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Retroviral nucleocapsid protein–metal ion interactions: Folding and sequence variants

(zinc/nucleic acid-binding protein/metal-binding domain)

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ABSTRACT **Retroviral nucleocapsid proteins contain one or two proposed metal-binding sequences of the form Cys-Xaa$_2$-Cys-Xaa$_4$-His-Xaa$_4$-Cys. Previously, we reported that an 18-amino acid peptide derived from the nucleocapsid protein of Rauscher murine leukemia virus (RMLV) binds metals such as Co^{2+} and Zn^{2+}. We have now synthesized the entire nucleocapsid protein from RMLV. We report here that the protein also binds Co^{2+} and Zn^{2+} and does so with a higher affinity than does the peptide. Limited proteolysis and circular dichroism studies reveal that metal binding induces folding of the metal-binding domain and, perhaps, the regions adjacent to it but the remainder of the protein remains in a relatively unstructured state. In addition, we have synthesized sequence variants of the metal-binding domain that correspond to viral mutations reported in the literature. In many cases, the metal-binding properties of these peptides correlate with the observed biological activity, providing further evidence for the importance of metal binding to nucleocapsid function.**

All infectious retroviruses contain a *gag* gene that encodes for the major structural proteins of the virus particle. One of these proteins, the nucleocapsid (NC) protein, is associated with the genomic RNA in the virion core (1). These proteins contain one or two sequences of the form Cys-Xaa$_2$-Cys-Xaa$_4$-His-Xaa$_4$-Cys, hereafter referred to as the CCHC box. Previously, one of us had proposed that these sequences may form metal-binding domains, in analogy with the zinc finger domains of transcription factor IIIA (TFIIIA) and related proteins (2). In support of this hypothesis, we have reported that an 18-amino acid peptide corresponding to the CCHC box of Rauscher murine leukemia virus (RMLV) does, indeed, bind metal ions (3). Furthermore, Summers *et al.* (4, 5) have demonstrated that a CCHC box peptide from human immunodeficiency virus (HIV) binds metal ions and have determined the structure of the peptide–zinc complex by NMR methods. It is important to bear in mind that the TFIIIA-like proteins and the CCHC box proteins may share the properties of binding metal ions and interacting with nucleic acids without being structurally or evolutionarily related in any other way.

The importance of the CCHC box sequences for retroviral replication has been demonstrated by site-directed mutagenesis studies in retroviral systems containing either one or two CCHC boxes. Deletion of either one or both CCHC boxes from the NC protein from Rous sarcoma virus resulted in the production of virus particles that had dramatically reduced infectivity (6). When examined more closely, the retroviruses produced were found not to have packaged their genomic RNA efficiently. Similar results were observed in all cases where the completely conserved cysteines or histidines were mutated to other amino acids (7–9). The CCHC box also contains other nearly invariant residues: there is a hydrophobic residue at one or the other of the two positions between the first two cysteines, a glycine residue immediately before the histidine, and a hydrophobic residue immediately following the histidine (3). In mutagenesis studies with Moloney murine leukemia virus (MoMLV), when the glycine preceding the histidine was changed to valine, a large reduction in packaging of genomic RNA was observed (9). The same phenotype was seen in this system when the tryptophan residue following the histidine was changed to glycine whereas a smaller reduction in genomic RNA packaging efficiency was observed when this residue was mutated to leucine. In some of these studies, packaging of heterologous RNA was also observed and the packaging of this heterologous RNA sometimes responded differently to mutations than did packaging of homologous RNA. Finally, when the tyrosine found before the second cysteine of the CCHC box of MoMLV was mutated to a serine, a particularly interesting phenotype was seen. Gorelick *et al.* (8) and Méric and Goff (9) found that viruses harboring this mutation packaged some of their RNA, yet the virus particles were not capable of replication. This suggests that the NC proteins may play another role in addition to that played in RNA packaging. Taken together, these mutagenesis results provide strong evidence for the importance of the CCHC boxes for viral RNA packaging and suggest that the NC proteins act by mediating specific protein–RNA interactions (8).

