Folding Determinants of LDL Receptor Type A Modules†

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ABSTRACT: To investigate how three disulfide bonds and coordination of a calcium ion cooperate to specify the structure of an LDL-A module, we studied the interdependence of disulfide bond formation and calcium coordination in the folding of ligand-binding module 5 of the LDL receptor (LR5). In variants of LR5 containing only a single pair of cysteines normally disulfide-bonded in the native polypeptide, the addition of calcium does not alter the effective concentration of one cysteine for the other. LR5 only exhibits a calcium-dependent preference for formation of native disulfide bonds and detectable calcium-induced changes in structure when the two C-terminal disulfide bonds are present. Furthermore, when the conformation of this two-disulfide variant of LR5 is probed by NMR in the presence of calcium, only the C-terminal lobe of the module, which contains the calcium coordination site, acquires a near-native conformation; the N-terminal lobe appears to be disordered. These findings contrast with studies of other model proteins, like BPTI, in which formation of a single disulfide bond is sufficient to drive the entire domain to acquire a stable, nativelike fold.

Low-density lipoprotein receptor A (LDL-A)[1] repeats are among the most common cysteine-rich modules in the protein sequence database and are found in a wide variety of cell-surface receptors (*1*). In the LDL receptor, where the modules were first identified (*2*), the LDL-A modules serve to bind lipoprotein ligands (*3*, *4*). Other receptors with LDL-A repeats participate in a wide range of biological processes, including lipoprotein uptake (*5*), brain development (*6*), and Wnt signal transduction (*7*−*9*).

Each LDL-A module is an autonomously folding unit that spans ∼40 residues. Structures have been determined for several LDL-A modules of the LDLR (*10*−*13*) and for two LDL-A modules of the LDLR-related protein-1 (*14*, *15*). Conserved structural features among these LDL-A modules include three disulfide bonds, a short antiparallel β-sheet, a single turn of 3_{10}-helix, and a high-affinity calcium-binding site (Figure 1A). Two loops define the N- and C-terminal "lobes" of the molecule, connected by the 3_{10}-helix at one end and by a disulfide bond between the second and fifth conserved cysteine residues at the other. The antiparallel β-sheet, together with a disulfide bond between the first and third conserved cysteines, serves as a scaffold for the N-terminal lobe of the module, positioning the conserved hydrophobic residues (Phe 10 and Ile 18) into the interior of the module, where they pack against each other. The C-terminal lobe is organized around the bound calcium ion and also contains the conserved disulfide bond between the fourth and sixth cysteines. The coordination sphere of this bound calcium ion is well-modeled in octahedral geometry, with four carboxylate oxygen atoms from the conserved acidic motif in one plane and the two carbonyl oxygen atoms completing the coordination sphere at the apices.

Despite the wealth of structural information about LDL-A modules, little is known about the relative influence of each of these structural features in forming and stabilizing the native fold of LDL-A modules. Understanding how LDL-A modules fold has implications for understanding mechanisms of misfolding of LDL-A modules in familial hypercholesterolemia (FH), a common genetic disorder associated with an increased risk for cardiovascular disease (see refs *16* and *17* for reviews). Ultimately, a better understanding of how LDL-A modules fold properly may assist in the design of strategies for rescuing folding defects found in patients with FH.

To investigate the principles which guide folding of LDL-A modules, we have chosen to study the fifth LDL-A repeat of the ligand binding domain of the LDLR (LR5) for three reasons. First, the LR5 sequence possesses the conserved sequence features characteristic of LDL-A modules: the six cysteines, which form three disulfide bonds, the four C-terminal acidic residues involved in calcium coordination, and the two conserved hydrophobic residues (*2*). Second, the structure of LR5 has been determined to 1.7 Å by X-ray crystallography, providing detailed information about the geometry of the calcium-binding site and the packing of hydrophobic residues (*11*). Finally, of the many FH mutations found in the ligand-binding domain of the LDLR, more are found in LR5 than in any other LDL-A module, and variants of LR5 which encode known FH mutations lose the ability to fold to a single-disulfide-bonded isomer (*18*). Thus, insight

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[1] Abbreviations: α-LA, α-lactalbumin; BPTI, bovine pancreatic trypsin inhibitor; β-VLDL, β-migrating very low-density lipoprotein; FH, familial hypercholesterolemia; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-pressure liquid chromatography; HSQC, heteronuclear single-quantum coherence; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; LR5, LDL-A module 5 of the low-density lipoprotein receptor; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; rp, reversed phase; TFA, trifluoroacetic acid.

