A Nitrogen 15 nmr Investigation of Zinc Cysteine Coordination

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The formation of zinc(II) cystinate complexes has been studied by a variety of workers [1–4]. Most of these studies have used classical techniques to ascertain the overall stoichiometry of the various complexes formed but have not pinpointed the actual ligand sites which participate in the binding. The titrimetric studies have, however, demonstrated the existence of a variety of protonated and polymeric forms for 2:1 molar ratio mixtures of cystein:zinc(II) at various pH's without describing the coordination details.

Zegzhda *et al.* did attempt to determine the mode of binding in zinc(II) cysteine by application of proton nmr, and infrared spectroscopies [5]. Their study indicated that the protonated complexes contain the $-NH_3^+$ group and, hence, coordination involves only deprotonated sulfur and carboxylate. At pH's where the complex was deprotonated, coordination of NH_2 was possible, but, based upon the ir absorption frequencies assigned to NH_2, they concluded that the nitrogen does not coordinate at high pH either.

The work reported here was undertaken to provide a more definitive answer to the question of zinc–cysteine bonding and to test a recently proposed ^{15}N nmr method for the assignment of nitrogen coordination details in zinc complexes [6].

Experimental

All nmr spectra were obtained on a JEOL FX90Q fourier transform spectrometer operating at 9.04 MHz. The samples were made up in 10 mm nmr tubes. A capillary tube containing 99.8% deuterium oxide or 99.8% hexadeuteroacetone, held in place by a vortex plug provided the nmr deuterium lock signal.

Cysteine was purchased from Aldrich Chemical Company and was used without further purification. Natural abundance zinc(II) oxide was prepared as previously described [6]. ^{67}Zn enriched zinc oxide was obtained from Oak Ridge National Labs at 89.68% isotopic purity. All complexes were prepared by mixing accurately weighed quantities of zinc oxide and cysteine in a molar ratio of 2:1 cysteine:zinc. The complex was dissolved in a small quantity of 30% glycerol/water solvent and the pH was adjusted with sodium hydroxide or nitric acid, as required. The pH was measured with a Corning 110 digital pH meter equipped with a graphic control combination electrode standardized against commercial buffers. A glycerol/water mixture was used as solvent for three reasons: to provide a high viscosity which minimizes ^{15}N T_1 relaxation [7], to slow chemical exchange [6] and to provide an antifreeze for low temperature studies. No attempt was made to correct solution pH's for the effect of the glycerol and it was assumed that the complex bonding was unaffected by the addition of the glycerol. The temperature of the sample at the nmr probe was calculated from the methanol resonance positions in the usual way [8].

Results and Discussion

Because the solubility of the neutral complex ($Zn(HL)_2$) is not great enough to obtain natural abundance ^{15}N spectra with the equipment used for this study and because of the formation of polynuclear complexes of somewhat uncertain composition at pH's below 8 (3), all the complex studies except one reported in this paper were performed at pH $\geq$ 9 in concentrated (~0.2 *M* solution). No spectral features or behavior were observed that indicated any polymeric complex formation. Because of the low signal-to-noise ratio encountered even after very long data acquisitions, small amounts of polymers or unusual isomers would not be observed. It is assumed in the analysis below that only the simple bis complexes $Zn(HL)_2$, $Zn(L)(HL)^-$ or $Zn(L)_2^{-2}$ or a weighted average of these forms is observed at any of the studied pH's. No more than one nitrogen resonance signal was observed at any pH. Although various isomeric forms of these complexes are possible, no spectral features which could be caused by isomeric differences were observed.

The results reported in Fig. 1 summarize a study of the ^{15}N chemical shift as a function of solution

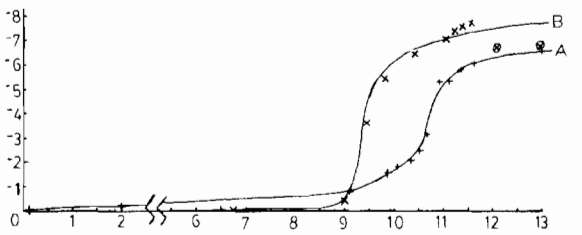

Fig. 1. Cysteine ^{15}N chemical shift versus pH. Vertical scale is ppm relative to low pH free ligand spectrum. The complex is insufficiently soluble below pH 7 to obtain spectra. (A) free ligand (B) 2:1 cysteine:zinc(II) complex. The circled points were obtained on a different solution than the uncircled points.