The *in vitro* assays that have been generally used for NC proteins involve nonspecific nucleic acid binding. Such assays cannot reflect the full biological activity of the NC proteins. Alkylation (10, 11) or oxidation (11) of the cysteines does not affect the behavior of the proteins in these assays, and neither does the presence of protecting groups on the cysteines in a synthetic protein (12). It is hard to reconcile these results with the striking conservation of the cysteine residues in these proteins and with the site-directed mutagenesis results discussed above. This strongly suggests that the NC proteins have some other activity that is important for their biological function. Another assay that has been developed involves the ability of NC proteins to facilitate certain nucleic acid annealing reactions, including the positioning of the replication primer tRNA onto the genomic RNA and genomic RNA dimerization (13, 14). The dependence of this assay on the presence of metal has not been reported, although zinc and reducing agents were present in the buffers used.

The ability of an 18-amino acid comprising a sequence derived from the CCHC box of RMLV to bind metal ions was previously demonstrated by us (3). We report here that a synthetic 56-amino acid protein having the sequence of the RMLV NC protein binds Co^{2+} and Zn^{2+} with high affinity. In addition, limited proteolysis studies demonstrate that the

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Abbreviations: NC, nucleocapsid; HIV, human immunodeficiency virus; RMLV, Rauscher murine leukemia virus; MoMLV, Moloney murine leukemia virus; TFIIIA, transcription factor IIIA.

addition of metal to the protein forms a stable core structure that is resistant to proteolytic attack. Circular dichroism (CD) spectra obtained for the protein and the metal-binding domain peptide in the presence and absence of zinc also demonstrate the ability of Zn^{2+} to induce folding of the metal-binding region. Finally, sequence variants of the metal-binding domain peptide corresponding to biologically characterized mutations have been synthesized. Studies of the metal-binding properties of these sequence variants revealed that many of the changes disrupt metal binding but that some do not. These results strongly support the hypothesis that metal binding is important for biological function and allow identification of residues that may be involved in stabilization of the metal complex and in specific interactions with viral RNA.

MATERIALS AND METHODS

The RMLV NC protein and the 18-amino acid peptide derived from its sequence were synthesized on a Milligen model 9050 Pepsynthesizer using *N*-fluorenylmethoxycarbonyl (Fmoc) amino acid pentafluorophenyl esters. The sequence variants were synthesized on a Milligen Excell synthesizer that employs benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate activation of Fmoc amino acids. All amino acids and reagents were purchased from Milligen/Biosearch except for dimethylformamide, which was Burdick and Jackson brand high-purity solvent. Cleavage of the protein and peptides from the resin, purification, reduction, and amino acid analysis were performed as described, as were the methods used to perform metal ion titrations.

The proteolysis studies of the protein were carried out in 20 mM Hepes/50 mM NaCl, pH 7.0, at room temperature. All digestions were performed under a dinitrogen atmosphere except for those on the oxidized protein. Each gel sample contained 6 μg of protein and either $ZnCl_2$ (to 2 mM), $CoCl_2$ (to 5 mM), or EDTA (to 10 mM) as indicated, and the sample was allowed to equilibrate for 30 min. The protein was digested for 3 min after the addition of 10 ng of trypsin or chymotrypsin. Reactions were stopped by the addition of 1 μg of phenylmethylsulfonyl fluoride. The samples were then analyzed on a 15% denaturing polyacrylamide gel (15). Oxidized protein was obtained by allowing the protein to stand overnight open to the air in pH 7.0 buffer. Whenever possible, reduced peptide and protein samples were handled in a Coy Laboratory Products (Ann Arbor, MI) anaerobic chamber under a 95% dinitrogen/5% dihydrogen atmosphere.

The samples to isolate the tryptic fragment from the protein–zinc complex contained 110 μg of protein in 20 mM Hepes/50 mM NaCl/2 mM $ZnCl_2$ buffer, pH 7.0. A total of 160 ng of trypsin was added to each tube, and the protein was allowed to digest for 3 min. Reactions were stopped by the addition of 20 μg of phenylmethylsulfonyl fluoride. The fragments were separated with reverse-phase high-performance liquid chromatography (HPLC) on an analytical Vydac C_4 column using a gradient of 0.1% trifluoroacetic acid/acetonitrile in 0.1% trifluoroacetic acid/water (0–35% in 55 min). The largest peak was collected and dried under vacuum. The first seven residues of the tryptic fragment were sequenced using an Applied Biosystems 475A sequencer and amino acid analyses were performed.

CD spectra were measured using an Aviv model 62DS spectropolarimeter. Spectra were recorded from 260 nm to 190 nm and averaged over seven scans. Protein and peptide samples were made up in 5 mM Tris (pH 7.2) to a concentration of 1 mg/ml, and $ZnCl_2$ was added as indicated.

