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Mutational Analysis of the Metal Sites in an LIM Domain*

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James W. Michelsen‡§, Andrew K. Sewell¶, Heather A. Louis‡, Jay I. Olsen‖, Darrell R. Davis‖, Dennis R. Winge¶, and Mary C. Beckerle‡‡‡**

From the Departments of ‡Biology, ¶Medicine and Biochemistry, and ‖Biochemistry and Medicinal Chemistry, University of Utah, Salt Lake City, Utah 84132

Site-directed mutagenesis was carried out to map the residues that form the two Zn(II) sites within a LIM domain. The C-terminal LIM domain derived from the cysteine-rich protein was utilized for this analysis and is referred to as LIM2. Seven cysteinyl residues and a single histidyl residue in the LIM2 sequence, $CX_2CX_{17}HX_2CX_2CX_{17}CX_2C$, comprise the conserved residues in the LIM consensus that are potential Zn(II) ligands. Two Zn(II) binding sites exhibiting tetrathiolate (S_4) and S_3N_1 Zn(II) coordination are displayed by LIM2 (Kosa, J. L., Michelsen, J. W., Louis, H. A., Olsen, J. I., Davis, D. R., Beckerle, M. C., and Winge, D. R. (1994) *Biochemistry* 33, 468–477). Site-directed mutagenesis was employed to generate three mutant LIM2 proteins with conversions of the second conserved cysteine to histidine (C2H), the fifth conserved cysteine to histidine (C5H), and the last conserved cysteine to aspartate (C8D). Metal coordination by the mutant proteins was evaluated by atomic absorption spectroscopy, Co(II) electronic spectroscopy, and ^{113}Cd NMR spectroscopy. The results permit discrimination between various models of metal ion binding and suggest that the LIM domain is comprised of a S_3N_1 site generated from the four N-terminal candidate ligands ($CX_2CX_{17}HX_2C$) and a S_4 site generated from the four C-terminal candidate ligands ($CX_2CX_{17}CX_2C$).

The LIM domain is a cysteine-rich, Zn-binding sequence found in a number of proteins that appear to be critical in development. The LIM motif sequence ($CX_2CX_{16-23}HX_2CX_2$-$CX_2CX_{16-21}CX_{2-3}$(C, H, or D)) was originally observed in three proteins, Lin-11, Isl-1, and Mec-3 (1, 2) from which the term "LIM" is derived (1). Since the initial description of the LIM motif, a number of other proteins that exhibit this sequence motif have been identified. Many of the known LIM domain proteins, including Lin-11, Isl-1, and Mec-3, also exhibit homeodomains which presumably mediate DNA binding (1–6). The LIM motif sequence does not itself appear to bind DNA (6). Indeed, it was recently reported that the LIM domains of both Isl-1 and Mec-3 inhibit the homeodomain-mediated DNA binding of these proteins (6, 7).

In addition to the LIM-homeodomain proteins, the LIM motif has also been reported in a number of proteins that lack obvious DNA-binding domains. These include the cysteine-rich intestinal protein (CRIP),[1] the oncogenic rhombotin family of proteins, and two cytoskeletal binding partners, zyxin and the cysteine-rich protein (CRP) (8–15). Although the biochemical function of the LIM domain is unresolved, evidence is accumulating in support of the notion that the LIM domain serves as an interface for protein-protein interactions (3, 6, 15).

Two LIM-domain proteins, CRP and zyxin, have been shown to be specific Zn-binding metalloproteins when purified from their endogenous sources (15, 16). In addition, CRP, which displays two copies of the LIM motif, and CRIP, which exhibits a single LIM motif have both been isolated as Zn(II)-binding proteins after expression in bacteria (16, 17). A single LIM domain binds two Zn(II) ions (16, 17). By analysis of the metal-binding properties of both CRIP and the C-terminal LIM domain of CRP (referred to as LIM2), we demonstrated that one of the two Zn(II) ions exhibits a tetrathiolate Cys_4 Zn(II)-binding site and the second Zn(II) ion has Cys_3-His_1 ligation (17). Toward the goal of elucidating the specific amino acid residues that constitute each Zn(II)-binding site, we carried out site-directed mutagenesis of LIM2 at three positions. The results predict that N-terminal half of the LIM domain forms the Cys_3-His_1 site and the C-terminal half constitutes the Cys_4 site.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis of LIM2—The LIM2 expression plasmid (17) encodes amino acids 108–192 of avian CRP (10). Mutations were engineered into the LIM2 gene by a restriction site elimination protocol (18). This protocol involved introducing specific mutations into the pAED4 plasmid containing the LIM2 sequences, pAED4-LIM2, which has been described previously (17). The pAED4 expression plasmid[2] is a derivative of the T7-based expression vector, pET-3a (19). The mutagenesis protocol is dependent on the target plasmid containing a unique restriction site that can be eliminated. Two mutagenic oligonucleotide primers of between 22 and 24 nucleotides were used, one primer to introduce the desired mutation in the LIM2 gene and the second contained a mutation to eliminate a unique restriction site in the pAED4 vector sequences. The two primers were annealed to heat-denatured, circular pAED4-LIM2 DNA, and a new second strand containing both primer sequences was synthesized with T4 DNA polymerase. After ligation the resulting DNA was transformed into the mismatch repair defective *Escherichia coli* strain, mut S. Amplified plasmid was recovered and treated with *Pvu*I that cuts the unique restriction site in unmodified pAED4 to linearize non-mutated vectors. After three sequential rounds of transformation to enrich for the mutagenized, circular plasmids, plasmid DNA was isolated from multiple individual colonies and sequenced to identify clones containing the desired mutation in LIM2. Plasmids containing the intended mutations were subsequently used for expression of mutant LIM2 proteins.