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pH for both free ligand and the bis complex with zinc(II). Examination of the free ligand pH dependence clearly shows the effect of protonation on the amine shift. The shape of the free ligand curve is consistent with previous studies of cysteine microscopic acid dissociation constants which have shown that the second and third breaks in the titration curve are due to partial deprotonation of both SH and NH_3^+ from the molecule [9, 10]. Assuming that the chemical shift is directly proportional to the fraction of time the amine is deprotonated (*i.e.* this assumes that the nitrogen shift is not a function of protonation of the sulfur) these data indicate that the -SH is deprotonated slightly before the NH_3^+ and that about ¼ of the NH_3^+'s are deprotonated by the time the SH is essentially completely deprotonated. Previous reports have suggested that these groups deprotonate approximately equally [9–11]. The difference between this work and the previous studies should not be viewed as a discrepancy but rather as a refinement since the early work relies on titration data which do not directly measure the state of protonation of any particular microscopic site. Thus, the ^{15}N nmr study of the free ligand is as expected.

The pH dependence of the complexed cysteine shown in Fig. 1 shows two features of the complexation: first at low pH, the protonated complex $Zn(HL)_2$ clearly has the proton on the nitrogen as suggested by Zegzhda, *et al.*. This is apparent from the fact that the nitrogen resonance shifts toward the free ligand protonated value as the pH is lowered, and there is no sign of a second break corresponding to protonation of the sulfur as observed in the free ligand case. Secondly, the apparent pK_a of the NH_3^+ has been decreased from about 10.8 in the free ligand to about 9.5 in the complex by the coordination. This decrease in pK_a could be caused either by coordination of the nitrogen, or simply by electrostatic repulsion by the positive charge of the zinc without nitrogen coordination. These data by themselves are not adequate to decide between the two possibilities. The previous study decided, on the basis of infra red measurements, that no nitrogen bonding occurred, even at high pH. It should be noted at this point, that the complexed nitrogen shifts are very nearly identical to the free ligand values in both protonated and deprotonated forms. Since significant shifts upon zinc coordination have been observed in some cases, one might conclude that coordination does not occur in this case [12]. Such a conclusion is not necessarily justified, however, as very small shifts have been observed in other cases [6].

In a previous paper, we have described a method for determining the existence of zinc-nitrogen bonds [6]. In this method, the effect of ^{67}Zn enrichment on the ^{15}N spectrum of the ligand is measured by acquiring the spectra with natural abundance zinc and with ^{67}Zn enrichment. If a zinc nitrogen bond exists, under conditions of slow chemical exchange, broadening of the nitrogen 15 signal can occur upon zinc 67 enrichment because of scalar coupling to the spin 5/2 ^{67}Zn if the ^{67}Zn quadrupolar relaxation is not too rapid. The conditions under which such broadening may be observed have been described theoretically and experimentally [13, 14].

This method was applied to the deprotonated complex in an attempt to ascertain whether deprotonation is accompanied by coordination. Since cysteine is a very unsymmetrical ligand, it is possible that coordination might not produce broadening [6, 14], but broadening cannot occur without coordination. Thus, negative results would be ambiguous while positive results would indicate not only that coordination occurs, but that this method can indicate coordination by even highly unsymmetrical ligands such as cysteine.

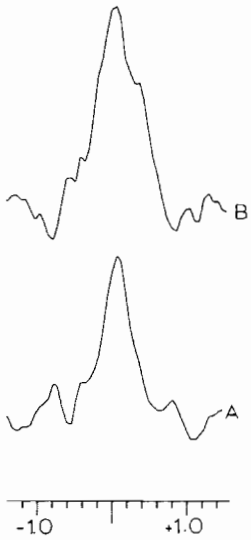

Fig. 2. Natural abundance ^{15}N nmr spectra of 2:1 cysteine: zinc(II) solutions at pH 10.1, −5 °C scale in ppm. Blow up of original spectrum acquired as 2 K real points, 2 K zeroes, 75° pulse, 1 KHz sweep width, pulse repetition rate 1.1 sec., approximately 20,000 scans. (A) Natural abundance (B) ^{67}Zn enriched.

Figure 2 shows the cysteine ^{15}N resonance line with natural abundance zinc and with ^{67}Zn enrichment. As can be seen from these data, ^{67}Zn enrichment results in an increase of line width from approximately 3.8 ± 0.4 Hz to 6.2 ± 5 Hz. The experimental uncertainties are estimated by measuring the uncertainty in full width at half height obtained by allowing for an uncertainty in the peak height of ±½ the peak-to-peak noise level. The observed broadening of 2.4 ± 1 Hz is outside of the experimental uncertainty.

These results, therefore, show that, contrary to the earlier report [5], deprotonation of the cysteine amine to $-NH_2$ does result in coordination to zinc. Also these results indicate that the broadening of ^{15}N resonances by coordinated zinc 67 is observable even when highly unsymmetrical ligands are employed. These results also indicate that ^{15}N chemical shifts are not necessarily reliable indicators of coordination.

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