Table VI. Solvent Isotope Effects for Reactions of Porcine Pancreatic and Human Leukocyte Elastase

| enzyme | substrate | $k_{H_2O}/k_{D_2O}$ | $\phi_{T_1}$[f] | $\phi_{T_2}$[f] | ref |
|---|---|---|---|---|---|
| PPE | Ac-ONP[a] | 2.45 | 0.47 | 1.00 | 27 |
| PPE | (Z)-Gly-ONP[a] | 2.45 | 0.47 | 1.00 | 29 |
| PPE | (Z)-Ala-ONP[b] | 1.75 | 0.57 | 1.00 | 28 |
| HLE | Suc-Ala-Ala-Ala-pNA[c] | 3.15 | 0.32 | 1.00 | g |
| HLE | Suc-Ala Ala-Val-pNA[d] | 2.22 | 0.45 | 1.00 | g |
| PPE | Ac-Ala-Pro-Ala-pNA[c] | 2.16 | 0.46 | 0.46 | 36 |
| HLE | MeOSuc-Ala-Ala-Pro-Val-pNA[e] | 3.34 | 0.55 | 0.55 | g |

[a] Kinetics of deacylation measured. [b] Kinetics of acylation measured ($[S]_0 < K_m; k_2 >> k_3$). [c] Kinetics of acylation measured ($[S]_0 > K_m; k_2 << k_3$). [d] Kinetics of acylation measured ($[S]_0 < K_m$). [e] Kinetics measured at $[S]_0 > K_m$. [f] Results of proton inventory experiments. [g] This study.

inventory for the HLE-catalyzed hydrolysis of the specific substrate MeOSuc-Ala-Ala-Pro-Val-*p*NA (Figure 6), which suggests *two* catalytically important protons. According to this view, the observed isotope effect of 3.3 arises from coupling of the charge-relay system's two protonic sites, with each generating an isotope effect of about 1.8. Proper functioning of HLE's charge-relay system is observed during the hydrolysis of MeO-Suc-Ala-Ala-Pro-Val-*p*NA as a consequence of this substrate's ability to form specific interactions with HLE in the catalytic transition state.

As observed during catalysis by other serine proteases,[27-29,36] the catalytic machinery of the charge-relay system is called into action only when quite specific substrate structural requirements are met, thus permitting an optimum transition-state fit. When substrates possess structural inadequacies a tight transition-state fit cannot be achieved, and the charge-relay system uncouples resulting in one-proton catalysis. Substrate structural requirements for the operation of the charge-relay system appear to vary with the protease. For example, chymotrypsin's requirements are not strict,[27,29] requiring only an aromatic amino acid residue at $P_1$ or peptide-like structural elements past $P_1$. In contrast, both porcine pancreatic elastase (PPE) and HLE impose more severe requirements on their substrates.[27-29,36] As shown in table VI, simply fulfilling the $P_1$ requirement for PPE with an alanine residue, achieved with (Z)-Ala-ONP, is not sufficient to couple the charge-relay system. PPE requires an "extended" substrate, such as Ac-Ala-Pro-Ala-*p*NA, to engage its catalytic machinery. For HLE, substrate requirements not only include an extended peptide structure but also the proper residue at $P_1$ (valine) and possibly the presence of a proline at $P_2$. Although we can *define* for some cases substrate structural requirements, at this time we are still unable to predict the structural features that will result in the intimate and precise transition-state interactions necessary for coupling and full functioning of the charge-relay system.

**Registry No.** I, 52299-14-6; II, 61043-47-8; III, 70967-90-7; HLE, 9004-06-2.

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# Equilibrium Species in Cobalt(II) Carbonic Anhydrase

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**Abstract:** By measurement of the $^1H$ NMR $T_1$ values of the 4 H proton of the coordinated histidine-119 of cobalt(II)-substituted human carbonic anhydrase B and bovine carbonic anhydrase B as a function of pH, it is proposed that the coordination number around the cobalt(II) ion in bovine carbonic anhydrase B is always four, whereas it is largely five in human carbonic anhydrase B in the low pH limit. This hypothesis is consistent with the different spectral properties and $pK_a$ values of the two isoenzymes.

