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Are there Other Acidic Groups Capable of Affecting the Electronic Spectra of Cobalt(II) Substituted Carbonic Anhydrase?

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The electronic spectra of NCS^- and I^- adducts of cobalt(II) human carbonic anhydrase I are pH dependent at pH values below 7. The pK_a of such equilibrium is dependent on the anion concentration and varies between 4.6 and 6.6. The ^1H NMR spectra show that the three histidine residues are bound to the metal ion over the entire pH range investigated. It is proposed that a Glu residue triggers the change in stereochemistry around the metal ion. It is possible that such a Glu residue is Glu 106 present in the active cavity.

Introduction

The catalytic rate constant of carbonic anhydrase (CA hereafter) is pH dependent [1, 2] presumably involving more than a single acid base equilibrium [3]. The electronic spectra of the cobalt(II) substituted CA derivatives are pH dependent in a similar fashion [4–6], providing evidence that at least two are the acidic groups capable of affecting the electronic spectra. The binding of binegative anions was shown to give rise to the release of two protons that were tentatively assigned to the metal coordinated water and to a histidine residue hanging in the cavity [3].

During a thorough study designed to find differences between cobalt(II) substituted human I and bovine II carbonic anhydrase (CoHCA I and CoBCA II respectively) we found that the electronic spectra of some inhibitor derivatives of CoHCA I are pH dependent, thus providing further information about the role of the acidic groups within the cavity. We wish to report the results of an investigation using electronic absorption spectroscopy and proton NMR measurements of CoHCA I in the presence of inhibitors like NCS^-, NO_3^-, I^- and NCO^-.

Experimental

Human carbonic anhydrase isoenzyme I was a gift from S. Lindskog and was used without further purification. It was demetallized by dialysis against 5×10^{-2} *M* 2,6-dipicolinic acid solution in phosphate buffer at pH 6.9 [7]; this was then followed by exhaustive dialysis against freshly bidistilled water, to remove excess chelating agent. The cobalt(II) derivative was prepared by dialyzing the apoprotein obtained by the above procedure against 10^{-3} *M* $CoSO_4$ solutions, and then against water several times; the pH of the final solution was always between 5.6 and 5.8. The enzyme concentrations and the cobalt(II) content were measured from the electronic spectra in the U.V. and visible regions respectively ($\epsilon_{280} = 49\,000\ M^{-1}\ cm^{-1}$ [8]; $\epsilon_{550} = 170\ M^{-1}\ cm^{-1}$ at pH 5.6 [6]).

The samples for electronic and NMR spectra were prepared by concentrating the enzyme solutions by ultradialysis under nitrogen pressure to a final concentration of $1–2 \times 10^{-3}$ *M*. The samples for NMR measurements were used as such for spectra in water solution, or diluted 10:1 with D_2O and concentrated again, repeating the procedure two times, for spectra in D_2O. The samples for electronic spectra were diluted with water or with HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) solutions as indicated in the caption to the figures. Alkaline samples were prepared by adding small amounts of concentrated solutions of NaOH to the above solutions.

The electronic spectra were recorded on a Cary 17D spectrometer, in the 0–0.1 absorbance range, using $1–5 \times 10^{-4}$ *M* solutions of the cobalt(II) substituted enzyme. The spectra of the inhibitor adducts were recorded on samples obtained by mixing unbuffered solutions of enzyme and inhibitor at the same pH, unless otherwise specified.

^1H NMR measurements were performed at 15 °C using an instrument based on a Bruker CXP 100 console and a Varian DA 60 1.4 T electromagnet equipped with an external lock circuit granting a ±1 Hz long term stability. Both D_2O and H_2O solution spectra were recorded in quadrature detection mode on a 50 KHz spectral width, using a modified DEFT pulse sequence [9] $90_x°–\tau–180_x°–\tau–90_x°$– acquisition, which was further phase alternated (Bruker standard PAPS program) to minimize build-up of coherent noise. Pulse lengths were typically

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3 μs for a 90° pulse, and were adjusted for each sample. Recycle times were around 50 ms to allow suppression of the slowly relaxing signals. T_1 values of the signals were measured with the same pulse sequence, measuring the signal height M_τ as a function of τ. The data were best fitted to the following equation [9]

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

with a non linear two parameter best fitting procedure. The estimated deviation is less than 10%.

