# $^1$H NMR Spectra of the Coordination Sphere of Cobalt-Substituted Carbonic Anhydrase

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**Abstract:** The $^1$H NMR spectra of some inhibitor derivatives of cobalt-substituted carbonic anhydrase are reported. It is shown that the signals of the coordinated imidazole rings are well shifted from the diamagnetic positions. The five-coordinated adducts display sharper signals and larger isotropic shifts than the pseudotetrahedral derivatives. The resonances of all the histidine protons have been identified, including the NH protons. In the pure enzyme the latter are shown to be present in the pH range 5.5–9, thus ruling out the possibility of their involvement in an acid–base equilibrium. However, their shifts are pH dependent according to a p$K_a$ of about 6.

The X-ray investigation of the native zinc-containing carbonic anhydrase has shown that the metal ion is bound to three histidine nitrogens, two of which bind through the 3-nitrogen and the third binds through the 1-nitrogen (Figure 1). The arrangement of the nitrogens is consistent with roughly tetrahedral geometry, the fourth coordination position being exposed to the solvent and probably occupied by a water molecule.[1] The cobalt(II) derivative, in which the native zinc(II) has been replaced by a cobalt(II) ion, is still quite active[2] and presumably is a rather close model of the native enzyme. Its electronic spectra in the near-infrared–visible regions are pH dependent.[3,4] One acidic group with p$K_a \simeq 6$ most dramatically influences the spectrum, although at least another group with p$K_a \simeq 7$ is capable of affecting the overall shape of the spectrum.[4] In principle, acidic groups capable of affecting the spectra with the above p$K_a$ values may be the coordinated water[5] and the NH groups of the coordinated histidines.[6,7] Both groups have very high p$K_a$ values, 14 and 14.2, respectively, which, however, can be lowered upon coordination. Several cobalt(II) complexes are known with a single water molecule coordinated to the metal ion.[8-11] In these cases the p$K_a$ has been found around 9. The hydrophobic properties of the active site cavity may account for a further lowering of the water p$K_a$. The complex [Co(tris(3,5-dimethyl-1-pyrazolylmethyl)amine)-$OH_2$]$(ClO_4)_2$ displays a pH dependence of the optical spectrum close to that of the cobalt enzyme.[11]

The pyrrole p$K_a$ of imidazole decreases from 14 down to around 10, 8, and perhaps 7 upon coordination.[12] Other acidic groups present in the cavity have been suggested to be capable of affecting the catalytic activity of the enzyme.[13,14]

We thought that recording the $^1$H NMR spectra of the groups close to the paramagnetic center in cobalt carbonic anhydrase would have been quite stimulating. The idea stems from the observation that high-spin cobalt(II) complexes may display very large proton isotropic shifts, i.e., large shifts from the diamagnetic positions, without severe broadening due to the coupling with the unpaired electrons.[15] If this is the case for the present system, as it has been shown to be for cobalt-substituted azurin,[16] proper use of an appropriate pulse NMR spectrometer may allow us to detect the $^1$H NMR signals of the protons shifted far away from the region of the diamagnetic protons. Such technique would provide a direct insight into the catalytic site of the enzyme, including its pH-dependent properties and the effects of inhibitor binding.

## Experimental Section

Bovine carbonic anhydrase was purchased from Sigma as a lyophilized powder; the isoenzyme B was obtained through chromatography on DEAE Cellulose,[17] depleted of zinc(II) by dialysis against 2,6-dipicolinate solutions[18] and exhaustively dialyzed against freshly bidistilled water. The cobalt(II) enzyme was prepared by dialysis of the apoenzyme against cobalt sulfate solutions and subsequent removal of excess metal. The solutions were concentrated by ultradialysis up to $(2-3) \times 10^{-3}$ M; enzyme concentrations were determined from the electronic spectra in the UV and visible regions ($\epsilon_{280} = 57\,000$ M$^{-1}$ cm$^{-1}$ and $\epsilon_{550} = 300$ M$^{-1}$ cm$^{-1}$ at pH 6.0[19,20]). The various inhibitors were added in such amounts as to ensure the complete formation of the adducts, as judged from the known values of the affinity constants[21] and checked through electronic spectroscopy. The electronic spectra of the noninhibited cobalt enzyme were also checked down to pH 5.5; no evidence of metal dissociation was found. $D_2O$ samples were prepared by twice lyophilizing and redissolving enzyme solutions in 99.95% $D_2O$. The pH values are given as uncorrected pH meter readings. The electronic spectra were recorded on a Cary 17D spectrophotometer.