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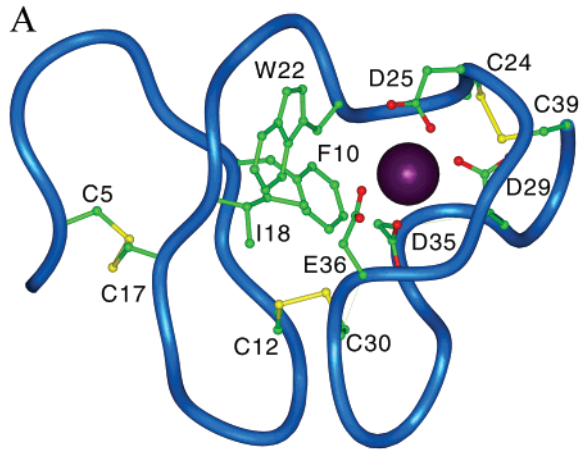

B

Mutant	Sequence
WT LR5	DSSPCSAFEFHCLSGECIHSSWRCDGGPDCKDKSDEENCA
C5-C17	DSSPCSAFEFHALSGECIHSSWRADGGPDAKDKSDEENAA
C12-C30	DSSPASAFEFHCLSGEAIHSSWRADGGPDCKDKSDEENAA
C24-C39	DSSPASAFEFHALSGEAIHSSWRCDGGPDAKDKSDEENCA
C5-C39	DSSPCSAFEFHALSGEAIHSSWRADGGPDAKDKSDEENCA
C17-C30	DSSPASAFEFHALSGECIHSSWRADGGPDCKDKSDEENAA
C5AC17A	DSSPASAFEFHCLSGEAIHSSWRCDGGPDCKDKSDEENCA
C12AC30A	DSSPCSAFEFHALSGECIHSSWRCDGGPDAKDKSDEENCA
C24AC39A	DSSPCSAFEFHCLSGECIHSSWRADGGPDCKDKSDEENAA

FIGURE 1: (A) Ribbon model of the LR5 crystal structure (PDB entry 1ajj; *11*). Conserved residues labeled as reference points include the three disulfide bonds (C5−C17, C12−C30, and C24−C39), four calcium coordinating residues (D25, D29, D35, and E36), and two hydrophobic residues (F10 and I18). (B) Amino acid sequences of wild-type LR5 and of LR5 variants constructed for these studies in which pairs of cysteines have been substituted with alanines. Cysteines still remaining in each LR5 mutant are labeled in red. Mutants which retain only two cysteines are named by explicit reference to the cysteines remaining (e.g., C5−C17), whereas forms of LR5 with four remaining cysteines are named by identification of the two cysteines replaced with alanine by mutagenesis (e.g., C5AC17A).

into the folding determinants of LR5 promises to enhance the current understanding of how FH mutations of LR5 lead to misfolding and disease.

In LR5, formation of the correct pattern of disulfide bonds is dependent on calcium coordination. In a redox buffer that allows disulfide rearrangement, reduced LR5 folds to a single-disulfide-bonded isomer only in the presence of calcium; without calcium, a mixture of isomers is obtained, which resembles the distribution of disulfide-bonded species formed when denaturant is added to the refolding buffer (*18*). However, the extent to which Ca^{2+} coordination favors formation of any individual disulfide bond or pair of disulfide bonds has yet to be investigated.

As a starting point for our investigations into the folding of LR5, we have focused on the cooperativity between disulfide bond formation and calcium coordination. First, we examined the structure of reduced LR5 in the presence of calcium to see whether the calcium coordination site could bind a calcium ion in the absence of any disulfide bonds. To determine whether individual disulfide bonds were

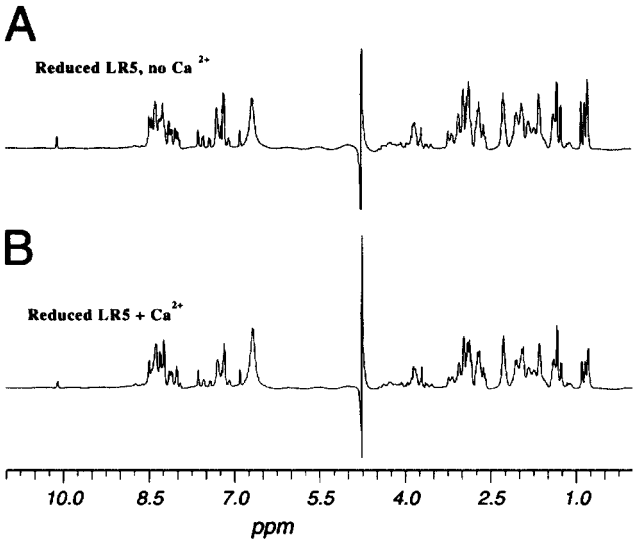

FIGURE 2: Reduced LR5 does not exhibit structural changes after addition of calcium. (A) One-dimensional ^{1}H NMR spectrum of reduced LR5 obtained in the absence of calcium. (B) One-dimensional ^{1}H NMR spectrum of the same sample of reduced LR5 after addition of calcium (10 mM).