Results

Addition of NCS^- to solutions of CoHCA I changes the electronic spectrum until a 1:1 adduct is formed. The formation of the adduct is almost quantitative ($K_{app} > 10^4$) at every pH between 5.4 and 7.5. Typical titrations are shown in Fig. 1. The spectra of the 1:1 adduct are, however, pH dependent, as shown in Fig. 2. The maximum molar absorbance increases from a value of 160 M^{-1} cm^{-1} at pH 7.5 to 270 M^{-1} cm^{-1} at pH 5.0. Owing to the impossibility of lowering the pH below 5 without loss of the metal ion, the spectrum of the limit low pH species and the pK_a for the acid base equilibrium involved cannot be determined. However from the pH dependence of the molar absorbance, the latter can be estimated to be lower than 5.5 and the limit maximum molar absorbance to be higher than 300 M^{-1} cm^{-1}. The spectrum of the 1:1 adduct at pH 7.5 is the same as that obtained at higher pH (*e.g.* 9.3) with excess of NCS^-.

Excess NCS^- is however still capable of affecting the electronic spectra of the acidic species (Fig. 3). The spectral change consists of a further increase in the molar absorbance of the adduct without any major alteration in the shape of the spectrum. Such change is almost complete in the presence of 0.7 M NCS^- at pH 5.8 (Fig. 3, inset). The electronic spectra of the enzyme in the presence of excess NCS^- are also pH dependent (Fig. 4), giving rise to a definite spectrum at low pH with molar absorbance of 540 M^{-1} cm^{-1}. At high pH the same spectrum reported in Fig. 1 for the 1:1 adduct at pH 7.5 is obtained. The pH dependence shows a well defined sygmoidal profile which can be fitted to a pK_a of 6.6 ± 0.1.

The ^1H NMR spectra of the NCS^- adducts under various experimental conditions are reported in Fig. 5. Spectrum A can be obtained either at pH 7.5 with a stoichiometric amount of NCS^- or at pH 9.3 with excess NCS^-. The sharp signal at around −70 ppm is assigned to the His 119 4H proton, in agreement with previous reports [10, 11]. Its T_1 value is 12 ms. The dotted signals disappear in D_2O solutions and are therefore assigned to the exchangeable NH protons of the three coordinated histidines. Spectrum B is