The ligands imidazole, 1-methylimidazole, 2-methylimidazole, and 4(5)-methylimidazole are commercially available. The cobalt(II) complexes were prepared following the procedures already reported.[22] The

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Table I. Analytical Data for High-Spin Cobalt(II) Complexes of Imidazole and Its Analgoues

| | C | | H | | N | |
|---|---|---|---|---|---|---|
| | calcd | found | calcd | found | calcd | found |
| $Co(imidazole)_6(ClO_4)_2$ | 32.4 | 32.0 | 3.6 | 4.0 | 25.2 | 25.5 |
| $Co(imidazole)_2Cl_2$ | 27.1 | 27.4 | 3.0 | 3.4 | 21.1 | 21.5 |
| $Co(1\text{-methylimidazole})_6(ClO_4)_2$ | 38.4 | 38.6 | 4.8 | 5.1 | 22.4 | 22.8 |
| $Co(1\text{-methylimidazole})_2Cl_2$ | 32.7 | 33.1 | 4.1 | 4.7 | 19.0 | 19.4 |
| $Co(5\text{-methylimidazole})_6(ClO_4)_2$ | 38.4 | 38.8 | 4.8 | 5.1 | 22.4 | 22.8 |
| $Co(2\text{-methylimidazole})_2Br_2$ | 25.1 | 25.1 | 3.1 | 3.2 | 14.6 | 15.0 |
| $Co(bis(4\text{-imidazolyl})methane)Br_2$ | 22.9 | 22.8 | 2.2 | 2.4 | 15.3 | 15.4 |

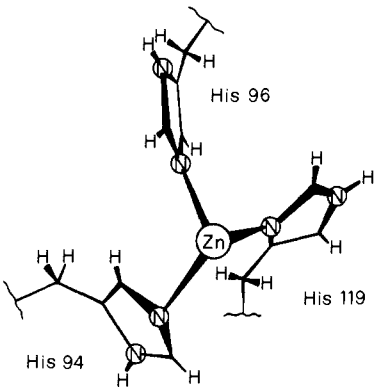


**Figure 1.** Protein ligands arrangement in the metal site of carbonic anhydrase.

ligand bis(4-imidazolyl)methane was synthesized as already described.[23] The Co(bis(4-imidazolyl)methane)$Br_2$ complex was prepared in acetone–ethanol solution by using stoichiometric amounts of the reactants. The solution was concentrated to a small volume, and then ether was cautiously added until crystals were formed. The analytical data are reported in Table I.

The NMR spectra were run with an instrument based on a Bruker CXP console and a low-resolution Varian DA 60 1.4-T electromagnet, equipped with an external lock circuit granting ±1-Hz long-term stability. The sample temperature was 32 °C. The system was usually operated in the high power mode; typical 90° pulse lengths were around 2.0 μs. The spectra were recorded in quadrature detection using the "modified DEFT" pulse sequence described by Hochmann and Kellerhals in ref 24. The use of such a sequence enables us to selectively reduce the intensity of signals with longitudinal relaxation times longer than those of signals of interest. The entire sequence described in ref 24 was further phase alternated to cancel coherent noise, and in some cases different dead times between pulse and acquisition were introduced in the subtract mode with respect to the add mode, properly chosen in order to obtain rephasing of the HDO sin wave which was therefore cancelled every second pulse, with minimal effects in the spectral region of interest. The modified DEFT sequence was found to work less satisfactorily on $H_2O$ samples, for which a "Redfield" pulse sequence was used,[25] again coupled with phase alternation and rephasing of the $H_2O$ sin wave.

For every sample an initial spectrum was taken over 125-kHz sweep width, which was subsequently reduced to 15–20 kHz according to the spreading of the observed signals. About $10^5$ scans were accumulated for each spectrum, with about 100-ms recycle time. $T_2$ values were obtained from the line widths at half-peak height, properly reduced for the line broadening introduced by exponential multiplication of the free induction decay (20 Hz), through the relation $T_2^{-1} = \pi\Delta\nu$. $T_1$ measurements were performed with a saturation recovery type of experiment,[24] using the "modified DEFT" sequence and an appropriate nonlinear least-squares fitting method.