favored in the presence of calcium, we next measured the effective concentration (C_{eff}) of pairs of cysteines from LR5 for each other with and without calcium. Then, we constructed variants of LR5 with four of the six native cysteine residues to see whether pairs of disulfide bonds could form their native linkage in response to added calcium. Finally, we investigated the conformation of the lone two-disulfide variant that folds in the presence of calcium by NMR. Our data show that the 18 C-terminal amino acids of the module can fold independently of the N-terminal residues, and that specific stabilizing interactions between the C- and N-terminal lobes of the module occur in response to calcium coordination and help couple folding between the two lobes of the module.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. Mutant forms of LR5 (Figure 1B) were generated either by Kunkel mutagenesis (C5−C17, C5−C39, C24−C30, C5AC17A, C12AC30A, and C24AC39A) (*19*) or with the Stratagene Quik-Change system (C12−C30 and C17−C30) using the pMMLR5 vector as a starting point (*18*). In the resulting plasmids, the mutant LR5 protein is expressed with an N-terminal $(His)_9$-trpLE tag (*18*). The unlabeled proteins were expressed in Luria Broth, while labeled proteins were expressed in M9 minimal medium using $^{15}NH_4Cl$ as the sole nitrogen source.

Mutant LR5 fusion proteins were expressed in *Escherichia* coli strain BL21(DE3). Recombinant protein synthesis was induced by addition of 0.4 mM IPTG at a measured OD_{600} of 0.8, and the cells were harvested 3 h after induction. Fusion proteins were isolated from inclusion bodies, cleaved, and purified as described previously (*20*). The masses of purified proteins were checked by MALDI-TOF mass spectrometry. Purified proteins were stored in reduced and lyophilized form over desiccant at 4 °C.

Effective Concentration Measurements. The reduced form of each LR5 variant was incubated at a concentration of 20

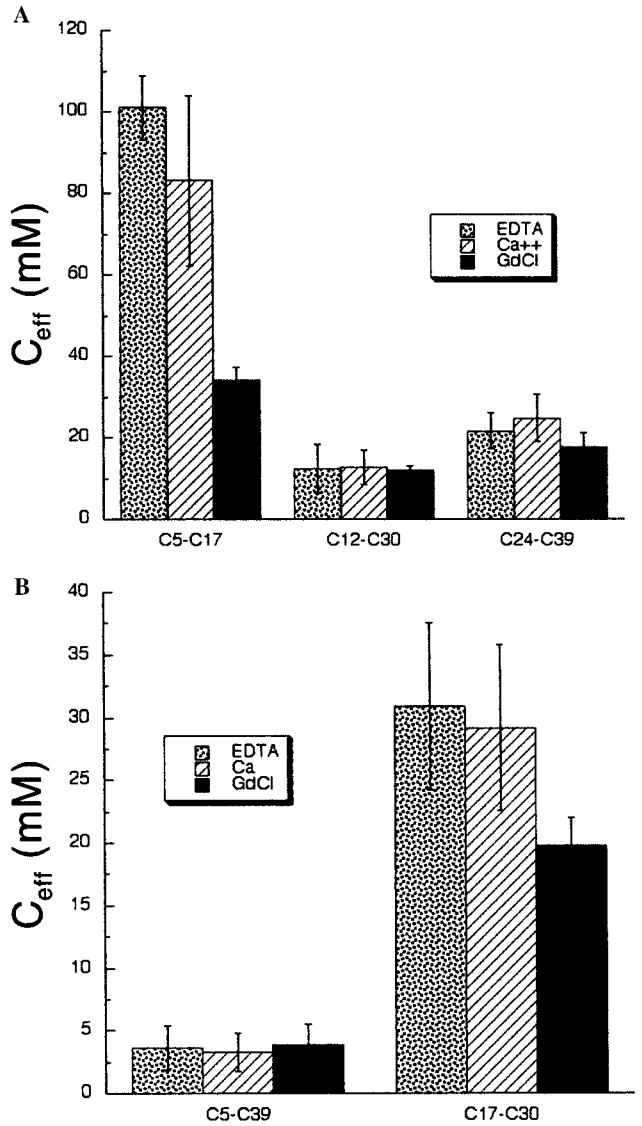

FIGURE 3: (A) Measured values of C_{eff} for the three native cysteine pairs in LR5. C_{eff} values were determined for each construct in redox buffers containing 100 μM EDTA (left, speckled bars), 10 mM calcium (center, hatched bars), or 6 M guanidine hydrochloride (right, black bars). (B) Measured values of C_{eff} for non-native cysteine pairs in LR5. C_{eff} values were determined for each construct in redox buffers containing 100 μM EDTA (left, speckled bars), 10 mM calcium (center, hatched bars), or 6 M guanidine hydrochloride (right, black bars).