A major difference between the high-activity (human C and bovine B) and the low-activity (human B) isoenzymes of carbonic anhydrase as native zinc enzymes is the $pK_z$ value of the group regulating the catalytic activity, which can be set around 6.5 and 7.5 for the two kinds of isoenzymes, respectively,[1-3] although there is not a single acid-base equilibrium.[4,5] If the cobalt(II) derivatives are considered, the difference in $pK_a$ is maintained;[4] additionally, while the high pH forms of both isoenzymes have very similar electronic spectra[4] and water proton relaxation capabilities,[6-9] the low pH form of the human B isoenzyme (CoHCAB hereafter) has sensibly lower molar absorbance in the visible region[4] and much lower water proton relaxing capability[4,6-9] than the corresponding high-activity bovine enzyme (CoBCAB hereafter). Recently a thorough nuclear magnetic relaxation dispersion (NMRD) study on this[10] and related systems,[11,12] showed that the dispersion curve is of the type expected on the basis of the Solomon theory:[13]

$$T_1^{-1} = KGf(\tau_c) \qquad (1)$$

$$K = \frac{[E]}{111} g^2\beta^2\gamma_1^2 S(S+1) \qquad G = \sum_i \frac{1}{r_i^6}$$

$$f(\tau_c) = \frac{3\tau_c}{1+\omega_1^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}$$

where $[E]/111$ is the enzyme/water proton molar ratio, $r_i$ is the distance of the $i$th proton from the metal, and $\tau_c$ is a correlation

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Table I. Longitudinal Relaxation Rates for the 4H His-119 Signal and Correlation Times for Cobalt(II) Derivatives of Bovine and Human Carbonic Anhydrases

| | CoBCAB | | CoHCAB | | CoHCAB + acetate, |
|---|---|---|---|---|---|
| | pH* 10.9, −62[c] | pH* 5.7, −60[c] | pH* 10.5, −66[c] | pH* 5.9, −66[c] | pH* 6.0, −70[c] |
| $T_1^{-1}$, s$^{-1}$[a] | 395 | 352 | 365 | 115 | 68 |
| $f(\tau_c)$, s[b] | $7.10 \times 10^{-11}$ | $6.30 \times 10^{-11}$ | $6.50 \times 10^{-11}$ | $2.06 \times 10^{-11}$ | $1.20 \times 10^{-11}$ |
| $\tau_c$, s[b] | $2.14 \times 10^{-11}$ | $1.82 \times 10^{-11}$ | $1.86 \times 10^{-11}$ | $2.50 \times 10^{-12}$ | $1.32 \times 10^{-12}$ |

[a] The estimated error is ±10%. [b] Calculated in the frame of eq 1 by assuming no ligand-centered contribution. [c] Shift, ppm, from $Me_4Si$.

time corresponding to the electronic relaxation time. We have also found that four-coordinate species have larger $\tau_c$ values and hence higher relaxing capability than five-coordinate species do.[6,11,12] The coordination number of carbonic anhydrase derivatives fits nicely with this scheme. No conclusion was drawn for the pure enzyme, especially for CoHCAB. Only the low relaxivity of the acidic form of CoHCAB has been attributed to the presence of five-coordinate species;[10] five-coordination would be reached through the coordination of two water molecules, which could add to the three histidine nitrogens of the protein. If this is true, the change in $\tau_c$ associated with the change in coordination number should be straightforward detected through the pH dependence of the $T_1^{-1}$ values of protons of the histidine ligands, without the problem present in NMRD measurements relative to the number of exchangeable protons and of their exchange rates.

