tetrahedral co-ordination.[44] An intermediate situation, between the acidic form of NiBCA and the azide complex, is displayed for both the alkaline form of NiBCA and the nitrate adduct.

Although ^1H T_1 data for nickel(II) metalloproteins are very scarce, recently T_1 values for Ni_2Zn_2 superoxide dismutase have been published.[46] In this derivative the nickel ion is five-co-ordinated and the T_1 values for the *meta*-like protons of the co-ordinated histidines are between 12 and 18 ms, whereas the *ortho*-like protons display values <2 ms. Thus our T_1 measurements are consistent with a five-co-ordinated stereochemistry for the nickel ion in the azide and cyanate adducts and probably also in the nitrate and acetate adducts, whereas in the case of NiBCA at neutral pH the smaller estimated T_1 value suggests a pseudo-octahedral co-ordination.

It may be concluded that in the acidic form of NiBCA II the metal ion displays a distorted-octahedral co-ordination in accord with a recent magnetic study,[41] whereas for its azide and cyanate derivatives, and also probably for the acetate and nitrate adducts, a five-co-ordinated geometry can be expected. For the alkaline form of NiBCA II five-co-ordination can be suggested but a distorted-octahedral stereochemistry would be not excluded.

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