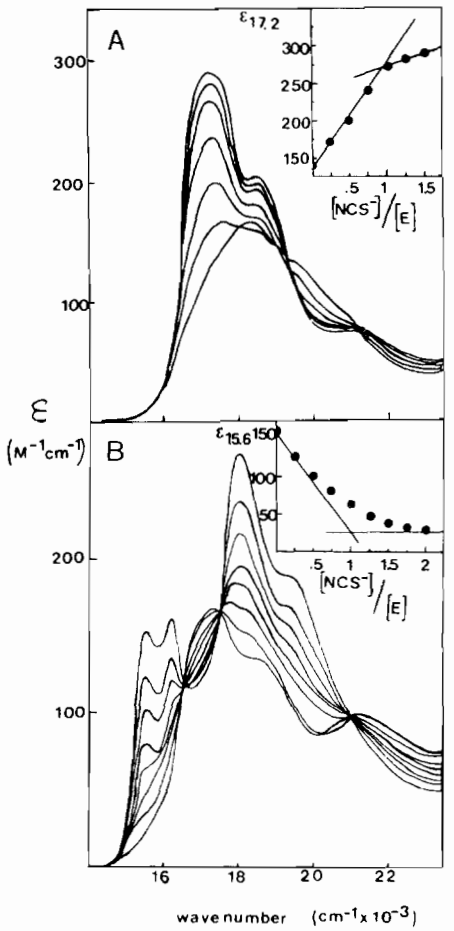


Fig. 1. A: Electronic absorption spectra of 1.9×10^{-3} M solutions of CoHCA I in 5×10^{-2} M HEPES buffer at pH 5.4 in the presence of 0, 4.9×10^{-4}, 9.2×10^{-4}, 1.4×10^{-3}, 1.9×10^{-3}, 2.4×10^{-3}, 2.9×10^{-3} M NCS^- in order of increasing molar absorbance at 17.2 $cm^{-1} \times 10^{-3}$. The spectra are corrected for the dilution. The final dilution was less than 10%. The inset shows $\epsilon_{17.2}$ as a function of the NCS^-: enzyme ratio. B: Electronic absorption spectra of 4.6×10^{-4} M CoHCA I solution at pH 7.5 in the presence of 0, 1.1×10^{-4}, 2.3×10^{-4}, 3.4×10^{-4}, 4.5×10^{-4}, 5.6×10^{-4}, 6.7×10^{-4}, 8.8×10^{-4} M NCS^- in order of decreasing absorbance at 15.6 $cm^{-1} \times 10^{-3}$. The spectra are corrected for the dilution. The final dilution was less than 5%. The inset shows $\epsilon_{15.6}$ as a function of the NCS^-: enzyme ratio.

obtained at pH 5.5 with 0.4 M NCS^-. The T_1 value of the His 119 4H signal is 3.6 ms. A pH titration of the latter sample from pH 5.5 to 9.3 shows that all three NH signals are present at low pH demonstrating that the three histidines are still coordinated to the paramagnetic cobalt(II) ion. Spectrum C is obtained at pH 5.5 with a stoichiometric amount of NCS^-: both isotropic shifts (inset of Fig. 5) and T_1 values of the His 4H signal (6.1 ms) are intermediate between those of the spectra A and B, indicating that spectrum C could be that of an equilibrium mixture between the high and low pH adducts. The effect of

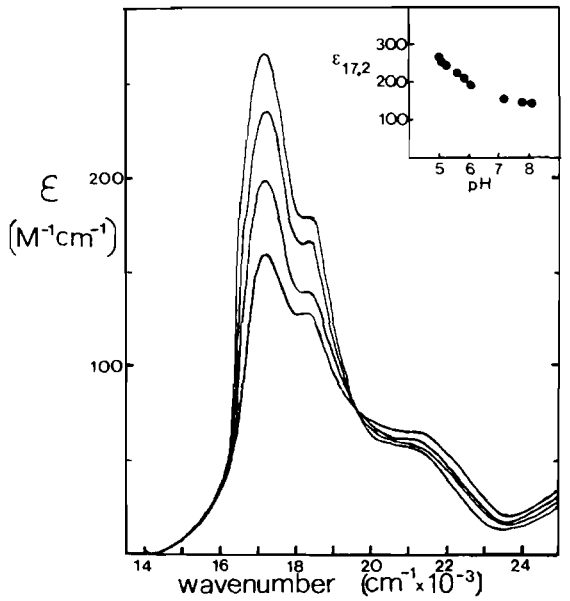


Fig. 2. pH dependence of the electronic specta of 2.7×10^{-4} *M* CoHCA I solution in 5×10^{-2} *M* HEPES buffer in the presence of 4.7×10^{-4} *M* NCS^-. The reported spectra are at pH 7.8, 6.1, 5.6, 5.3 in order of increasing molar absorbance at 17.2 $cm^{-1} \times 10^{-3}$. The inset shows the pH dependence of $\epsilon_{17.2}$.

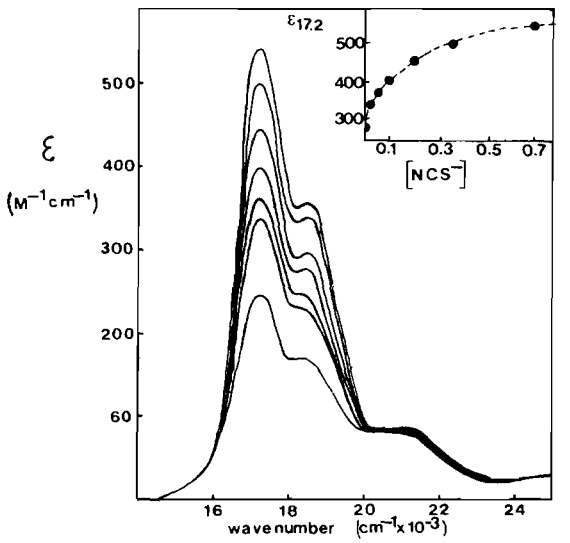


Fig. 3. Electronic absorption spectra of 5.1×10^{-4} *M* CoHCA I solution in 5×10^{-2} *M* HEPES buffer at pH 5.8 in the presence of 5.1×10^{-4}, 2.4×10^{-2}, 4.9×10^{-2}, 9.6×10^{-2}, 0.19, 0.36, 0.73 *M* NCS^- in order of increasing molar absorbance at 17.2 $cm^{-1} \times 10^{-3}$. The inset shows $\epsilon_{17.2}$ as a function of NCS^- concentration. The curve is calculated for an apparent affinity constant of 10 (see discussion).