Some spectra were also recorded at Bruker AG, Karlsruhe, on a CXP 300 instrument at 7-T magnetic field.

## Results and Discussion

**The Assignment of the Spectra.** The $^1H$ NMR spectra of $H_2O$ and $D_2O$ solutions containing CoCA and several inhibitors have been recorded between −180 and +120 ppm from $H_2O$ or HDO, which is about 4.8 ppm downfield from $Me_4Si$ (Figure 2). The spectra show weak and broad signals spread over the above range, except that in the range ±10 ppm from $H_2O$ where very strong residual resonance signals due to the diamagnetic protein part of the molecule and to $H_2O$ are present. The weak signals have the shape of the signals shifted through hyperfine interactions. The spectra of some inhibitor derivatives are better resolved, the signals being well spread out over the entire range investigated, whereas in other cases the signals are ill resolved. The inhibitor derivatives which had been described as five-coordinated on the basis of the electronic absorption[21] and ESR[26] spectra show spectra of the former type. Any attempt of assignment should be based on these "high quality" spectra.

When the spectra of the five-coordinated $Au(CN)_2^-$, $NCS^-$, and $I^-$ derivatives in $D_2O$ solutions are examined, some common features are apparent (Figure 2). Five broad signals of the same intensity (taken as one) are present in any case, four of them downfield in the region −40 to −140 ppm and one upfield, around +30 ppm for $Au(CN)_2^-$ and $NCS^-$ and at +51 ppm for $I^-$. There are several narrow signals more or less well resolved in the region −30 to +30 ppm; finally, a narrow signal of intensity 1 exhibiting a large downfield shift (−70 to −90 ppm) is present in all of the three spectra. The longitudinal relaxation times ($T_1$) of the above signals have also been measured for the iodide derivative; the values obtained (Figure 2) parallel the $T_2$ values obtained from the observed line widths, although the latter are generally smaller.

From the known structural data (Figure 1) six signals are expected from the imidazole CH protons, five of which are adjacent and one is in position β with respect to the coordinated nitrogen. If the longitudinal relaxation times are dominated by dipolar contributions, as it has been shown to be reasonable,[27] the ratios between the observed $T_1$ values for the various signals should be equal to the sixth power of the metal–nuclei distance ratios.[28] The calculated distance ratios are close to 1 for the five protons exhibiting broad signals and 1.4 for the proton responsible for the narrower resonance.

In the absence of analogously rigid-model complexes and of simple tools of chemical substitution, the assignment of the signals should be proposed with some caution. If the relaxation data are taken as a criterion for the assignment, the five broad signals may be ascribed to the α-protons and the narrow signal at −70 to −90 ppm to the β-proton with respect to the coordinating nitrogens. This assignment is consistent also for the oxalate derivative (Figure 2) which, however, shows an additional signal at +47 ppm, somewhat narrower than the five signals assigned to the α-protons. When the spectra are recorded in $H_2O$, three further signals are observed in every case (Figure 2), whose line widths are similar to that of the signal assigned to the β-proton. This is consistent with the β-position of the three NH protons of the coordinated imidazole groups. Although the latter protons are completely exchanged with deuteron in $D_2O$ solution, they exchange slowly on the NMR time scale: from their isotropic shift values the exchange rate can be estimated to be smaller than $5 \times 10^3$ $s^{-1}$.

A number of narrow signals are observed between −30 to +30 ppm and outside the diamagnetic region. These signals may be tentatively assigned to the β-$CH_2$ protons of the coordinated