Purification of LIM2 and Site-specific Mutants of LIM2—The LIM2 protein was expressed in *E. coli* BL21(DE3) as described previously

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‡‡ Supported by the American Heart Association and the National Institutes of Health and is a Established Investigator of the American Heart Association. To whom correspondence should be addressed. Tel.: 801-581-4485 (M. C. B.) or 801-585-5103 (D. R. W.); Fax: 801-581-4668 (M. C. B.) or 801-585-5469 (D. R. W.).

[1] The abbreviations used are: CRIP, cysteine-rich intestinal protein; CRP, cysteine-rich protein.

[2] D. Doering and P. Matsudaira, unpublished results.

(17). Expression was achieved by incubating cultures at an $A_{600\ nm}$ of 0.7–0.9 with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C in the presence of 1 mM $ZnSO_4$. The harvested cells were washed in 10 mM potassium phosphate, pH 7.2, containing 10 mM KCl, and 10 mM dithiothreitol and were lysed by a freeze-thaw cycle followed by sonication. The clarified supernatant was applied to a CM-52 cation-exchange column equilibrated in the same buffer. Elution of LIM2 was achieved with a linear gradient of 0–250 mM KCl in the column buffer. Fractions enriched in LIM2 were identified by zinc analysis, atomic absorption spectroscopy, and SDS-polyacrylamide gel electrophoresis. The pooled LIM2 fractions were concentrated by lyophilization and chromatographed on Sephadex G-50 in 50 mM KCl, 20 mM potassium phosphate, 1 mM dithiothreitol. Homogeneity of the LIM2 samples was shown by SDS-polyacrylamide gel electrophoresis and amino acid analysis. Purification of the mutant LIM2 molecules was carried out according to the same protocol described above for native LIM2. To confirm that the isolated proteins exhibited the expected mass, electrospray mass spectrometry was performed on a Fisons Instruments Trio 2000 mass spectrometer (VG Biotech; Chesire, United Kingdom). The instrument data system consisted of an IBM compatible PC which ran the LAB BASE software provided with the mass spectrometer. This analysis was conducted on samples at pH 3.5 to generate data on the unmetallated protein.

Metal Ion Reconstitution—Apo-proteins were prepared by chromatography of the Zn-containing LIM molecules on Sephadex G-25 equilibrated at pH 1.6. The Zn(II)-depleted proteins were found to contain reduced sulfhydryl groups. The concentration of each protein was quantified by amino acid analysis. Co(II) and Cd(II) binding studies were carried out by anaerobically mixing apo-proteins in 0.025 N HCl with a set mole equivalency of Co(II) or Cd(II) in 0.01 N HCl followed by neutralization with Tris base to pH 7.5. The concentrations of the Co(II) and Cd(II) stock solutions were verified by atomic absorption spectroscopy.

Spectroscopic Analyses—Ultraviolet absorption spectroscopy was carried out on a Beckman DU spectrophotometer. Intrinsic fluorescence measurements were made on a Perkin-Elmer 650–10S fluorimeter. ^{113}Cd NMR spectroscopy was performed on a Unity 500 Varian spectrometer operating in the Fourier transform mode at 110.89 MHz. Spectra were recorded on ^{113}Cd samples (6–10 mg of protein/ml) containing ^2H_2O as a field lock.

RESULTS

Generation of Mutations in the LIM2 Polypeptide—From analysis of more than 20 LIM domain sequences, the following consensus sequence has been defined: $CX_2CX_{16-23}HX_2CX_2$-$CX_2CX_{16-21}CX_{2-3}$ (C, H, D) (15). We have been interested in determining the metal-binding properties of the LIM domain. We have utilized the LIM2 protein as a model polypeptide for studying the structural features of the LIM domain. LIM2 displays a single LIM domain derived from CRP (10), can be expressed as a fully metallated protein *in vitro*, and can be readily purified in quantities sufficient for spectroscopic analysis. LIM2 exhibits a conventional LIM sequence: $\mathbf{C^1X_2C^2X_{17}H^3X_2}$-$\mathbf{C^4X_2C^5X_2C^6X_{17}C^7X_2C^8}$. It displays eight candidate metal liganding residues that are designated with superscripts beginning with the most N-terminal potential ligand.