RESULTS

The NC protein from the RMLV, which has the sequence (16) Ala-Thr-Val-Val-Ser-Gly-Gln-Arg-Gln-Asp-Arg-Gln-Gly-Gly-Glu-Arg-Arg-Arg-Pro-Gln-Leu-Asp-Arg-Asp-Gln-Cys-Ala-Tyr-Cys-Lys-Glu-Glu-Lys-His-Trp-Ala-Lys-Asp-Cys-Pro-Lys-Lys-Pro-Arg-Gly-Pro-Arg-Gly-Pro-Arg-Pro-Gln-Ala-Ser-Leu-Leu, has been synthesized using solid-phase peptide synthesis methods. The HPLC trace of the crude protein contains one major peak, the amino acid analysis of which is consistent with the above sequence. The presence of three free thiols per protein was confirmed with the use of 5,5′-dithiobis(2-nitrobenzoic acid) (17). The protein can be kept in the reduced state by minimizing exposure to air.

The protein binds Co^{2+} and Zn^{2+} with high affinity. Addition of Co^{2+} to the protein at pH 7.0 produces an absorption spectrum that is entirely consistent with tetrahedral coordination of the Co^{2+}. The absorption spectrum of the protein–Co^{2+} complex is very similar to that found for the 18-amino acid peptide–Co^{2+} complex previously reported, except that the protein spectrum is slightly sharper, as shown in Fig. 1. A least squares analysis of data obtained from the titration of 14 nmol of protein with Co^{2+} yielded a dissociation constant, K_d^{Co}, of 2×10^{-8} M for the protein–Co^{2+} complex, as shown in Fig. 2. The titration curve for a similar amount of peptide, which has a dissociation constant of 1×10^{-6} M (3), is plotted on the same figure, graphically demonstrating the higher affinity of the protein for Co^{2+}. Zn^{2+} binds more tightly than does Co^{2+}. The ratio of K_d^{Co}/K_d^{Zn} has been found by titrating the protein–Co^{2+} complex in the presence of a large excess of Co^{2+} with Zn^{2+}. The ratio of dissociation constants was found to be $\approx 2 \times 10^4$. This indicates that K_d^{Zn} is $\approx 1 \times 10^{-12}$ M, revealing that this protein binds Zn^{2+} with great avidity. The corresponding value for the CCHC box peptide is 6×10^{-10} M.

To test the idea that metal binding imparts stability to the protein, proteolytic digests were performed on metal-bound protein and apoprotein using trypsin and chymotrypsin, as shown in Fig. 3. The presence of either Zn^{2+} and or Co^{2+} inhibited digestion of the protein. To determine which region of the protein was protected from trypsin digestion by metal binding, a larger amount of protein with added Zn^{2+} was digested, and the fragments were purified. Based on a determination of the sequence of the first five amino-terminal residues and amino acid composition, the sequence of the protected fragment was Arg-Arg-Pro-Gln-Leu-Asp-Arg-Asp-Gln-Cys-Ala-Tyr-Cys-Lys-Glu-Glu-Lys-His-Trp-Ala-Lys-

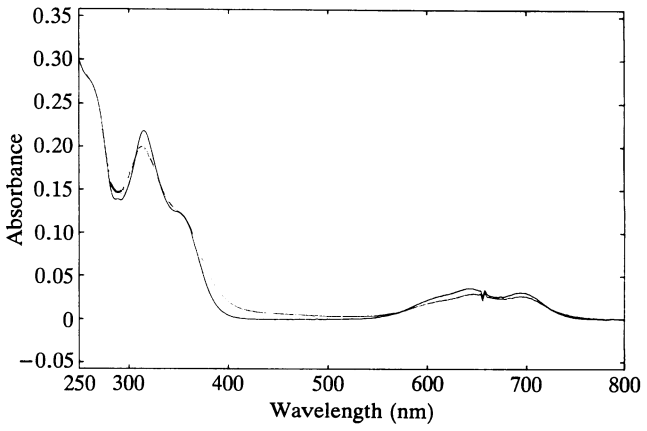

FIG. 1. Absorption spectrum of the cobalt(II) complex of the RMLV NC protein. The lighter line shows the spectrum of the cobalt(II) complex of the 18-amino acid peptide that corresponds just to the CCHC box metal-binding domain of this protein (3).