## Experimental Section

All the chemicals used were analytical grade. Human carbonic anhydrase B (HCAB) was a gift of S. Lindskog, and bovine carbonic anhydrase was purchased by Sigma Chemical Co. The former was used as such, while the latter was chromatographed on DEAE-cellulose[14] to obtain the B isoenzyme (BCAB). Both enzymes were demetallized by equilibrium dialysis against 0.1 M 2,6-dipicolinic acid in phosphate buffer, pH 6.9,[15] followed by exhaustive dialysis against freshly doubly distilled water. The cobalt derivatives (CoHCAB and CoBCAB) were obtained by dialyzing apoenzyme solutions against unbuffered $10^{-3}$ M cobalt solution and finally dialyzing out the excess metal ions. The pH of the resulting cobalt enzyme solutions was always around 5.7.

The samples for NMR measurements were concentrated up to (1–2) $\times 10^{-3}$ M by ultradialysis. The samples were either used as such or diluted 10:1 with $D_2O$ and concentrated again repeating the procedure twice. The pH dependence of the NMR parameters was followed by increasing the pH of the starting sample by adding small amounts of concentrated solutions of NaOH. No buffers were used; however, on one occasion alkaline samples were brought back to pH 5.7 by adding solid Hepes [*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid), and the spectral and NMR parameters were the same as those of the starting enzymes at low pH.

The electronic spectra were recorded on a Cary 17D spectrometer. $^1H$ NMR measurements were performed at 15 °C with an instrument based on a Bruker CXP 100 console and a Varian DA60 1.4T electromagnet equipped with an external lock circuit granting a ± 1 Hz long term stability. The spectra of both $D_2O$ and $H_2O$ solutions were recorded in quadrature detection on a 50-kHz spectral width by using the pulse sequence (modified DEFT)[16]

$$90°_x\text{–}\tau\text{–}180°_x\text{–}\tau\text{–}90°_x\text{–acquisition}$$

which was further phase alternated (Bruker standard *PAPS* program) to minimize buildup of coherent noise. The pulse angles were adjusted for each sample. Typical 90° pulse lengths were around 3 μs. The $\tau$ value was chosen around 30 ms, and the recycle time was around 50 ms. Such choice allows suppression of the slowly relaxing signals ($H_2O$, diamagnetic signals from the protein) without affecting the fast relaxing signals from the coordinated histidines. The $T_1$ values of the 4 H signal of His-119 were obtained with the same pulse sequence by measuring the signal height $M_\tau$ as a function of $\tau$. The data were best fitted to the equation[16]

$$M_\tau = M_\infty(1 - 2e^{-\tau/T_1} + e^{-2\tau/T_1}) \quad (2)$$

with a nonlinear two-parameter best-fitting procedure. The estimated deviation is less than ±10%. Two representative sets of data are shown in Figure 1.

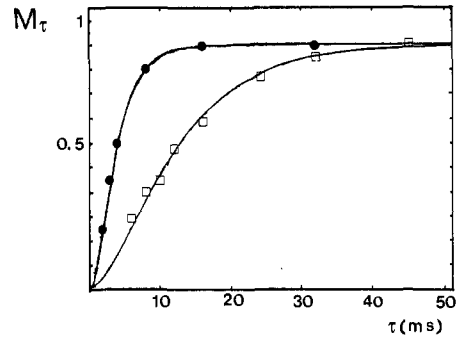


**Figure 1.** Two parameters best fitting of the $M_\tau$ values for the 4H His-119 signal of CoHCAB at pH* 5.9 (□) and 10.0 (●) by using eq 2.

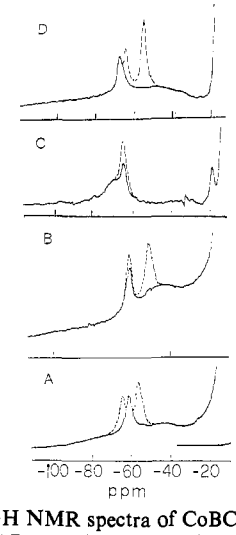


**Figure 2.** 60-MHz $^1H$ NMR spectra of CoBCAB at pH* 5.9 (A) and 10.0 (B) and CoHCAB at pH* 5.9 (C) and 10.0 (D). The dashed lines show the three additional histidine NH signals observed in $H_2O$ solutions.