excess thiocyanate would then simply be to shift the pK_a for the acid base transition in the adduct towards higher values. Under this assumption, if the low pH electronic spectrum of the system containing excess

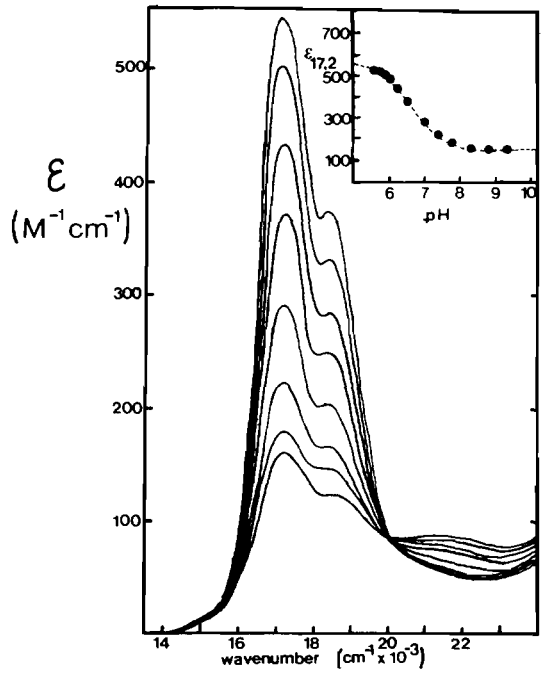


Fig. 4. pH dependence of the electronic spectra of the NCS^- adduct of CoHCA I in the presence of 0.4 *M* NCS^-. The reported spectra are at pH 9.3, 8.3, 7.4, 7.0, 6.5, 6.25, 5.9, 5.6 in order of increasing molar absorbance at 17.2 $cm^{-1} \times 10^{-3}$. The inset shows the pH dependence of $\epsilon_{17.2}$; the data can be best fitted to a pK_a of 6.6 ± 0.1.

thiocyanate is taken as the low pH limit spectrum of the NCS^- derivative without ligand excess, then the pK_a for the spectral variation of the latter can be set at around 4.6. In Fig. 6 the apparent pK_a is shown as it depends on the NCS^- concentration.

The iodide derivative shows a analogous behavior. A titration at pH 5.8 with increasing amounts of I^- is shown in Fig. 7. The formation of the adduct is not stoichiometric, the apparent affinity constant being around 500 M^{-1} cm^{-1}. Further addition of I^- again increases the molar absorbance of the spectrum (Fig. 7 and inset A). Again the spectrum of the adduct is pH dependent, as shown in Fig. 7, inset B, for a CoHCA I solution containing 0.4 *M* I^-; a pK_a can be settled at 6.1 ± 0.2.

At variance with the two above inhibitors, nitrate and cyanate do not show appreciable variations of the electronic spectra of their enzyme adducts with pH even in the presence of excess ligand.

Discussion

The results indicate that for every concentration of NCS^-, the NCS^- derivative of CoHCA I has pH dependent spectral properties with a pK_a varying from about 4.6 for the system containing stoichiometric amounts of NCS^- to 6.6 in the presence of a