As mentioned previously, the LIM2 protein binds two Zn(II) ions. Although recent work has revealed that the metal ions are tetrahedrally coordinated at Cys_4 (S_4) and Cys_3-His_1 (S_3N_1) sites, the specific amino acid residues that participate in the coordination of the two metal ions have not been defined. Assuming that potential ligands that are separated by only 2 amino acids will ligate the same metal ion, there are three models for the metal sites in LIM2 (Fig. 1). The model shown in Fig. 1*A* is a "double finger" configuration that was initially proposed by Liebhaber and colleagues (9, 20). In this model, the LIM domain displays an S_4 site generated by C^5, C^6, C^7, and C^8 and a S_3N_1 site generated by C^1, C^2, H^3, and C^4. Alternatively, as illustrated in Fig. 1*B*, the S_4 site could be generated by C^1, C^2, C^5, and C^6 with the remaining ligands contributing to the S_3N_1 site. Finally, as suggested in Fig. 1*C*, the S_4 site could be comprised of C^1, C^2, C^7, and C^8 with the central four ligands (H^3, C^4, C^5, and C^6) generating the S_3N_1 site.

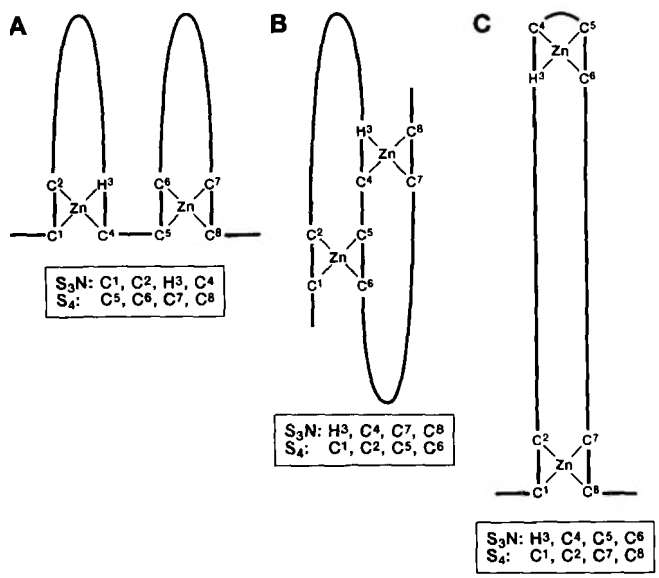

FIG. 1. **Three models for metal coordination by the LIM domain.** A LIM domain binds two Zn(II) ions. One metal site exhibits tetrathiolate (S_4) coordination and the second metal is liganded by 3 cysteinyl residues and 1 histidyl residue (S_3N_1). For each of the three models, the potential metal liganding residues expected to participate in Zn(II) coordination at each of the two sites are shown in the *boxes*.

In order to distinguish between the three possibilities represented in Fig. 1, site-directed mutagenesis was employed to convert certain candidate cysteinyl ligands to potential histidyl or aspartyl ligands. Recently this strategy was successfully employed to analyze the metal binding sites of the *E. coli* Ada protein (21). In our study, we generated single Cys to His amino acid substitutions at ligand positions 2 and 5 (designated C2H and C5H) and a Cys to Asp substitution at ligand position 8 (C8D) (Fig. 2). The C8D mutation was designed to test directly the popular hypothesis that aspartyl residues can serve as metal ligands at position 8 in the LIM domain (15, 20). We reasoned that analysis of the mutated LIM2 molecules using electronic spectroscopy of Co(II) complexes and ^{113}Cd NMR would indicate whether any changes in the ligand fields had occurred as a result of the amino acid substitution, thus allowing us to define the residues involved in coordinating each metal ion. These spectroscopic approaches can be used to differentiate between sulfur and nitrogen or oxygen liganding atoms. So, for example, if the model in Fig. 1*B* were correct, the S_4 metal site would not be perturbed in the C8D mutant; in contrast, if models 1*A* or 1*C* were correct, the S_4 site would be converted to an S_3O_1 resulting in predictable changes in both the electronic spectrum of Co(II) complexes and the ^{113}Cd NMR spectrum of the mutant protein compared to wild-type LIM2.

Purification of the LIM2 Mutant Proteins—Wild-type LIM2 as well as the C2H and C8D mutants were expressed efficiently in bacteria and were purified by a combination of cation-exchange chromatography and gel filtration. Each isolated protein was resolved as a single band by SDS-polyacrylamide gel electrophoresis and the apo-protein exhibited the expected mass by electrospray mass spectrometry. Purified wild-type LIM2 displayed a mass of 8693 by electrospray mass spectrometry, consistent with the interpretation that the purified protein lacked the N-terminal methionine. The N-terminal methionine also appears to be absent in the mutant proteins. The observed masses of the C2H and C2D mutant proteins were 8724 and 8703, respectively, compared to masses of 8727 and 8705 predicted if the N-terminal methionine was absent. Only low levels of the C5H protein could be recovered using the bacterial expression system. Although the minimal quantities