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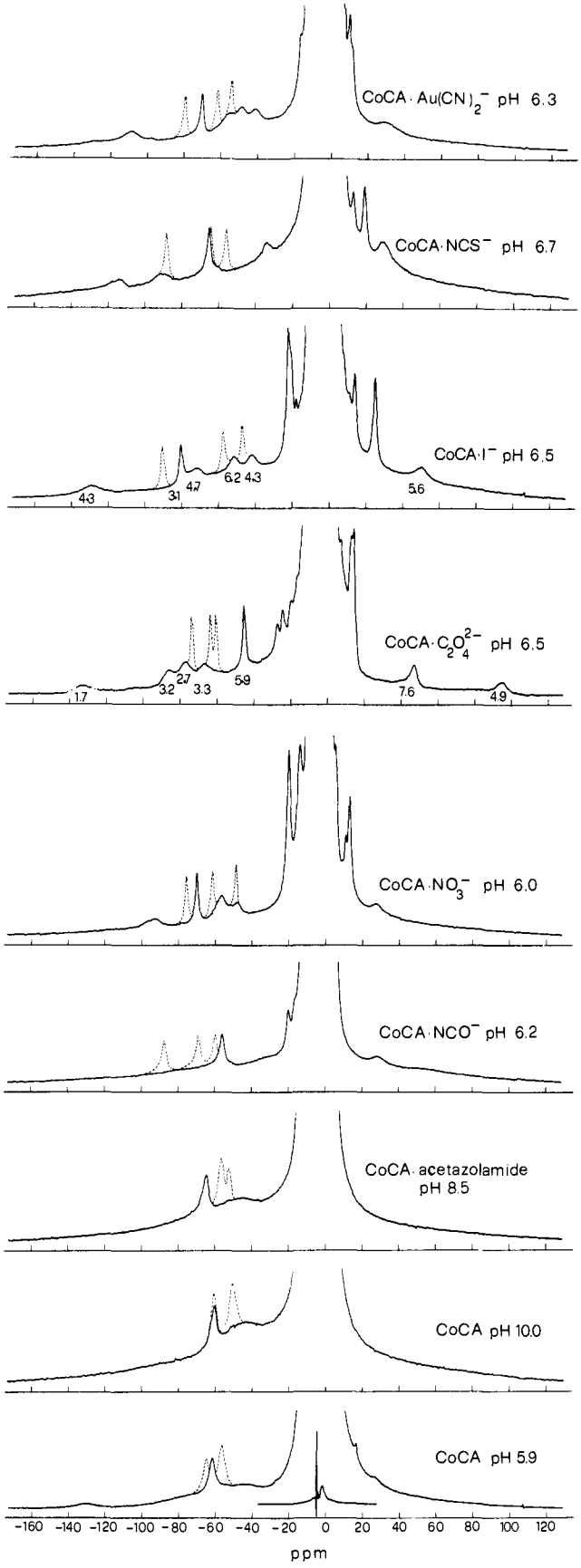
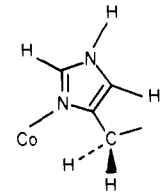



**Figure 2.** 60-MHz $^1H$ NMR Spectra of cobalt(II) bovine carbonic anhydrase B (CoCA), at pH 5.9 and 10.0, as well as of some of its inhibitor derivatives. Enzyme concentrations were (2–3) × $10^{-3}$ M; inhibitor concentrations were about $10^{-2}$ M for $NCS^-$, $NCO^-$ and acetazolamide and $10^{-1}$ M for the others. The dotted lines refer to the imino protons of the cobalt-bound histidines which are observed in $H_2O$ solution. The $T_1$ values (ms) for some signals in the iodide and oxalate derivatives are also reported.

histidines. The signal at +47 ppm in the oxalate derivative belongs to a proton experiencing a relative proximity to the paramagnetic center and may be assigned to a proton of the $\beta$-$CH_2$ group of His-119 which from X-ray data seems directed toward the metal according to I.



I

High-spin cobalt(II) complexes are known to display both contact and dipolar contributions to isotropic shifts.[15] The former are believed to dominate in complexes with monodentate ligands of the type $CoL_6$, where the dipolar contribution due to the magnetic anisotropy is washed out by rapid dynamic rearrangement of the magnetic axes of the chromophore,[15,29-31] or in tetrahedral complexes, where the magnetic anisotropy is usually smaller.[32] In Table II the proton isotropic shifts in several imidazole complexes of cobalt(II) are reported. In all cases the isotropic shifts for ring protons fall in the range −20 to −70 ppm;[31] alkyl substituents in any ring position experience much smaller isotropic shifts. In the present enzyme systems the ring signals are spread over a much wider range, suggesting the presence of sizeable up- and downfield contributions from the dipolar mechanism which add up to a downfield contact term. The NH and the $\beta$-H signals are somewhat more grouped (−50 to −90 ppm) than those of the $\alpha$-protons, consistent with their larger distance from the metal ion which would reduce the dipolar contributions; the contact contribution is likely to be similar in both cases.