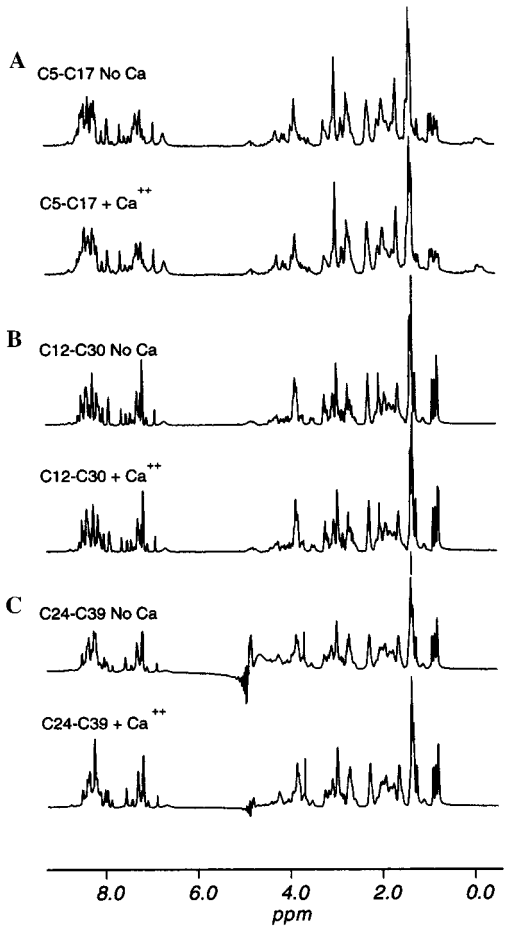

FIGURE 4: One-dimensional ^{1}H NMR spectra of oxidized single-disulfide LR5 variants obtained before (top) and after (bottom) addition of calcium (10 mM): (A) C5−C17, (B) C12−C30, and (C) C24−C39. None of these single-disulfide-bonded variants of LR5 exhibits significant conformational changes upon addition of calcium.

μM, in a redox buffer [20 mM Tris (pH 8.5)] containing calcium and a mixture of reduced and oxidized glutathione in molar excess. In each case, the experiment was repeated for all combinations of at least three different reducing potentials ($[GSH]^2/[GSSG]$ ratios ranging from 0.1 to 80) and five different calcium concentrations (ranging from 0 to 500 mM). Each sample was allowed to equilibrate for 48 h at 20 °C. At 24 and 48 h, aliquots of each refolding reaction mixture were acidified [by addition of 5% (v/v) acetic acid] to halt disulfide exchange. Oxidized and reduced peptide species were separated by reversed-phase (rp) HPLC on an analytical Vydac C-18 column using water with 0.1% TFA and a 90% acetonitrile/10% water mixture with 0.1% TFA as solvents A and B, respectively. A linear gradient of solvent B, beginning around 24% B and increasing by 0.1% per minute, was used to separate the reduced and oxidized peptides. The starting point of the linear gradient was optimized for each LR5 variant that was analyzed. The ratio between oxidized and reduced peptide concentrations at equilibrium was combined with the reducing potential to calculate an effective concentration (C_{eff}) for formation of each disulfide bond using eq 1.

$$C_{eff} = [\text{ox. peptide}][GSH]^2/[\text{red. peptide}][GSSG] \quad (1)$$

The reducing potentials employed for each mutant were optimized to yield substantial oxidized and reduced peaks at equilibrium. Effective concentration data obtained after equilibration for 24 and 48 h were compared to confirm that that equilibrium was attained.

NMR Experiments. For NMR spectroscopy, all peptides were first purified by rpHPLC in reduced form. To obtain the unlabeled oxidized single-disulfide LR5 variants, each purified reduced peptide was taken up in 20 mM Tris (pH 8.5) and stirred for 48 h at 4 °C exposed to air. The oxidized peptide was then repurified by preparative HPLC over a Vydac C18 column. Unlabeled and ^{15}N-labeled wild-type LR5 and ^{15}N-labeled C5AC17A were purified in oxidized form by refolding the purified reduced peptide. Each protein was dialyzed exhaustively against a buffer made up of 20 mM Tris (pH 8.5), 10 mM $CaCl_2$, 0.5 mM GSH, and 0.25