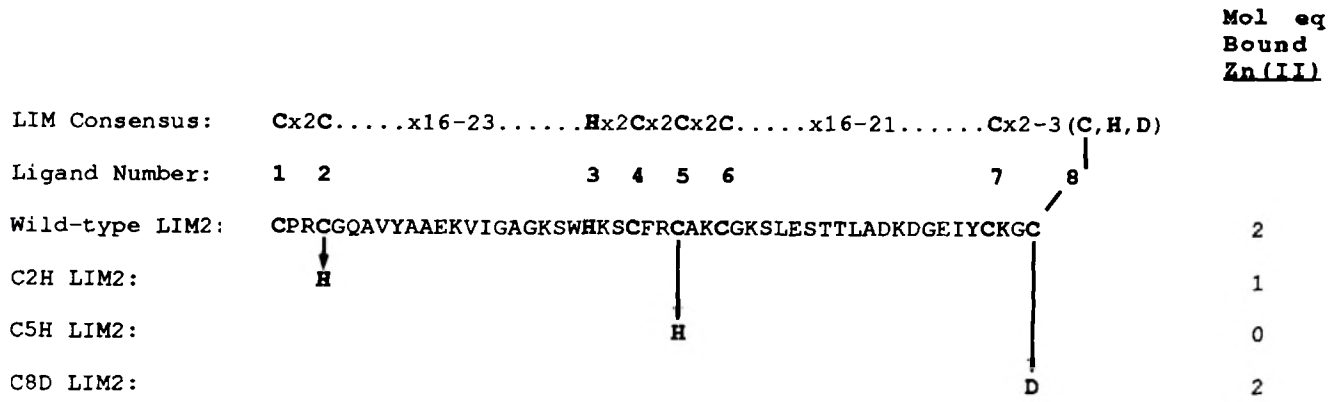

FIG. 2. **Site-specific mutations in LIM2.** The consensus sequence for the LIM motif is shown. The *numbers* used to designate each metal liganding residue in the LIM consensus are illustrated. The sequence of wild-type LIM2 is shown in relation to each of the site-specific amino acid substitutions which are marked with *arrows*. The maximal metal binding capacities for LIM2 as well as each of the mutant proteins are listed on the *right*.

of C5H protein obtained precluded the use of gel filtration, the isolated C5H protein was estimated to be >95% pure after chromatography on CM-cellulose.

Metal Content of LIM2 Mutant Proteins—Wild-type LIM2 contains 2 mol eq Zn(II) (17). Interestingly, the substitution of a histidyl residue for a cysteinyl residue at position 5 (the C5H mutant) yielded protein that was devoid of bound Zn(II). Because the C5H protein did not bind metal, it was not useful in our effort to define the metal ligands in the LIM domain. However, as will be discussed below, the inability of the C5H protein to coordinate Zn(II) may shed some light on the folding pathway for the LIM domain.

In contrast to the C5H mutant protein, the C2H and C8D mutants were purified as Zn(II)-binding metalloproteins. The C2H and C8D mutant proteins were found to contain 1.0 and 1.8 mol eq bound Zn(II), respectively. We have used reconstitution studies to evaluate whether the low Zn(II) content in the C2H protein is a true reflection of the maximal metal binding capacity of the C2H protein or results from oxidation of the protein during isolation as is known to occur with intact smooth muscle CRP (16). To insure maximal metal incorporation, the apo-C2H protein was reduced prior to titration studies with Cd(II). The addition of 5 mol eq Cd(II) to apo-C2H followed by dialysis in 40 mM Tris-Cl, pH 7.5, containing 40 mM KCl and 0.1 mM EDTA resulted in the recovery of Cd-C2H protein with 0.9 mol eq Cd(II) bound. Under these reconstitution conditions 2 mol eq Cd(II) are bound to wild-type LIM2. Likewise, addition of 2 mol eq Co(II) to the reduced apo-C2H protein followed by dialysis as described above yielded 0.9 mol eq Co(II) bound. Therefore the maximal metal binding capacity of the C2H protein approaches 1 mol eq. In contrast, addition of 2 mol eq Co(II) to either apo-LIM2 or the apo-C8D mutant under the same conditions led to 2.0 or 1.7 mol eq Co(II) bound, respectively.

By monitoring the metal ligand charge transfer transitions in the ultraviolet by absorption spectrometry, we previously observed a shoulder feature at 295 nm in fully metallated LIM2 protein samples. This Trp-related electronic transition was observed in Zn_2LIM2 as well as Cd_2LIM2, but not in apo or singly metallated LIM2 (17). This feature suggests that the Trp residue encounters a more apolar environment in a fully metallated LIM domain. The sole Trp residue in the LIM2 molecule is adjacent to the metal liganding His residue in position 3 of the LIM consensus. Thus, for LIM2, it appeared that the engagement of metal by His^3 occurred upon occupancy of the second metal site and that binding was reflected in the absorption spectrum (17). Ultraviolet absorption spectra were recorded to determine whether the singly metallated C2H mutant protein exhibited the shoulder transition (Fig. 3). No Trp-related transition was observed in the absorption spectrum of a Zn(II) complex of the C2H mutant, whereas the shoulder feature was apparent in the absorption spectra of Zn(II) complexes of both wild-type LIM2 and the C8D mutant protein.