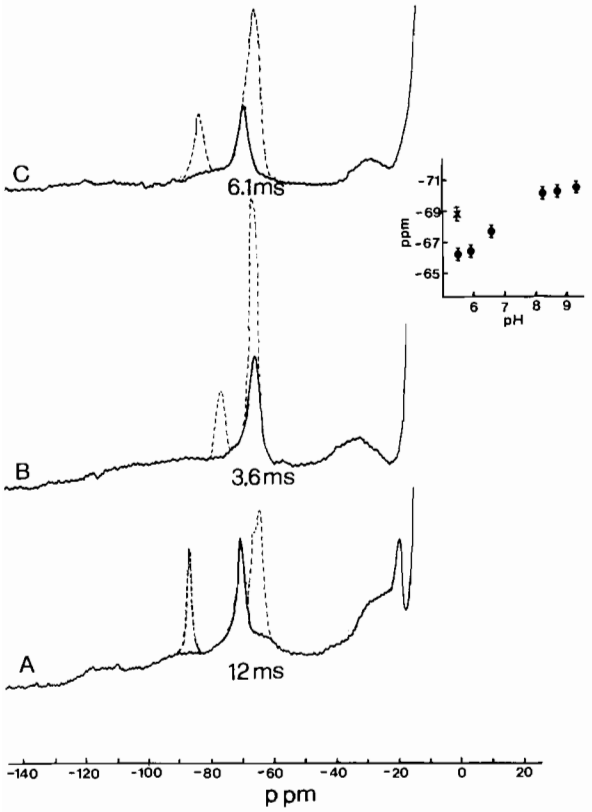


Fig. 5. 60 MHz ^1H NMR spectra and T_1 values of the His 119 4H signal at 15 °C of: A) CoHCA in the presence of stoichiometric amounts of NCS^- at pH 7.5 or in the presence of 0.4 *M* NCS^- at pH 9.3. B) CoHCA I in 5×10^{-2} *M* HEPES buffer at pH 5.5 in the presence of 0.4 *M* NCS^-. C) CoHCA I in the presence of stoichiometric amounts of NCS^- at pH 5.5. The dashed signals disappear in D_2O solution. The inset shows the pH dependence of the isotropic shift (ppm from TMS) of the His 119 4H signal of CoHCA I in the presence of 0.4 *M* NCS^- (•), the limiting values being those of spectra A and B. × represents the isotropic shift for the same signal of CoHCA I in spectrum C.

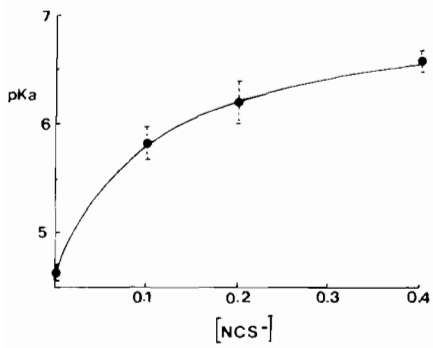


Fig. 6. pK_a of the electronic spectra variations for the system CoHCA I + excess NCS^- as a function of NCS^- concentration. The curve is calculated for an apparent affinity constant of 10 (see discussion).

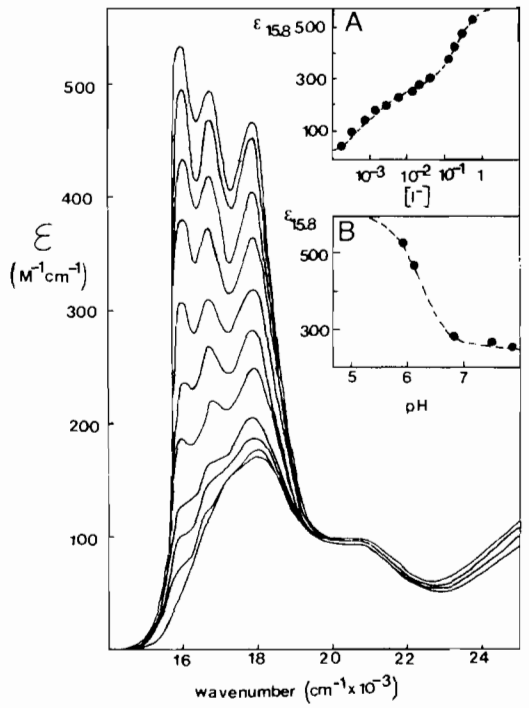
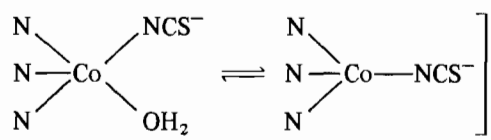