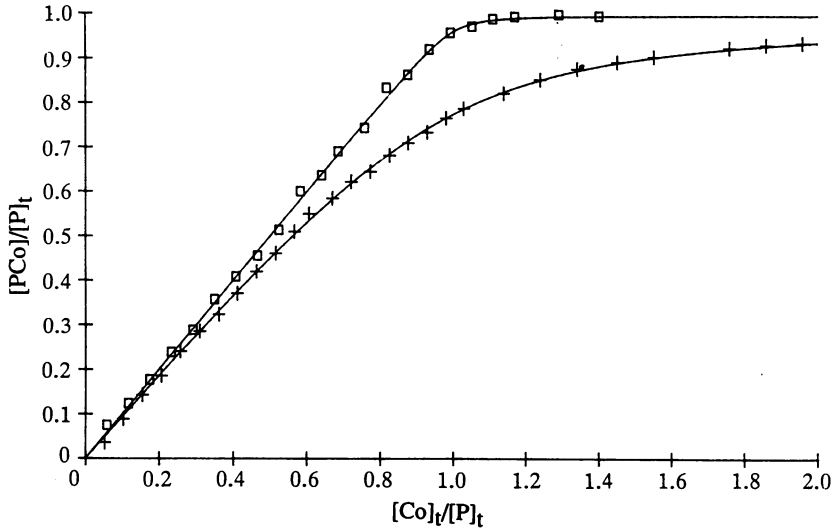

FIG. 2. Cobalt titration curves for the RMLV NC protein (□) and its CCHC box peptide (+) in 20 mM Hepes/50 mM NaCl at pH 7.0. The intact protein binds cobalt more tightly as the curve for the protein could be fit with a dissociation constant of 2×10^{-8} M, whereas that for the peptide could be fit with a dissociation constant of 1×10^{-6} M (3).

Asp-Cys-Pro-Lys-Lys-Pro-Arg. As can be seen, the putative metal-binding region is protected, suggesting that metal binding produces a stable, folded structure, that is less susceptible to proteolysis. In addition, potential tryptic cleavage sites on each side of the metal-binding domain are also protected. This suggests that either the adjoining regions also become more structured upon metal binding or that the sites are made inaccessible by their proximity to the metal-binding domain.

CD spectroscopy was also used to monitor the folding of protein and peptide induced by the addition of Zn^{2+}. CD spectra of apo- and metal-bound protein and peptide are shown in Fig. 4. A dramatic change was seen in the peptide spectrum on the addition of metal, whereas a much smaller change was seen for the intact protein. The changes that were observed between the apoprotein and Zn^{2+}-bound protein are similar to those seen in the spectra recently published by Roberts *et al.* (18).

Having established that the CCHC box-containing core forms a stable structure unit upon metal binding, we elected to try to obtain further evidence for the functional importance of the CCHC box region by synthesizing sequence variants that correspond to biologically characterized mutants. The metal-binding properties of these sequence variants were compared to those of the protein and the 18-amino acid peptide reported previously. The sequence of the 18-amino acid wild-type peptide and of the sequence variant prepared

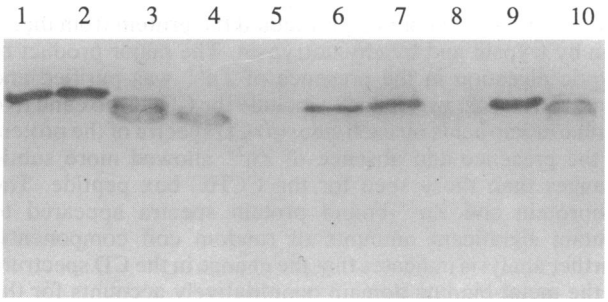

FIG. 3. Proteolysis of the RMLV NC protein in the presence and absence of metal ions. Lanes: 1, reduced protein alone; 2, reduced protein plus zinc; 3, reduced protein plus zinc plus trypsin; 4, reduced protein plus cobalt plus trypsin; 5, reduced protein plus EDTA plus trypsin; 6, reduced protein plus zinc plus chymotrypsin; 7, reduced protein plus cobalt plus chymotrypsin; 8, reduced protein plus EDTA plus chymotrypsin; 9, oxidized protein alone; 10, oxidized protein plus zinc plus trypsin. Concentrations are given in the text.