The presence of dipolar shifts, which so nicely spread the signals in such a wide frequency range, makes the assignment of the $\alpha$-protons signals difficult in the absence of detailed structural information and detailed knowledge of the magnetic anisotropy. From the ESR spectra of cobalt carbonic anhydrase and its inhibitor derivatives, a large pseudotetragonal anisotropy of the magnetic tensor may be inferred;[26] furthermore, the observed hyperfine coupling constant in the case of the iodide derivative has been interpreted as indicative of a roughly square-pyramidal structure. Within such a frame, a histidine could be axial and the other two equatorial, in order to account for the different behavior of an $\alpha$-proton signal with respect to the other four. Indeed, in tetragonal high-spin cobalt(II) complexes upfield dipolar shifts are usually observed in axial ligands and downfield shifts in equatorial ligands.[15,33] Since only one proton is found at high fields, it could be proposed that His-119, being the only one having a single $\alpha$-proton (Figure 1), is axial, and this could be consistent also with the upfield shift of a proton of the $\beta$-methylene group in the oxalate derivative. However, the fact that neither the $\beta$-proton nor any of the NH protons is upfield with respect to the average values for protons reported in Table II is somewhat puzzling, unless (1) they experience larger downfield shifts in the enzyme with respect to the model complexes or (2) some peculiar orientation of one histidine residue whose $\alpha$-proton experiences upfield shift is proposed with respect to the axes of the magnetic tensor.

The pseudotetrahedral $NCO^-$ and acetazolamide derivatives (Figure 2) give rise to smaller isotropic shifts and broader signals; although fewer signals than those in the five-coordinated deriv-

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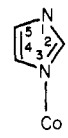
Table II. $^1$H Chemical Shift Values (ppm)[a] for High-Spin Cobalt(II) Complexes of Imidazole and Its Analogues in Acetone-$d_6$ Solutions



| | 1 | 2 | 4 | 5 |
|---|---|---|---|---|
| Co(imidazole)$_6$(ClO$_4$)$_2$ | *b* | −31 | −44[c] | |
| | | (−23) | (−37) | |
| Co(imidazole)$_2$Cl$_2$ | −69 | −45 | −50[c] | |
| | (−56) | (−37) | (−43) | |
| Co(1-methylimidazole)$_6$(ClO$_4$)$_2$ | −8 (CH$_3$) | −32[d] | −47[d] | −52 |
| | (−4) | (−24) | (−40) | (−45) |
| Co(1-methylimidazole)$_2$Cl$_2$ | −12 (CH$_3$) | −55 | −71 | −52 |
| | (−8) | (−48) | (−64) | (−45) |
| Co(5-methylimidazole)$_6$(ClO$_4$)$_2$ | −48 | −33 | −44 | −7 (CH$_3$) |
| | (−36) | (−25) | (−37) | (−5) |
| Co(2-methylimidazole)$_2$Br$_2$ | −63 | +1 (CH$_3$) | −76[e] | −45[e] |
| | (−52) | (+3) | (−69) | (−38) |
| Co(bis(4-imidazolyl)methane)Br$_2$ | −60 | *b* | −10 (CH$_3$) | −44 |
| | (−48) | | (−7) | (−37) |

[a] Negative shifts are downfield with respect to Me$_4$Si; isotropic shifts in parentheses. [b] Not observed. [c] Degenerate owing to rapid protomeric equilibrium. [d] The assignment of 2 H and 4 H signals is only tentative and is based on the analogy with the 5-methylimidazole derivative. The diamagnetic-paramagnetic exchange rate is slow on the NMR time scale. [e] The protomeric equilibrium is slow on the NMR time scale.

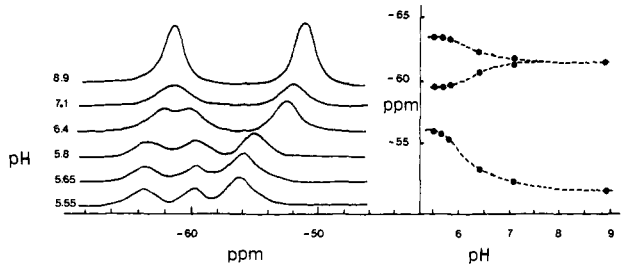



**Figure 3.** pH dependence of the chemical shifts for 300-MHz $^1$H NMR signals in H$_2$O solution of histidines bound to the cobalt(II) ion in the active site of carbonic anhydrase.

atives are resolved, the relatively narrow resonance around −60 ppm can be assigned to the $\beta$-proton of His-119, in analogy with the assignment discussed above. When the spectra are recorded in H$_2$O, additional signals of about the same line width are again apparent (Figure 2), consistent with their assignment to the imidazole NH protons. Smaller dipolar shifts and larger dipolar broadening are expected in the above derivatives since the orbitally nondegenerate ground state in tetrahedral complexes reduces the orbital contribution to the magnetic susceptibility and hence the magnetic anisotropy. For the very same reason the electronic relaxation times are longer than those in complexes with larger magnetic susceptibility values.[34] Therefore the observed isotropic shifts are probably largely contact in nature.