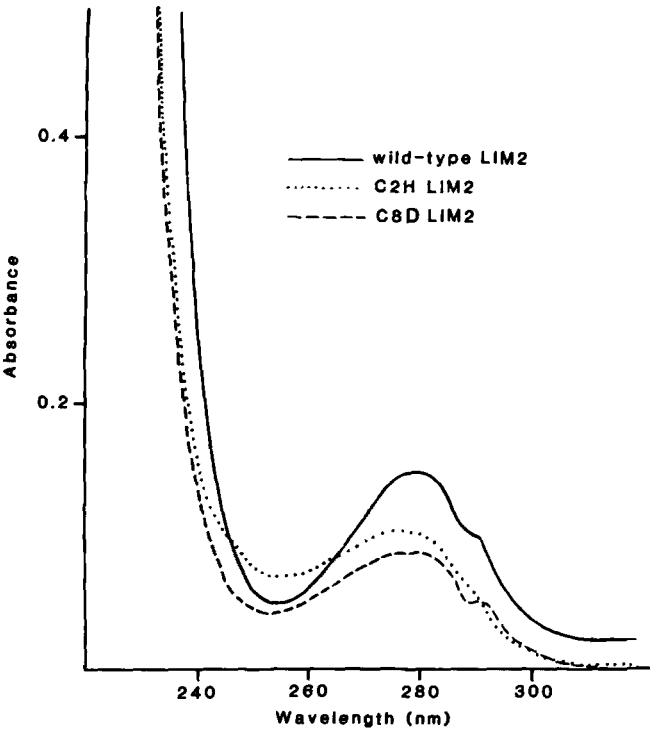

FIG. 3. **Ultraviolet absorption spectra of wild-type and mutant forms of LIM2.** The absorption spectra of wild-type LIM2 (——), C2H LIM2 (·····), and C8D LIM2 (– – –) were recorded at pH 7.5 at protein concentrations of 130 μg of LIM2/ml, 95 μg of C2H LIM2/ml, and 90 μg of C8D LIM2/ml.

Intrinsic fluorescence measurements of wild-type LIM2 revealed a metal dependence in the energy of the Trp fluorescence. The presence of the single Trp residue in LIM2 yielded maximal fluorescence at 355 nm in the metal-free protein but at 335 nm in the fully metallated LIM2 protein (17). We demonstrated that the energy shift occurred predominantly in the binding of the second metal ion (17). Fig. 4 shows the intrinsic fluorescence of the mutant LIM2 proteins reconstituted with

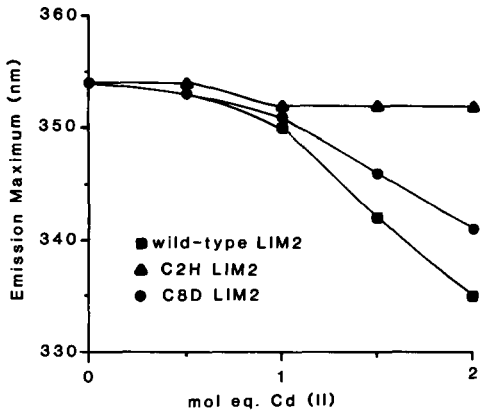

FIG. 4. **Tryptophan fluorescence of wild-type and mutant LIM2 proteins as a function of added Cd(II).** Wild-type and mutant LIM2 proteins were reconstituted with increasing quantities of Cd(II) as shown and the wavelength of maximal emission was determined after excitation at 270 nm.

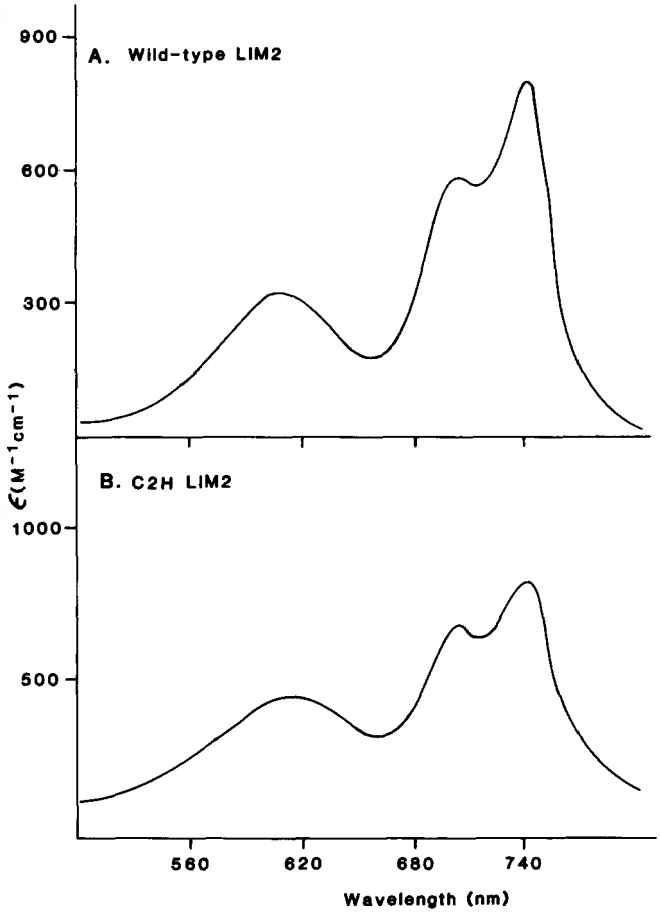

FIG. 5. **Electronic spectrum of Co(II) complexes of wild-type LIM2 and the C2H LIM2 mutant proteins.** Apo-C2H LIM2 was reconstituted with 2 mol eq Co(II) under anaerobic conditions to a final pH of 7.5 and spectrum was recorded. *Panel A* shows apo-LIM2 (wild-type) reconstituted with 1 mol eq Co(II).

increasing quantities of Cd(II)(0–2 mol eq). With increasing Cd(II), the C8D mutant protein exhibited an energy shift similar to that of wild-type LIM2, whereas the fluorescence of the C2H mutant protein was minimally perturbed by the addition of Cd(II) ions supporting our previous suggestion that the second metal site in the C2H protein is unoccupied despite the presence of excess Cd(II) ions.