Fig. 7. Electronic absorption spectra of 1.4×10^{-3} *M* CoHCA I solutions at pH 5.8 in the presence of 0, 2.5×10^{-4}, 4.9×10^{-4}, 9.8×10^{-4}, 1.9×10^{-3}, 7.5×10^{-3}, 5.84×10^{-2}, 0.11, 0.22, 0.43, 0.79 *M* I^- in order of increasing absorbance at 15.8 $cm^{-1} \times 10^{-3}$. Inset A shows $\epsilon_{15.8}$ as a function of I^- concentration; the curve is best fitted with two apparent affinity constants of 486 and 2.3 (see discussion). Inset B shows the pH dependence of $\epsilon_{15.8}$ in the presence of 0.4 *M* I^-; the data can be fitted to a pK_a of 6.1 ± 0.2.

large excess of NCS^-. The spectral variations are indicative of an equilibrium between two stereochemistries around the central metal ion. The high pH form of the NCS^- adduct shows spectra of low intensity and long ^1H NMR T_1 value of the His 119 4H signal. These data are consistent with pentacoordination around the cobalt(II) ion [10–15], as already suggested for the NCS^- derivative of the bovine II isoenzyme [10, 12, 15]. The low pH form displays much more intense electronic spectra and shorter ^1H NMR T_1 value of the same proton signal. These data are consistent with those of the pseudotetrahedral adducts of CA [10–15]. Since the ^1H NMR measurements show that the three histidine residues are always bound and since NCS^- is presumably bound to the cobalt(II) ion, it is reasonable to propose an equilibrium of the type



high pH low pH

Since this equilibrium does not directly involve the H^+ species, the removal of the water molecule from the coordination sphere should be a consequence of a conformational change triggered by the dissociation of an acidic group. The pK_a of such an acidic group in the absence of excess NCS^- is around 4.6 and presumably it is also around this value in the pure enzyme. The only reasonable residues with such a pK_a are the glutamic acid residues.

NCS^- ions may interact with accessible arginines with apparent affinity constants of 10^3 [16, 17]; The dependence of the pK_a of the acidic group responsible for the spectral changes in the present system on the NCS^- concentration (Fig. 6 and inset of Fig. 3) indicates an apparent affinity constant of NCS^- ions for the protein of about 10. This is consistent with the values reported for the interaction of NCS^- with His and Lys residues in proteins [16, 17].

The fact that excess iodide ions show a less marked effect than NCS^- on the electronic spectra, and NO_3^- and NCO^- do not show any appreciable effect, is consistent with this weak interaction of anions with positively charged groups in the protein, which is known to follow the Hofmeister lyotropic series [18, 19].

This interaction has to be responsible for an increase in the pK_a of the residue assigned as a Glu. It is known that a change in the overall charge on a protein alters the pK_a of acidic groups in the protein itself according to an empirical relationship of the type [20]:

$$pK_{obs} = pK_{int} - 0.868\ w\bar{Z}$$

where w is a constant whose value is related to the kind of acidic group under consideration (w = 0.025 for carboxylic groups) and $\bar{Z}$ is the overall charge of the protein. However, the observed increase of pK_a from 4.6 to 6.6 upon addition of excess NCS^- would imply binding of about 90 negative ions on the protein. Such a number greatly exceeds the total number of arginine, lysine and histidine residues available in the present enzyme; furthermore, not all of these groups are accessible to solute molecules. Although electrostatic contributions may be present, such a large shift in pK_a is more likely to depend on some kind of conformational change induced by NCS^- binding on some of the positive residues mentioned above; the proposed conformational change might stabilize a hydrogen bonding between the protonated carboxylic moiety of the Glu residue and another group. Since such a Glu residue is capable of changing the coordination number around the metal ion, it is tempting to propose that it is the Glu 106 present in the cavity which can interact through hydrogen bonding with the nearby Thr 199 [21].

The conformational changes associated with Glu 106, or any other Glu residue deprotonation, as well as the possible formation of hydrogen bonding which affects the pK_a of the above group are presumably quite accidental as far as the enzymatic mechanism is concerned; indeed, the high activity bovine II isoenzyme does not show any pH dependence of the spectra of the NCS^- derivative under any circumstance.

This research points out the role which the flexibility of the polypeptidic chain may play in the structural properties of the active sites of metalloproteins.

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