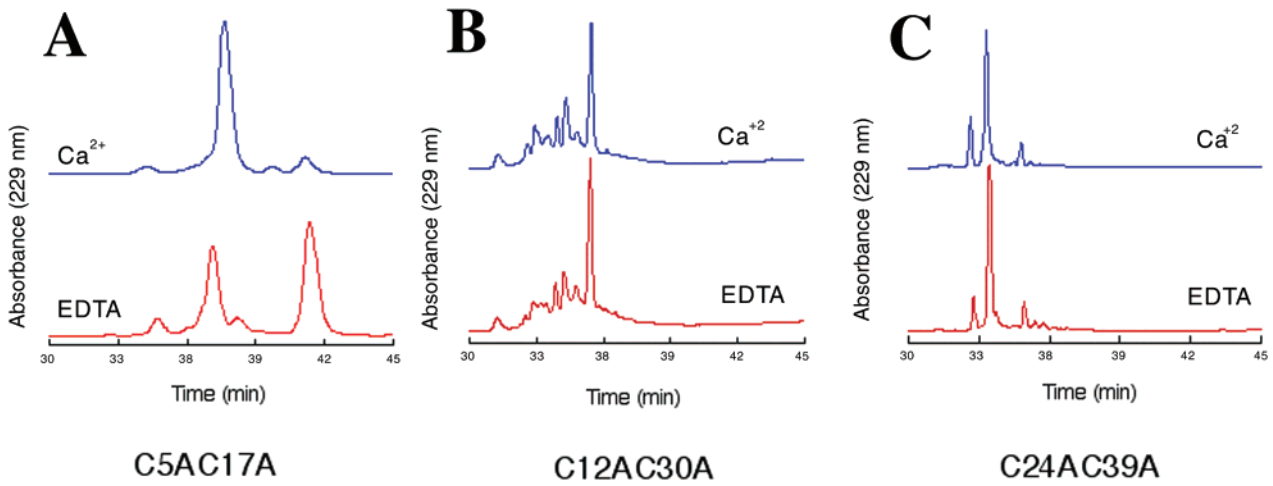

FIGURE 5: HPLC chromatograms of products formed after folding of (A) C5AC17A, (B) C12AC30A, and (C) C24AC39A for 24 h under conditions that permit disulfide rearrangement. Folding of each mutant in the absence of calcium (A−C, bottom trace, red) produces a distribution of disulfide-bonded species. Folding of C5AC17A (A, top trace, blue) in the presence of calcium leads to a predominant peak in which both native disulfide bonds have formed. In contrast, folding of the C12AC30A and C24AC39A mutants (B and C, top traces, blue) in the presence of calcium yields a distribution of disulfide-bonded species similar to that observed in the absence of calcium.

mM GSSG at 4 °C. The resulting fully oxidized and refolded peptide was purified by preparative HPLC on a Vydac C18 column.

All NMR spectra were obtained at pH 5.5 in 10% D_2O without additional buffer or salt, on a Varian 500 MHz spectrometer at 25 °C. For one-dimensional spectra of unlabeled proteins, 4096 points were acquired with a sweep width of 7000 Hz. For HSQC spectra of wild-type and C5AC17A LR5, 1024 × 64 complex points were obtained with a sweep width of 7000 Hz in D_1 (^{1}H) and 1800 Hz in D_2 (^{15}N). A 90°-shifted sine function was applied to the data in D_1 and D_2. All spectra were processed using Felix 97.0 software (MSI Inc.) and/or NMRpipe (*21*).

Fluorescence Assay. The Ca^{2+} affinity constants of wild-type LR5 and C5AC17A in oxidized forms were obtained by observing Ca^{2+}-dependent changes in the fluorescence emission spectrum of tryptophan 22, as described previously (*20*).

RESULTS

NMR Experiments Demonstrate the Necessity of both Calcium Coordination and Disulfide Bond Formation for Folding. When ^{1}H NMR spectra of fully reduced LR5 are acquired in the absence or presence of calcium at 100 mM, no differences are detected (Figure 2). This finding shows that in the absence of any disulfide bonds, the LR5 polypeptide backbone does not possess detectable affinity for calcium and indicates that organization of the calcium coordination site does not occur in the absence of disulfide bond formation.

Effective Concentration Measurements. To investigate whether cooperativity exists between the formation of any individual disulfide bond and calcium coordination, we measured the effective concentration for the formation of individual disulfide bonds using glutathione as a reference thiol. We first compared the C_{eff} values for individual disulfide bonds in native buffer and guanidinium hydrochloride (GdCl). In 6 M GdCl, native and nonspecific interactions are weakened or eliminated; the C_{eff} is dominated by the chain statistics of the polypeptide backbone and serves as a baseline for comparison.

Under native and denaturing conditions, the effective concentration for formation of two native disulfide bonds (C12−C30 and C24−C39) and the non-native disulfide controls are identical within experimental error (Figure 3). This observation suggests that polypeptide collapse and the formation of hydrophobic packing interactions play very little role in stabilizing the tertiary fold of the C-terminal half of the module. In contrast, the effective concentration for formation of the disulfide bond between C5 and C17 increases 3-fold when measured in native buffer (Figure 3), indicating that the formation of specific side chain contacts or polypeptide collapse contributes, at least in part, to stabilization of the amino-terminal part of the module.

To measure directly any cooperativity between formation of any single-disulfide bond and calcium coordination, we next proceeded to compare the effective concentration for the formation of each of the three native disulfide bonds in the presence and absence of calcium. Remarkably, the effective concentration for each native disulfide bond, as well as for two non-native controls, is the same in the absence and presence of calcium at concentrations up to 100 mM (Figure 3).

If the C_{eff} values for the native single-disulfide variants are truly independent of calcium, then the oxidized form should not detectably bind calcium. To test this prediction, we acquired ^{1}H NMR spectra on the 5−17, 12−30, and 24−39 single-disulfide variants. In each case, the spectra acquired in the presence and absence of 10 mM calcium are indistinguishable (Figure 4). Together, the C_{eff} measurements and NMR data demonstrate that none of the single-native disulfide variants of LR5 are capable of stably organizing a calcium coordination site or acquiring the native LR5 fold.