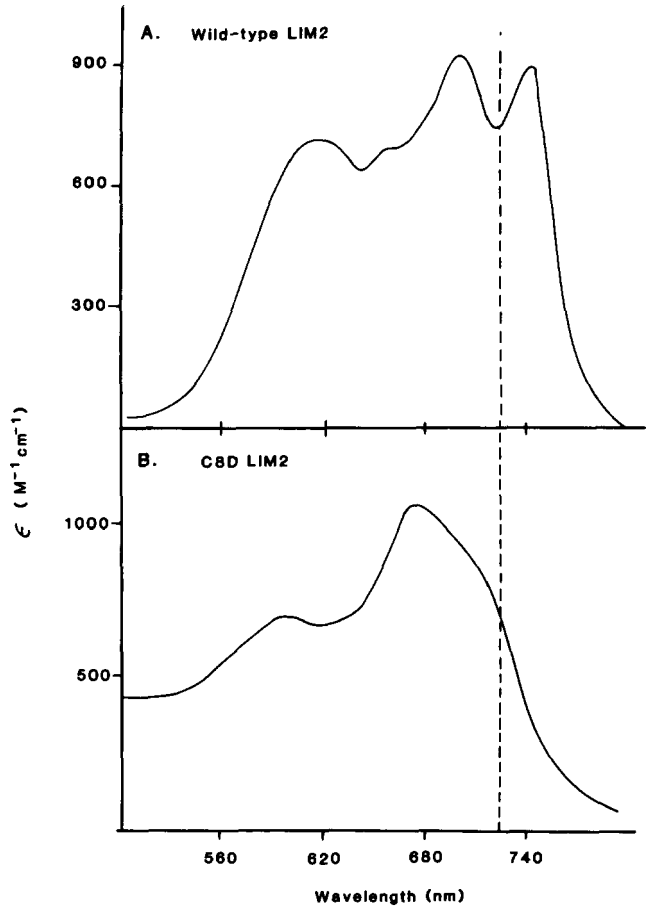

FIG. 6. **Electronic spectrum of Co(II) complexes of wild-type LIM2 and the C8D LIM2 mutant proteins.** Spectra were collected of apo-proteins of each were reconstituted with 2 mol eq Co(II) under anaerobic conditions. The *dashed line* is to show the loss of the 745-nm transition in the C8D mutant protein.

Electronic Spectroscopy of Co(II) Complexes of LIM2 Mutant Proteins—Co(II) complexes were prepared with wild-type LIM2 as well as the C2H and C8D mutant forms of LIM2. The *d-d* transition envelope of Co(II) complexes is sensitive to the ligand types. Sulfur-Co(II) complexes exhibit transitions at lower energies compared to nitrogen-Co(II) or oxygen-Co(II) complexes (22). The prediction with the mutant proteins was that a change in the S_4 Zn(II) site to a S_3N_1 or S_3O_1 site would result in an energy shift of the *d-d* envelope to higher energies or lower wavelengths. As mentioned above, the C2H protein maximally exhibited a single bound metal ion even when an excess of Co(II) was available for binding. The electronic spectrum of a Co(II) complex with the C2H mutant protein revealed a prominent 745-nm component in the *d-d* envelope showing that the tetrathiolate site was unperturbed in the singly metallated protein (Fig. 5*B*). The spectrum was similar to that of wild-type LIM2 with a single Co(II) ion bound (Fig. 5*A*).

The Co(II) complex of the C8D mutant protein revealed a marked blue shift in the *d-d* envelope compared to the Co(II) complex of wild-type LIM2 (Fig. 6). The 745-nm transition seen in Co_2LIM2 (Fig. 6*A*) was not present. The absence of the 745-nm transition is consistent with the loss of a tetrathiolate site and conversion to a S_3O_1 site. The centroid of the *d-d* envelope for an S_4 site is near 680 nm, whereas the centroid of the envelope of an O_4 site is blue-shifted toward 630 nm (22). No significant change in the extinction coefficient of the C8D mutant was expected in comparison with wild-type LIM2.