is as follows: $\overset{1}{\text{Asp}}$-$\overset{2}{\text{Gln}}$-$\overset{3}{\text{Cys}}$-$\overset{4}{\text{Ala}}$-$\overset{5}{\text{Tyr}}$-$\overset{6}{\text{Cys}}$-$\overset{7}{\text{Lys}}$-$\overset{8}{\text{Glu}}$-$\overset{9}{\text{Lys}}$-$\overset{10}{\text{Gly}}$-$\overset{11}{\text{His}}$-$\overset{12}{\text{Trp}}$-$\overset{13}{\text{Ala}}$-$\overset{14}{\text{Lys}}$-$\overset{15}{\text{Asp}}$-$\overset{16}{\text{Cys}}$-$\overset{17}{\text{Pro}}$-$\overset{18}{\text{Lys}}$.

C3S: Cys^3 to Ser
Y5S: Tyr^5 to Ser
G10V: Gly^{10} to Val
H11Q: His^{11} to Gln
W12L: Trp^{12} to Leu

The peptide–Co^{2+} complex absorption spectra for the variants C3S, G10V, and H11Q are different from that of the wild-type peptide, with the bands broadening and losing definition, as shown in Fig. 5. The Cys to Ser variant C3S was able to bind Co^{2+}, but the peptide was not saturated by the addition of 20 equivalents of Co^{2+}, indicating that the metal

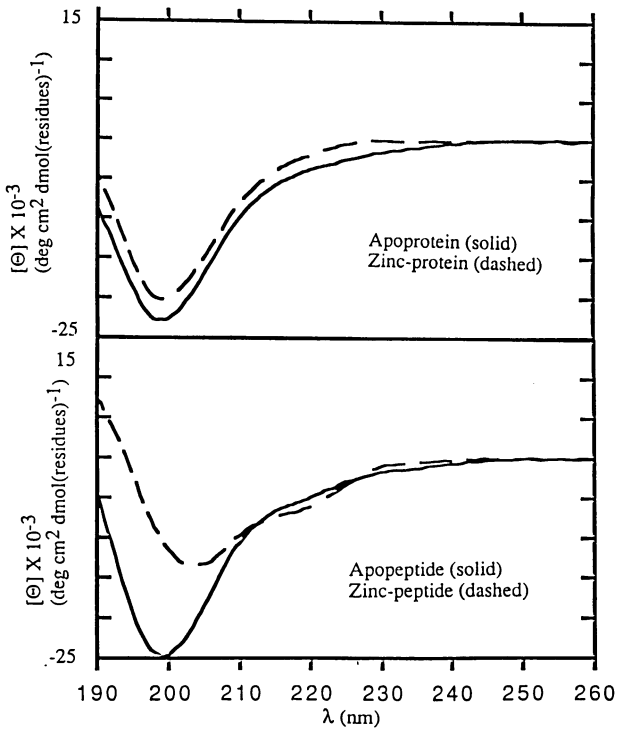

FIG. 4. CD spectra of the RMLV NC protein and its CCHC box peptide in the absence and presence of zinc. The large changes observed for the peptide upon binding zinc account for the changes seen for the protein from 190 to 210 nm, which appear smaller due to scaling to the total concentration of residues.

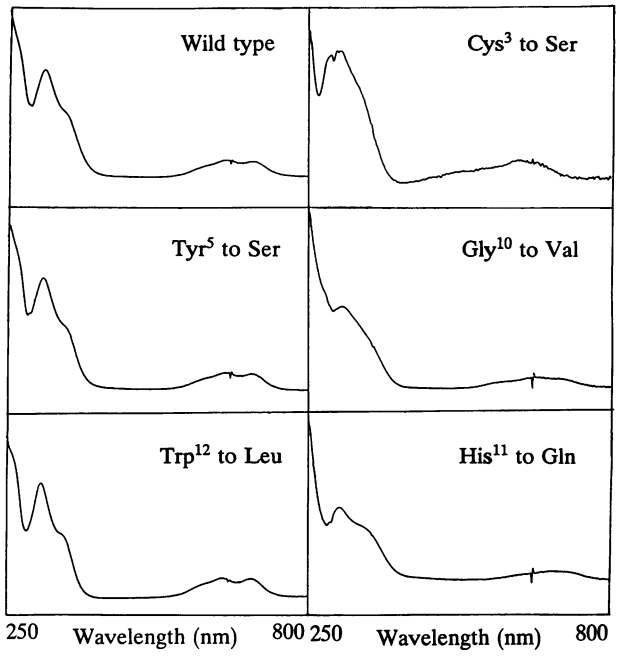

FIG. 5. Absorption spectra of the cobalt(II) complexes of the 18-amino acid CCHC box peptide and its sequence variants. The three spectra on the left side show features very similar to that of the wild-type peptide complex, whereas those on the right side are broadened and often shifted relative to that of the wild type, indicating that the sequence change has affected the structure or homogeneity at the cobalt(II) center.