HPLC Analysis of Disulfide Reshuffling Experiments on Two-Disulfide LR5 Variants. To ascertain whether pairs of native disulfide bonds could cooperate to organize the calcium coordination site and thereby allow calcium-dependent folding of LR5, we generated variants containing two of the three native disulfide bonds present in the wild-

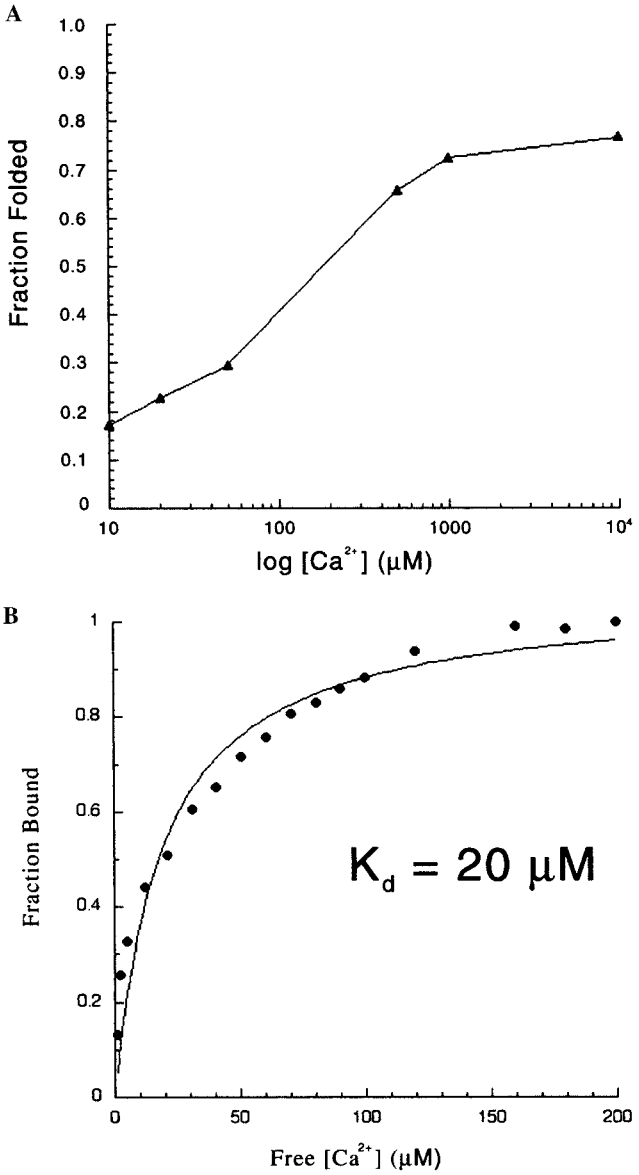

FIGURE 6: (A) Semilog plot illustrating the fraction of C5AC17A molecules that acquire native disulfide connectivity as a function of calcium concentration. (B) Measurement of the calcium affinity of purified, folded C5AC17A by tryptophan fluorescence. The calcium dissociation constant was calculated by curve fitting to a single-binding site model using the software program Kaleidagraph (Synergy Software).

type module. The cysteines which make up the third disulfide bond were mutated to alanines. These variants are named such that, for example, the LR5 variant retaining cysteines which form the 12−29 and 24−39 disulfide bonds but which can no longer form the 5−17 bond because the required cysteines have been mutated to alanines, is denoted C5AC17A.

HPLC analysis of the products of oxidative refolding of the two-disulfide variants of LR5 demonstrates that neither C12AC30A nor C24AC39A exhibits calcium-dependent folding. However, when the N-terminal 5−17 disulfide bond is removed by mutagenesis (C5AC17A), the module retains its ability to fold to a predominant-disulfide-bonded isomer in the presence of calcium (Figure 5). Refolding experiments carried out in the presence of gradually increasing amounts of calcium demonstrate that the C5AC17A variant folds in a calcium-dependent manner, and that the effect is saturable (Figure 6A). The K_d of C5AC17A for calcium, determined