^{113}Cd NMR of LIM2 Mutants—^{113}Cd(II) complexes were pre-

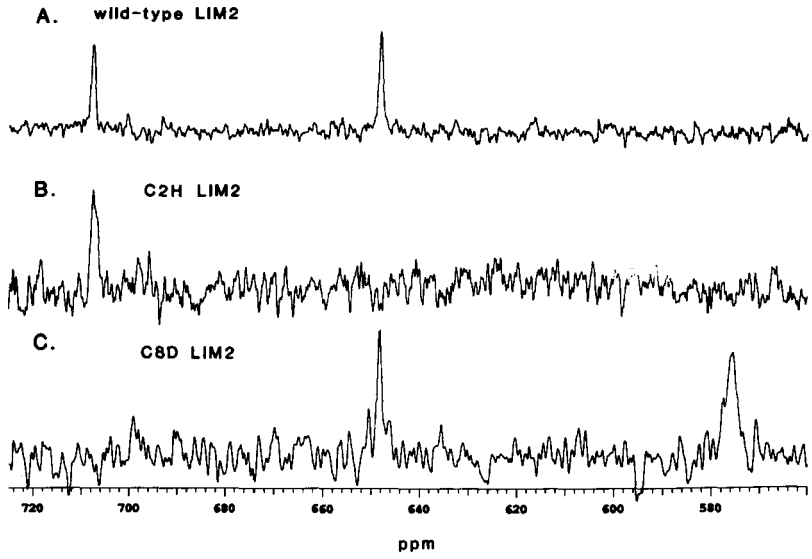

FIG. 7. **^{113}Cd NMR of wild-type and mutant forms of LIM2.** ^{113}Cd(II) complexes of wild-type LIM2 (10 mg/ml), C2H (7 mg/ml), and C8D (6 mg/ml) mutant LIM2 proteins were prepared as described previously (17). NMR spectra were recorded of samples containing ^2H_2O as a field lock. Chemical shifts are relative to 1 M $Cd(ClO_4)_2$. The number of transients and spectral width for each sample were 7,500 and 750→550 ppm for wild-type LIM2; 21,632 and 800→500 for C2H mutant LIM2; and 44,672 and 750–400 ppm for C8D mutant LIM2. An acquisition time of 0.8 s was used for each sample. Increasing the acquisition time to 1.6 s for the C8D LIM2 sample did not enhance the observed signal intensity. In each case a pulse width of 60° was used and either 40- or 80-Hz line broadening was applied for spectral enhancement.

pared for each of the mutant LIM2 proteins to investigate further the ligand fields in the mutant proteins. ^{113}Cd is widely utilized as a spectroscopic probe for Zn(II) sites in metalloproteins (23). Wild-type LIM2 showed two ^{113}Cd resonances at 707 and 648 ppm (Fig. 7*A*) (17). The same two signals are observed in the intact CRP molecule with four bound ^{113}Cd(II) ions (16). The 707-ppm signal is diagnostic of a tetrathiolate site (24). Considering the results of the electronic spectroscopy of Co(II)-substituted protein which suggested that the S_4 site was retained in the C2H mutant and eliminated in the C8D mutant, we predicted that the single ^{113}Cd(II) ion in C2H would exhibit a 707-ppm signal (or perhaps a slightly shifted resonance if the chemical environment of a mono-metallated LIM2 is different from a fully metallated protein). Furthermore, we predicted that in the C8D mutant protein the 707-ppm signal would be shifted significantly upfield with no change in the 648-ppm signal derived from the S_3N_1 site. NMR results for the two mutant proteins confirmed the predictions (Fig. 7). The C2H mutant protein did indeed exhibit a single ^{113}Cd resonance at 707 ppm (Fig. 5*B*), whereas the C8H mutant protein yielded ^{113}Cd resonances at 648 and 575 ppm. The upfield shift from 707→575 ppm is consistent with a conversion of a S_4 site to a S_3O_1 site. Upfield shifts correlate with greater shielding and shielding increases in moving from sulfur to oxygen ligands (23, 25).

DISCUSSION

The conserved LIM residues in the LIM2 protein that are potential Zn(II) ligands include 7 cysteinyl residues and a single histidyl residue in the sequence $CX_2CX_{17}HX_2$-$CX_2CX_2CX_{17}CX_2C$. We previously demonstrated that LIM2 binds two Zn(II) ions with S_4 and S_3N_1 coordination (17). In the present study, site-directed mutagenesis was used to map the residues forming the two Zn(II) sites in LIM2. In designing the ligand mapping strategy, we made the assumption that CX_2C pairs will ligate the same metal ion. This has been found to be true in a myriad of Zn(II)-binding proteins (26–29). A common CX_2C structural motif in metal-binding proteins is a half-turn with the two sulfur atoms in van der Waals contact with a stabilizing backbone amide→sulfur hydrogen bond (26, 30).

Three models for the Zn(II) sites in a LIM domain are illustrated in Fig. 1. In model 1*A*, the tetrathiolate site is comprised of C^{5-8}; in model 1*B* it is comprised of C^1, C^2, C^5, and C^6; and in model 1*C*, it is comprised of C^1, C^2, C^7, and C^8. We used spectroscopic methods to examine the fate of the S_4 site in the mutant LIM2 proteins. Mutagenesis of C^8 to Asp (C8D) eliminated the S_4 site as evidenced by the absence of a 745-nm *d-d* transition of the Co(II)-C8D complex and the shift of the 707-ppm ^{113}Cd resonance to 575 ppm. A 575-ppm ^{113}Cd resonance is consistent with a replacement of a sulfur ligand by an oxygen atom (23). In the *E. coli* Ada protein mutagenesis of a liganding Cys residue to His resulted in 55 ppm upfield shift in the ^{113}Cd signal (21). One caveat of such an experiment is that the chemical shift of the ^{113}Cd nucleus is highly influenced by the chemical environment, such that a chemical shift difference may occur by a structural alteration and not a ligand-type switch. We feel that this is unlikely in LIM2 as the 648-ppm signal occurs in both the wild-type and C8D mutant LIM2 molecules. One implication of this finding is that the chemical environment of the S_3N_1 site is apparently unaffected by the change in the other site. Thus, there appears to be marked independence of the two metal sites within a LIM domain. The fact that no 745-nm *d-d* transition is seen in the Co(II) complex in the C8D mutant corroborates the ^{113}Cd NMR data which suggest that the change in the C8D mutant arises from a change in the ligand-type (S→O) rather than a structural alteration.