ion affinity was reduced by several orders of magnitude. Changing the His to Gln had a less drastic effect on metal ion affinity, increasing the dissociation constant by a factor of 6, but changes in the absorption spectrum indicated that the coordination environment of the Co^{2+} ion had changed. The Gly to Val change also significantly affected the ability of the peptide to bind metal, as changes in the shape of the absorption spectrum were seen when titration of the peptide was attempted. This precluded the determination of the dissociation constant for the Co^{2+} complex of this peptide.

In contrast, the absorption spectra of the Co^{2+} complexes for the Y5S and W12L sequence variants did not change from that found for that of the wild-type peptide, with the absorption spectrum of the Trp to Leu variant being as sharp as that seen for the intact protein–Co^{2+} complex. In titration experiments, the W12L variant was found to bind Co^{2+} with slightly greater affinity than does the wild-type peptide (K_d^{Co} decreased by a factor of 2), whereas Y5S showed slightly lower affinity (K_d^{Co} increased by a factor of 3).

DISCUSSION

The experiments described herein were directed toward three questions. First, do retroviral NC proteins bind metal ions through the conserved CCHC boxes? Previous work from this and other laboratories had focused on studies of peptides that included the CCHC box itself with two additional amino acids on each side. Results from these investigations had revealed that such peptides bind metal ions such as Co^{2+} and Zn^{2+} via their cysteinate and histidine residues (3–5). Our studies of a RMLV CCHC box peptide have shown that such units do bind metal ions with affinities comparable to the TFIIIA-type zinc finger peptides (3) whereas the structural studies of Summers and coworkers (4, 5) on the Zn^{2+} complex of a peptide derived from the HIV NC protein have revealed that such peptides form a well-defined structure in the presence of metal ions. We have now extended these studies to the entire mature NC protein of RMLV. We find that this 56-amino acid protein binds Co^{2+} and Zn^{2+} very tightly, with dissociation constants that are 50 times smaller than were found for the CCHC box peptide. The absorption spectra for the Co^{2+} complexes of the protein and of the CCHC box peptide are very similar except that the protein complex spectrum is slightly sharper. While this manuscript was in preparation, Roberts *et al.* (18) reported similar spectroscopic results for the RMLV NC protein synthesized independently. These observations demonstrate that intact NC proteins are, indeed, capable of binding metal ions through the CCHC boxes and the previous results with smaller peptides are not artifactual. It has been reported that the NC protein purified from avian myoblastosis virus (AMV) does not bind zinc with high affinity and that addition of zinc did not affect the CD spectrum of the protein (7). One explanation for the difference between these observations and those with RMLV NC protein involves the possibility that the AMV NC protein may have been air oxidized during preparation. Some evidence for internal disulfides has been reported (19). Our experience suggests that such air oxidation is quite facile and that metal-binding activity is lost upon oxidation. Finally, it has been noted by two groups that it has not been possible to detect large quantities of zinc in purified virions (7, 8). This observation is surprising in light of the high affinity of the mature protein for Zn^{2+} we have observed *in vitro*. However, several explanations for this apparent contradiction appear plausible, including loss of zinc during virion purification, strong zinc binding by only a small fraction of the NC protein during viral maturation (8), or nondetection of zinc for technical reasons.