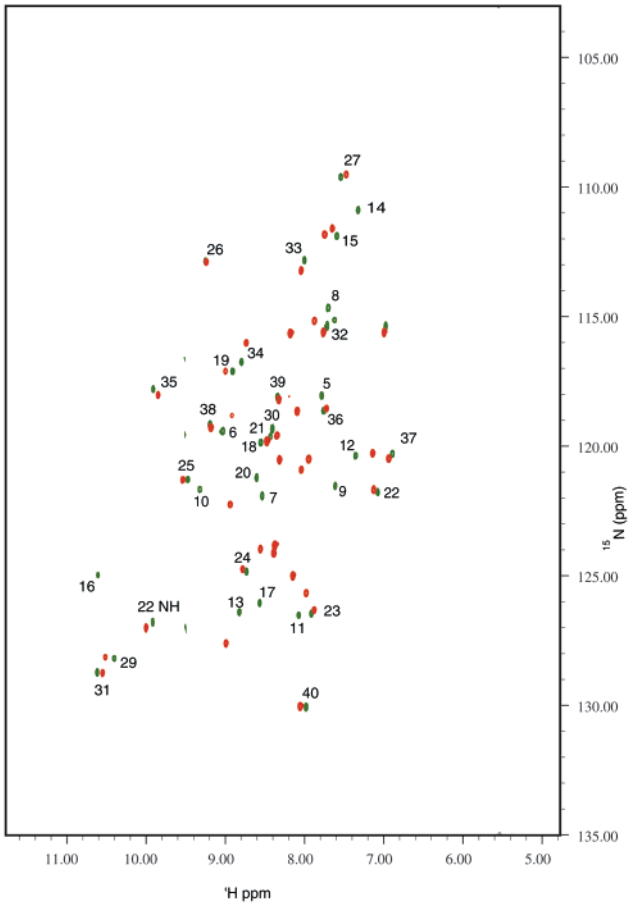

FIGURE 7: Comparison of the $^{15}N-^{1}H$ HSQC spectra of wild-type LR5 (red) and C5AC17A (green) in an overlay plot. Spectra were obtained at pH 5.6 in 10 mM Ca^{2+} at 25 °C.

by fluorescence, is 20 μM, a value 500-fold weaker than the calcium affinity of native LR5 (Figure 6B). These data indicate that the organization of the calcium coordination site and, thus, the sensitivity to calcium of disulfide bond formation in LR5 is encoded not in any individual disulfide bond but rather in the combination of the 12−30 and 24−39 disulfide bonds.

Structural Characterization of the C5AC17A Module by NMR. To determine whether the affinity of C5AC17A for calcium is weaker because of altered geometry at the calcium-binding site and to investigate further which residues of the C5AC17A mutant retain a nativelike conformation, the conformation of ^{15}N-labeled C5AC17A was probed in a $^{15}N-^{1}H$ HSQC experiment. When the HSQC spectrum of the C5AC17A mutant is compared to the HSQC spectrum of wild-type LR5, peaks in C5AC17A can be identified at positions near the resonances for the 18 C-terminal residues of the wild-type module (Figure 7). These data strongly support a model in which the C-terminal portion of the C5AC17A variant is in a native conformation when calcium is present and the native disulfide bonds are formed. In contrast, most of the resonances of the first 22 residues of C5AC17A do not appear near the corresponding peaks of the wild-type module. Instead, poorly resolved amide proton signals are clustered in the region from 8 to 8.5 ppm. These data suggest that the N-terminal portion of the module is poorly ordered. Thus, in the presence of calcium, the 18 C-terminal residues of LR5 are capable of folding indepen-

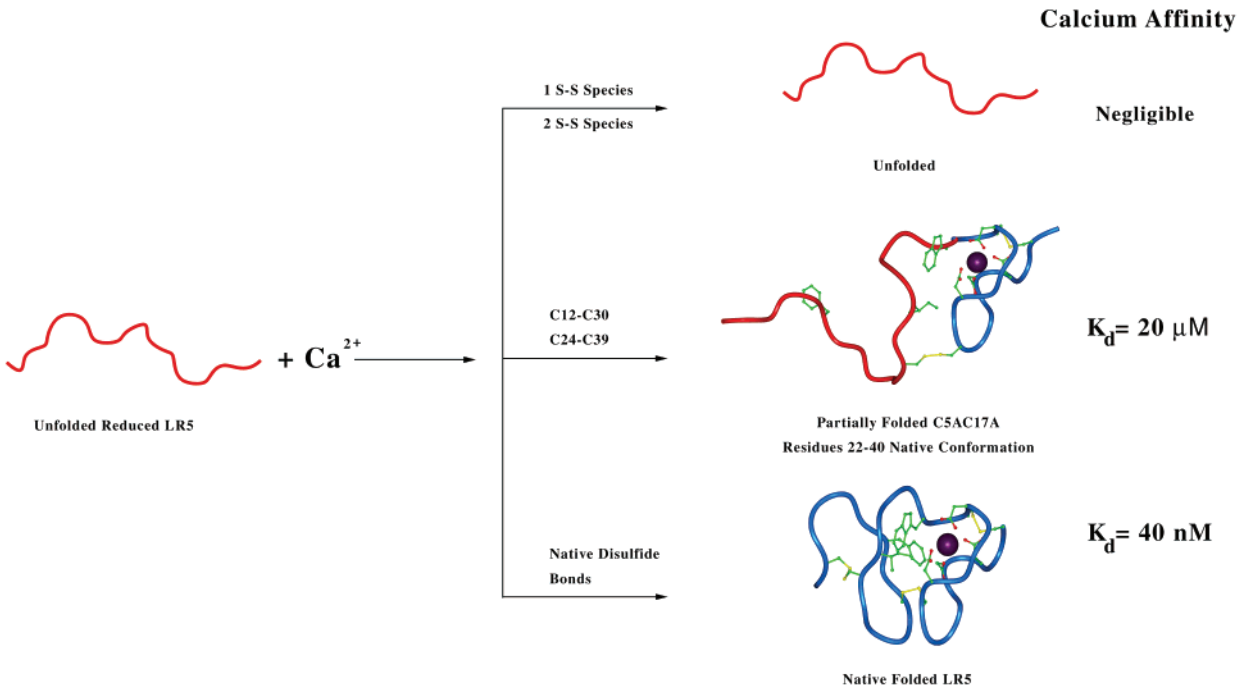

FIGURE 8: Model illustrating how calcium coordination and disulfide bond formation combine to specify conformational features of the native fold of LR5. The C-terminal lobe acquires nativelike structure when the C12−C30 and C24−C39 disulfide bonds are present and a calcium ion is bound at the coordination site. Native structure within the N-terminal lobe requires the C5−C17 disulfide bond in addition to a properly folded C-terminal lobe. Further details of the model are discussed in the text.

dently of the 22 N-terminal residues and may be considered to be a "subdomain".