Consistent with its assignment as tetrahedral the spectrum of the noninhibited cobalt enzyme in D$_2$O (Figure 2) again shows only one signal reasonably sharp around 60 ppm and assigned to the $\beta$-proton; very broad absorptions are present in the range −100 to −20 ppm, and a further weak signal is present at −130 ppm in the acidic sample, arising from the $\alpha$-protons. In H$_2$O solutions at pH 5.5 two NH signals are observed at −64 and −56 ppm, the latter being of intensity 2. In Figure 3 the NMR spectra of pure cobalt carbonic anhydrase in H$_2$O are reported as a function of pH; the spectra have been recorded at 300 MHz in an attempt to increase resolution. Although two NH signals are degenerate over the entire pH range investigated, no decrease in intensity of the signal occurs; the $\beta$-proton and the third NH proton signals also collapse with increasing pH, the spectrum at pH 8.9 showing only two resonances of intensity 2 at −62 and −51 ppm. The chemical shift variations with pH can be fitted to a single acid–base equilibrium with a p$K_a$ of 6.05 ± 0.15. The line widths of the above signals decrease with increasing temperature in both the acidic and basic limits of the pH range investigated, as expected for protons exchanging much more slowly than required to be sensitive to exchange broadening effects.

## Conclusion

The five-coordinated complexes of high-spin cobalt(II) derivatives of bovine carbonic anhydrase B provide reasonably well-resolved $^1$H NMR spectra. In particular, all the signals expected from the metal-coordinated imidazole residues are apparent in the spectra. The spreading of the signals is attributed to the concurrence of dipolar and contact mechanisms in the hyperfine coupling, while their reasonable sharpness is ascribed to the short electronic correlation times of five-coordinated cobalt(II), which have been estimated through water proton relaxation studies at variable frequency to be in the range (3–6) × $10^{-12}$ s.[38]

$^1$H NMR spectroscopy may thus provide an important tool in the study of the active site of cobalt(II)-substituted metalloproteins in which it is reasonable to assume that cobalt(II) undertakes five-coordination. In the case of tetrahedral coordination the resolution is much poorer, owing to a decrease in dipolar contributions to the shifts and to a larger dipolar broadening. The electronic relaxation times for the tetrahedral derivatives of cobalt carbonic anhydrase have indeed been found to be larger than $10^{-11}$ s.[38] However, even in the latter case signals of protons in $\beta$-position with respect to the coordinating nitrogens such as the NH protons experience enough isotropic shift (mainly contact) to be easily resolved from the diamagnetic region of the spectrum and are far enough away from the paramagnetic center to have reasonable line widths. In the present case it has been possible to follow the shifts of the NH protons of the coordinated imidazoles in cobalt(II) carbonic anhydrase with pH. It has been shown that the shifts of the NH protons are sensitive to an acid–base equilibrium with p$K_a \simeq 6$, corresponding to the p$K_a$ of the group whose dissociation mostly affects the electronic spectra.[4] However, all three of the NH protons are observed in the pH range 5.5–9, ruling out the possibility that in this range one of the coordinated imidazoles

(34) The magnetic moments reported for a series of CoCA derivatives[35,36] are presumably affected by an error larger than the difference expected between four- and five-coordinated high spin Co(II) complexes. The former are reported to have magnetic moments of 4.3 and the latter of 4.7 $\mu_B$.[37]

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ionizes to an imidazolate ion. This is quite conclusive evidence, which was needed after the report that coordinated imidazoles may indeed ionize with low $pK_a$ values,[6] and is in agreement with what was induced from $^1H$ NMR spectra of the whole diamagnetic zinc enzyme.[39] Another important point in the present research is that the three imidazoles are indeed regularly coordinated, despite the apparent irregularities in bond distances from the X-ray structural data[1] and that they are not replaced by any of the inhibitors investigated.