## Results and Discussions

The use of a particular NMR pulse sequence[16] has recently allowed us to detect the isotropically shifted signals of protons of the cobalt ligands in CoBCAB and its derivatives and to measure their $T_1$ values.[17] In Figure 2 the NMR spectra are reported for both cobalt derivatives; the 4 H signal of the only histidine residue (His-119) bound to the metal ion through the $N_1$ nitrogen is unambiguously assigned[17] as the only relatively sharp signal present in $D_2O$ solutions. Such sharp signal is particularly suitable for measuring $T_1$. The $G$ parameter of eq 1, however, may contain ligand-centered contributions according to the relationship[18]

$$G = 1/r^6 + \rho^2\Delta$$

where $\rho$ is the fractional spin density on the attached carbon, which is proportional to the proton contact shift. Since the 4 H isotropic shifts are within 60–70 ppm downfield for all the compounds investigated (Figure 2 and Table I), variations in $\rho$ have been

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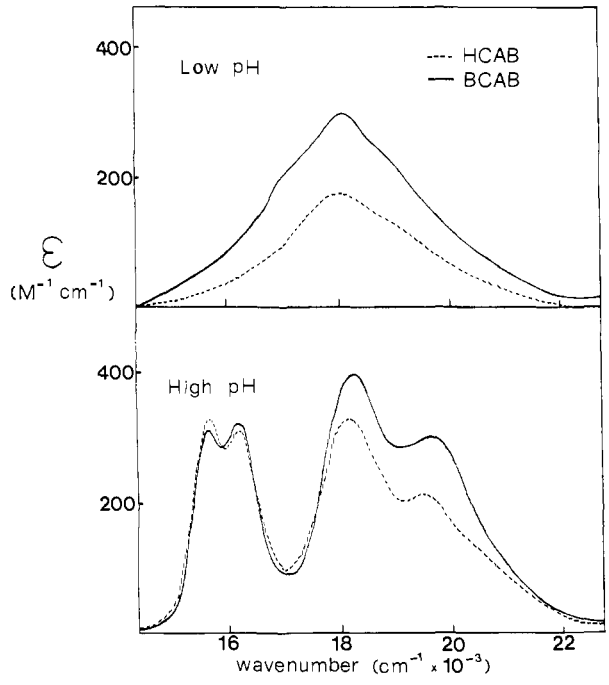


Figure 3. Visible electronic spectra of CoHCAB (---) at pH 5.7 and 10.5 and CoBCAB (—) at pH 5.5 and 10.8.

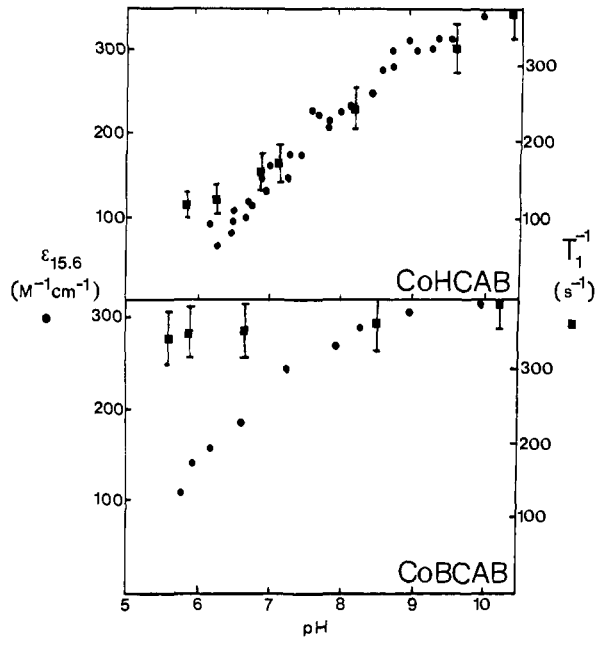


Figure 4. pH dependence of the molar absorbance at 640 nm ($\epsilon_{15.6}$) (●) and of the $T_1^{-1}$ values of the 4 H His-119 signal (■) for CoHCAB and CoBCAB. The scales on the left- and right-hand sides are such that the experimental $\epsilon_{15.6}$ and $T_1^{-1}$ values at high pH coincide.

neglected during this analysis. On the other hand, a variation from 530 to 540 pm on the Co–4 H distance that could arise from pH changes or change in the coordination number would result in only 12% variation in $T_1^{-1}$. Under these conditions, the $T_1^{-1}$ values are considered to be essentially proportional to $f(\tau_c)$, which only depends on the electronic relaxation times.