Second, what effect does metal binding have on the structure of the protein? Several lines of evidence reveal that metal binding induces significant structural changes in the CCHC box itself. The first evidence came from NMR studies of a CCHC box peptide from HIV, which reveal that the peptide is not structured in the presence of metal ions but folds to a unique structure upon metal binding (4). We have found that this folding is also manifested in the CD spectra of the RMLV CCHC box peptide. The CD spectrum of this peptide in the absence of Zn^{2+} is indicative of a random coil. Addition of Zn^{2+} caused a large decrease in the negative ellipticity at 200 nm as well as several other changes suggestive of structure formation. The absence of more dramatic changes in the CD spectrum is consistent with the structure of the HIV CCHC box complex determined by NMR, which contains only β turns as secondary structural units (5). The effects of metal binding on the structure of the intact mature protein have been probed by two methods. (*i*) Limited proteolysis studies revealed that metal binding protected the protein from digestion by trypsin and by chymotrypsin. The major product of tryptic digestion in the presence of Zn^{2+} was purified and characterized. It was found to include the CCHC box and five to nine amino acids on each side. (*ii*) CD spectra of the protein in the presence and absence of Zn^{2+} showed more subtle changes than those seen for the CCHC box peptide. The apoprotein and Zn^{2+}-bound protein spectra appeared to contain significant amounts of random coil components. Further analysis indicates that the change in the CD spectrum of the metal-binding domain quantitatively accounts for the difference between the apoprotein and the protein–Zn^{2+} complex from 190 to 210 nm, although not for the small differences observed at longer wavelengths. Taken together, the limited proteolysis and CD results suggest that metal binding induces structure formation in the CCHC box region and, perhaps, regions immediately adjacent to it but that the amino- and carboxyl-terminal regions of the protein remain relatively unstructured even in the presence of bound metal ion.

Finally, is metal binding important for biological activity? Roberts *et al.* (18) recently reported that zinc binding did not have a significant effect on nonspecific nucleic acid binding by intact, reduced RMLV NC protein, but the difficulties in interpreting results from this assay in terms of biological function have been noted. Since no *in vitro* assay appears to account for the full activity of the NC proteins, we resorted to synthesis and characterization of peptide sequence variants that corresponded to biologically characterized viral mutants. In site-directed mutagenesis studies, when any of the conserved cysteines or histidines are mutagenized, the virus is no longer capable of packaging its genomic RNA (7–9). Not surprisingly, when peptides containing replacements for either cysteine or histidine were examined, the structure of the metal-binding domain had changed, as evidenced by the changes in the absorption spectra of the peptide–Co^{2+} complexes, and the ability of the peptide to bind metal had been impaired. Interestingly, however, significant changes in metal-binding properties were also seen for the Gly^{10} to Val sequence variant. This glycine is nearly, although not completely, invariant in known CCHC box sequences. Mutation of this glycine to valine in MoMLV resulted in a profound reduction in RNA packaging efficiency (9). Our results suggest that the strong phenotypic change resulting from this mutation may result from a defect in metal binding by the NC protein. This is further supported by the observations of Summers *et al.* (5) that the HIV CCHC box peptide complex may not have room to accommodate a non-hydrogen side chain at this position. Two peptide sequence variants did not show drastic changes in metal binding properties. In viral mutagenesis studies, changing the tryptophan residue corresponding to that at position 12 in the CCHC box peptides greatly reduced but did not completely block RNA packaging (8, 9). Replacing the tyrosine corresponding to position 5 by serine had more modest effects on homologous RNA packaging and infectivity and actually appear to increase packaging of heterologous RNA in one study. These observations and our results are consistent with the hypothesis that these conserved hydrophobic residues do not play a central role in metal binding but instead act by mediating interactions with RNA. Again, supporting evidence comes from the structure of the HIV CCHC box complex, which reveals that these two residues are solvent exposed and spatially close in the folded structure (5). Thus, the results of our studies of the sequence variants of the CCHC box peptide correlate well the biological activities of retrovirus mutants with the corresponding sequence changes, providing further, albeit indirect, evidence that metal binding is required for full biological function.

These studies of the intact mature NC protein from RMLV and of sequence variants of the metal-binding core as well as the structural studies of one CCHC box peptide–metal complex have yielded compelling evidence that the CCHC box forms a metal-binding domain and that this unit is required for the full biological function of the NC proteins. But what is the mechanism of action of these proteins in promoting efficient RNA packaging? Gorelick *et al.* (8) suggested that the CCHC box–metal complexes may mediate specific protein–RNA interactions—that is, recognition of a specific packaging signal on the viral RNA. This hypothesis appears to be consistent with the extant data. Support for the ability of CCHC box-containing proteins to selectively recognize single-stranded nucleic acids comes from the discovery of a protein that binds to a sterol regulatory element in humans (20). This protein contains seven CCHC box sequences and has been shown to selectively bind one single-stranded oligonucleotide corresponding to the sterol regulatory element but not the complementary strand nor the double-stranded form (20). Development of similar *in vitro* assays for specific NC protein–nucleic acid interactions should provide a major advance in our understanding of the function(s) of the NC proteins.

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