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# Effective Enhancement of Valinomycin-Mediated Potassium Uptake in Organic Phase by Uncouplers of Oxidative Phosphorylation Detected by $^1H$ NMR

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**Abstract:** $^1H$ NMR studies demonstrated the existence of a ternary complex of the electrogenic ionophore valinomycin, $K^+$, and an anion, such as a protonophoric uncoupler of oxidative phosphorylation, when these components were partitioned between an organic solvent and water. The stoichiometry of the ternary complex was found to be 1:1:1 when the acid was an uncoupler such as SF 6847, FCCP, 2,4-dinitrophenol, or picric acid. However, when 8-anilinonaphthalene-1-sulfonate (ANS) or benzoic acid was used as gegenion, no ternary complex could be detected. Studies on the NMR spectra of valinomycin and organic acids and quantitative determination of potassium showed that potent uncouplers greatly enhance valinomycin-mediated potassium uptake. This effect of uncoupler was well correlated with their uncoupling activity in mitochondria. The synergistic actions of valinomycin and uncoupler in the transport of potassium ion and proton across membranes is suggested.

Uncouplers of oxidative phosphorylation and ionophores, such as valinomycin, are very useful for manipulating the electrochemical potential across biological and model membrane systems. Recently, the synergistic effects of ionophore and uncouplers or other anions have been proposed to be important in the process of alkali-metal cation and proton translocation through the membrane and in the action of uncouplers.[1-6] From the spectral change of the uncoupler[4,5] and kinetic treatment of $K^+$ or $H^+$ transport,[1,5,6] the formation of a 1:1:1 complex of valinomycin, $K^+$, and a monovalent organic anion, such as an uncoupler, is considered to be responsible in these processes. However, there is no direct evidence for the formation of the ternary complex, except for the crystallographic study on the complex of valinomycin-$K^+$ with an inorganic anion.[26] Further, little is known about the role of anions in valinomycin-mediated potassium transport, though the movement of potassium has been found to depend on the kind of anion acting as gegenion.[7]

This paper reports direct evidence for the existence of the ternary complex obtained by $^1H$ NMR spectroscopy and describes the molecular basis of the synergistic actions of uncoupler and valinomycin in the transport of $K^+$ and $H^+$. This study may shed light on the mechanism of the action of uncoupler on biomembranes.

## Experimental Section

**Materials.** SF 6847[8] and PCP[8] were gifts from Dr. Y. Nishizawa, Sumitomo Chemical Industry, Osaka (Japan), and FCCP[8] was kindly supplied by Dr. P. Heytler, E. I. DuPont de Nemours and Co., Wilmington, DE. Valinomycin was purchased from Sigma Chemical Co., St. Louis MO. Other reagents were commercial products and were used without further purification.

**Two-Phase Extraction for NMR Measurement.** A volume of 0.5 mL of $CCl_4$ containing 3 mM of valinomycin was equilibrated with an aqueous solution (1 mL) of 1 N KOH containing an anion (30 mM), such as an uncoupler of ANS,[8] by shaking for 10 min at 20 °C. After equilibrium, the NMR spectrum of the $CCl_4$ phase was measured with a JEOL FX-100 NMR spectrometer, using the pulsed Fourier transform method. $^1H$ NMR spectra were usually collected as 200 transients after 45° pulses (10 μs) with the interval of 3.5 s.

**Two-Phase Extraction for Measurement by Flame Photometry.** One milliliter of chloroform, which had been equilibrated with 10 mM LiOH, was added to 3 mL of an aqueous solution of 10 mM LiOH and 100 μM KCl. Valinomycin was added to the chloroform phase at 300 μM and an uncoupler or other acid was added to the aqueous phase at 0, 100, 300, and 500 μM. The two phases were equilibrated by shaking gently overnight at 4 °C. Then the concentration of $K^+$ in the aqueous phase was determined by flame photometry in a Hitachi flame photometer, Model 205. LiOH was added to the aqueous phase to maintain a constant pH and also as an internal standard in assay of $K^+$ by flame photometry. The reference experiment in the absence of valinomycin was carried out in the same way.

## Results

The $^1H$ NMR spectra of valinomycin in $CCl_4$ under various conditions are shown in Figure 1. The line assignments were made on the basis of the results reported previously.[9-12] Figure 1a is

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