The $T_1^{-1}$ values for the 4 H signal of CoBCAB and CoHCAB derivatives are reported in Table I. The high-pH form of CoHCAB and the high- and low-pH forms of CoBCAB show very similar $T_1^{-1}$ values, indicating essentially the same geometry around the metal site. The high nuclear relaxing capability of these systems confirms their pseudotetrahedral geometry. On the other hand, the CoHCAB·acetate adduct, which was shown to be five-coordinate,[19] shows a much smaller $T_1^{-1}$ value; finally, the relaxation rate of the low-pH form of CoHCAB shows an intermediate value, although much closer to that of the acetate adduct than to those of four-coordinate derivatives. This, by itself, accounts for the lower water proton relaxivity of the latter system. By comparing the $T_1^{-1}$ values of the low-pH form of CoHCAB with those of the high pH form of CoHCAB and of its acetate adduct, and taking these latter as representative of pure four- and five-coordinate chromophores, respectively, we can state that the low-pH form of CoHCAB is largely five-coordinated. The overall equilibrium would then be

$$\text{Co}\begin{matrix}\diagup \text{OH}_2 \\ \diagdown \text{OH}_2\end{matrix} \rightleftharpoons \text{Co—OH}_2 \rightleftharpoons \text{Co—OH}$$

If the electronic spectra are taken into consideration, the lower molar absorbance of CoHCAB with respect to CoBCAB at low pH (Figure 3) is now fully accounted for on structural basis. According to the spectroscopic criterion developed to discriminate between four- and five-coordination in cobalt(II)-substituted enzymes,[6,20,21] the molar absorbance of the low pH form of CoHCAB indeed suggests the presence of a sizable fraction of five-coordinate species. However, one could think that the lower $pK_a$ of bovine isoenzyme does not allow the low pH species to fully develop at the investigated pH values, thus leaving the possibility that the two isoenzymes behave in a similar fashion, i.e., both the acidic species are largely five-coordinated. In Figure 4, the pH dependence of the absorption at 640 nm is shown as compared to the $T_1^1$ values. Whereas the two curves show the same pattern for the human isoenzyme, they diverge for the bovine isoenzyme, consistent with the idea that in the latter case there is no change in coordination number.

Coming back to the analysis of the longitudinal relaxation rate, if we consider only the metal-centered contribution to the $G$ factor, and take the distance between the 4 H proton of His-119 and the paramagnetic center as 530 pm,[22] we can calculate $f(\tau_c)$ and $\tau_c$ for all the derivatives investigated (Table I). The values are in good agreement with those obtained from the $^1$H relaxation rates of water.[9-11] If some ligand-centered contribution to the $G$ factor is taken into account, shorter $\tau_c$ values are obtained, but a large difference remains in any case between the $\tau_c$ values of the four-coordinated species on one hand and that of the low-pH form of CoHCAB and of the five-coordinate acetate adduct on the other.

The existence of a predominance of five-coordinate species in the low-pH form of CoHCAB, postulated before[10] and now given experimental support, is consistent with the higher $pK_a$ value for the activity-linked ionization with respect to the high-activity isoenzymes. Indeed, the acidity of a coordinated water molecule is expected to decrease on passing from four- to five- to six-coordinate adducts in an homologous series of compounds.

**Acknowledgment.** Thanks are expressed to Prof. S. Lindskog for his kind gift of HCAB and to V. Piccinotti for technical assistance.

**Registry No.** Carbonic anhydrase, 9001-03-0.

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