DISCUSSION

Proteins harboring LDL-A modules transport ligands into cells (*5*), transduce signals from the external environment (*22*), and act as receptors for pathogenic viruses (*23*, *24*). To investigate how disulfide bonds and calcium ligation combine to define the structure of a prototype LDL-A module, we studied the conformational preferences of forms of LR5, the fifth ligand-binding module of the LDLR, that retained zero, one, or two disulfide bonds in the presence and absence of calcium.

Our findings demonstrate that there is an intimate relationship between calcium ligation and the native disulfide bonds in specifying and stabilizing the fold of LR5. Features of the interplay between formation of native disulfide bonds, calcium coordination, and native structure are summarized in the model presented in Figure 8.

When fewer than two disulfide bonds are present, calcium ligation is highly unfavorable, and LR5 cannot stably acquire native structural features in either its N-terminal or C-terminal lobe. Calcium does not have any detectable impact on the conformation of LR5 variants with only a single disulfide bond, as assessed by both effective concentration measurements and proton NMR experiments.

Organization of the calcium site only becomes energetically favorable when formation of both C-terminal disulfide bonds occurs. When these two disulfide bonds are present, the conformational diversity of unliganded LR5 is restricted enough for the free energy of calcium coordination to overcome the entropic cost of organizing the calcium-binding site. Calcium coordination then constrains the C-terminal lobe sufficiently to allow it to acquire nativelike structure. Because conformational folding requires the presence of at least two disulfide bonds, LR5 folding appears to be a "quasi-stochastic" process, as defined by Scheraga and colleagues (*25*).

Formation of the 5−17 disulfide bond plays a similar role in driving the folding of the N-terminal lobe into its native structure in the presence of a nativelike C-terminal lobe. When the 5−17 disulfide bond cannot form and the accompanying conformational restriction is not present, the N-terminal lobe prefers to be disordered because the free energy gained from packing interactions in the native structure is outweighed by the increased entropic cost that is incurred. Hence, the N-terminal lobe of C5AC17A is disordered, while the C-terminal lobe is in the native wild-type geometry. When the 5−17 disulfide bond of native LR5 is added, packing interactions with the C-terminal lobe become favorable because the disulfide bond restricts the conformational diversity of the N-terminal lobe in the unfolded state. Because the calcium affinity indirectly reports on the stability of the module, this model also explains why C5AC17A, despite having a calcium-binding site with wild-type geometry, binds calcium 500-fold more weakly than native LR5.

The paucity of hydrophobic residues and secondary structural elements in LR5 contrasts with structural properties of other disulfide-bonded proteins used as models for protein folding, like α-lactalbumin (α-LA) and BPTI, and thus demands a different solution to the folding problem. In both α-LA and BPTI, hydrophobic−polar patterning and packing of secondary structural elements provide much greater contributions in dictating the tertiary fold.

A variant of α-LA, in which each cysteine has been replaced with alanine, still exhibits near-native helical content under native conditions (*26*), and the C_{eff} for the 28−111

disulfide bond of α-LA is 10^5-fold greater in physiologic buffer than in denaturant (*27*). Thus, the primary sequence of α-LA encodes the secondary structural elements necessary to position these cysteines in a near-optimal geometry for disulfide bond formation in the absence of any of the other disulfide bonds (a "folded precursor" mechanism according to the Scheraga formalism; *25*). Although reduced BPTI is unfolded, formation of either one of the 30−51 or 5−55 disulfide bonds is sufficient to drive folding to a near-native structure, as judged by NMR (*28*−*30*) and model peptide studies (*31*).

Because LR5 lacks extensive secondary structure, the structural constraints upon residue type at nonconserved sites may be minor. Thus, all that may be required to specify the LR5 fold are the six cysteines, four acidic residues for calcium coordination, two conserved hydrophobic residues, and appropriate spacers for maintaining these determinants in appropriate register with one another. In this model, conserved sequence motifs in LDL-A repeats act purely as local structural constraints, an assumption consistent with the observed lack of intermodule contacts in LDL-A module pairs (*20*, *32*). In contrast, the sequence of BPTI must encode both helices and strands and specify packing interactions among these secondary structural elements. This combination of requirements is considerably more complex than those for LR5, yet recently published data have shown that replacement of 29 of the 58 residues of BPTI with alanines can be accomplished without major deviation from the native structure (*33*). Thus, if replacement of 50% of the wild-type sequence with alanines does not cause major structural deviations in BPTI, an even higher percentage of replacement might very well be successful in LR5.

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