The loss of the tetrathiolate site in the C8D mutant allows us to eliminate model 1*B* in which C^8 does not participate in generating the S_4 site. The analysis of the C8D mutant did not allow us to distinguish between models 1*A* and 1*C*, but the characteristics of the C2H mutant allowed us to achieve this goal. Mutagenesis of C^2 to His (C2H) failed to eliminate the S_4 metal site as would be expected if models 1*B* (eliminated by other criteria as noted above) or 1*C* were correct. The single ^{113}Cd resonance at 707 ppm and the 745-nm Co(II) *d-d* transition were indications that the S_4 site was populated in the monometallated C2H mutant protein. The fact that the ^{113}Cd chemical shift in the C2H protein is the same as in the wild-type LIM2 protein suggests that the chemical environment of the S_4 site is unaffected by whether the S_3N_1 site is populated.

Collectively, the results presented here suggest that the S_3N_1 Zn(II) in LIM2 consists of C^1, C^2, H^3, and C^4 and the S_4 site consists of the last 4 conserved Cys residues in the LIM sequence (Fig. 1*A*). Thus, the LIM domain can be considered a double finger as was predicted previously (20). The 2-residue linker between the two Zn(II) modules of the LIM domain is short compared to the spacing between adjacent fingers in the classical Zn(II) finger motif (27). Preliminary structural analysis of LIM2 suggests that its structure is distinct from that of

the classical Zn(II) finger motif,[3] so a double finger designation, although useful as a two-dimensional model, is not an accurate description of the LIM2 structure.

Although only a minimal biophysical analysis could be performed on the C5H mutant protein since our ability to recover the protein from bacteria was limited, the small amounts of protein did permit the demonstration that the protein exists in a metal-depleted state. The presence of a bulky histidyl group at position 5 in the S_4 site or a second histidyl group in the S_3N_1 site, as in the C2H mutant protein, may prevent folding of the domain to a stable state. Consequently, the goal of converting a sulfur ligand to a nitrogen ligand was not realized, presumably due to steric or electrostatic constraints. The metal sites may not have the necessary inherent flexibility for accommodation of the bulkier histidyl group.

We previously demonstrated that the S_4 metal site is the first to be populated in wild-type LIM2 (17). Addition of 1 mol eq Co(II), Cd(II), or Zn(II) results in tetrathiolate coordination by LIM2 (17). The lack of bound Zn(II) in the C5H mutant protein is consistent with the results of the previous study in which a sequential folding pathway was observed. The inability of the C5H mutant protein to bind metal suggests that population of the S_4 site may be required for metal binding in the N-terminal S_3N_1 site. Disruption of the S_4 site by substitution of a histidyl residue at position 5 eliminates the S_3N_1 site as well. Intramolecular interactions between the polypeptide segments enfolding the two Zn(II) sites may be necessary to stabilize the fold of the S_3N_1 subsite.

A considerable number of LIM domain proteins including Lin-11, Isl-1, LH-2, Rhombotin-1, zyxin, Apterous, and Xlim-1 have aspartyl residues in the eighth conserved ligand position in one or more of their LIM domains (1, 2, 5, 6, 15, 31, 32). The eighth candidate ligand position is Cys, Asp, or His in all known LIM domain proteins. It is clear from the results with the C8D mutant LIM2 that the presence of an aspartyl residue in the last candidate ligand position yields an oxygen liganding atom to Zn(II). Aspartate is one of the most common Zn(II) ligands in known Zn(II) metalloproteins, so this observation is not surprising. One implication of the residue variability at the eighth position is that some polypeptide flexibility must be permitted at this position to accommodate a significant change in residue volume and chemistry. A second type of variation observed near the eighth candidate ligand position is variation is the spacing between the last 2 residues. Most LIM domain proteins have a spacing of 2 residues (C*XX*Z where Z specifies the His, Cys, and Asp variability in position eight), yet CRIP exhibits a spacing of 3 residues separating 2 cysteinyl residues (C*XXX*C).

In summary, the mutational analysis presented here provides evidence that the LIM domain is comprised of a S_3N_1 site generated from C^1, C^2, H^3, and C^4 and an S_4 site generated from C^{5-8}. This coordination gives rise to a double finger as was first suggested by Liebhaber and colleagues (20). We further demonstrated that an aspartyl residue in the eighth ligand position can function in Zn(II) coordination in the LIM domain.

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[3] G. C. Perez-Alvarado, C. Miles, J. W. Michelsen, H. A. Louis, D. R. Winge, M. C. Beckerle, and M. F. Summers, manuscript in preparation.

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