Scrambled Isomers as Key Intermediates in the Oxidative Folding of Ligand Binding Module 5 of the Low Density Lipoprotein Receptor

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The ligand binding module five (LA5) of the low density lipoprotein receptor is a small, single-domain protein of 40 residues and three disulfide bonds with a calcium binding motif that is essential for its structure and function. Several mutations in LA5 have been reported to cause familial hypercholesterolemia by impairing a proper folding of the module. The current study reports the oxidative folding and reductive unfolding pathways of wild type and mutant LA5 modules through kinetic and structural analysis of the trapped intermediates. Wild type LA5 folding involves an initial phase of nonspecific packing where the sequential oxidation of its cysteines gives rise to complex equilibrated populations of intermediates. In the presence of calcium, a scrambled isomer (termed Xa) constitutes the global free energy minimum of the folding process. Xa and the native form are stable, inter-convertible species whose relative populations at equilibrium appear displaced in disease-linked mutants toward the scrambled form. Because stable scrambled isomers such as Xa avoid the exposition of reactive cysteines in misfolded modules, they might constitute a strategy to prevent wrong interactions with other domains during folding of the receptor. Comparison of the folding pathways of wild type and mutant LA5 provides the molecular basis to understand how LA modules fold into a functional conformation or upon mutation misfold and lead to disease.

The low density lipoprotein receptor (LDLR)6 plays a primary role in the homeostatic control of blood cholesterol by mediating the uptake of cholesterol-containing lipoprotein particles from the circulation into cells (1, 2). Loss-of-function mutations in the LDLR gene cause familial hypercholesterolemia (FH), an autosomal dominant disorder affecting ~1 in 500 individuals worldwide (3). There are over 1000 mutations believed to cause FH, including numerous insertions, deletions, and point mutations. In heterozygotes, FH results in elevated concentrations of LDL and cholesterol in blood and therefore in an increased risk of atherosclerosis and coronary heart disease. Homozygotes develop severe atherosclerosis at a very early age, which leads to death. The mature LDLR is a single-pass transmembrane, multidomain glycoprotein of 839 residues that comprises seven different regions (see Fig. 1): seven tandem LDLR type A (LA) modules at the N terminus responsible for binding of lipoproteins, two epidermal growth factor (EGF) repeats, a YWTD domain, another EGF-like repeat, an aminoterminal domain, and a C-terminal cytoplasmic tail (4).

LA3 to LA7 modules are essential for binding of LDL, while LA5 is also crucial for high affinity binding of β-VLDL (very low density lipoprotein) (5, 6). The LA modules contain ~40 residues that are cross-linked by three disulfide bridges with the following connectivity: C1–C3, C2–C5, and C4–C6 (Fig. 1). The solution structures of LA1, LA2, and LA6 together with the crystal structure of LA5 reveal a low content of secondary structure: a short β-hairpin and two 310 helices (7–10). According to the crystal, the LA5 structure is organized around a calcium ion coordinated through the side chains of residues Asp-25, Asp-29, Asp-35, and Glu-36 and the backbone carbonyls of Thr-22 and Gly-27. The structural analysis of LA5 also allows the classification of FH mutations in two categories: elimination or misplacement of direct Ca2+ ligands (W22X, G27D, D29X, D35E, E36X), and removal of hydrogen or disulfide bonds that disrupt the backbone topology (C5X, C12Y, C30X, C39X, E16K, D32X, S34X) (9).

6 The abbreviations used are: LDLR, low density lipoprotein receptor; LA, type A module of LDLR; AEMTS, 2-aminoethylmethyliithiosulfonate; DTT, dithiothreitol; FH, familial hypercholesterolemia; GdnHCl, guanidine hydrochloride; GdnSCN, guanidine thiocyanate; GSH, reduced glutathione; GSSG, oxidized glutathione; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; N, native form; R, fully reduced/denatured form; RP-HPLC, reversed-phase high performance liquid chromatography; TCEP, Tris-(2-carboxyethyl)phosphine; WT, wild type.

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9 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6.

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The LDLR is an outstanding example of multidomain protein for which the in vivo folding seems to take place non-vectorially in a posttranslational manner (11) and whose LA modules have been shown to fold independently (12). Flexible linker regions connecting the individual modules allow the ligand binding repeats of the receptor to adjust their relative position in order to bind a variety of heterogeneous LDL particles of different shape and diameter (13, 14). Several studies have been focused on the influence of FH mutations on the conformation of the LA5 domain. The abundant mutations that affect the calcium binding motif of this module have been shown to prevent, in many cases, the attainment of a native structure leading to misfolding of the domain and disease (15). Despite all this information, little is known about the folding landscape of individual LA modules from the reduced/unfolded ensemble to the native state, the influence of calcium in shaping it, or the precise effect of FH mutations on the LA folding pathway.

FIGURE 1. Three-dimensional structure of LA5. a, schematic organization of the LDL receptor, illustrating the position of the LA5 module. EGF, epidermal growth factor. b, ribbon plot of the native structure of LA5 (Protein Data Bank access code 1AJJ). The disulfide bonds are shown as black sticks. The calcium ion is represented inside the structure by a dark gray sphere. N and C indicate, respectively, the N and C terminus of the protein. The figure was prepared with the program PyMOL. c, amino acid sequence of LA5. The regular secondary structure elements and the disulfide pairings of this module are depicted above the sequence. The mutants constructed and analyzed in this work are indicated below.

EXPERIMENTAL PROCEDURES

Expression and Purification of LA5—The cDNA encoding for human LA5 was amplified by PCR and cloned into the pGEX-4T-3 vector (GE Healthcare), allowing the expression of recombinant LA5 as a glutathione S-transferase fusion protein in Escherichia coli strain BL21(DE3). Typically, 10 liters of culture were grown at 37 °C in Luria Bertani medium until an A600 of 0.6 and then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (final concentration) at 18 °C. The soluble fusion protein was extracted from the cell pellet by sonication in an ice bath, purified by glutathione-agarose affinity chromatography (GE Healthcare), and subsequently cleaved with bovine thrombin (Sigma) to release the LA5 module (10 units of thrombin/1 mg of glutathione S-transferase-LA5). The thrombin cleavage site resulted in the modification of the first amino acid of LA5 (D1G). After cleavage, the protein was refolded under conditions permitting disulfide exchange by exhaustive dialysis at 4 °C against 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM CaCl2, 0.5 mM cysteine, and 2 mM cysteine. The resulting LA5 was purified to homogeneity by RP-HPLC on a preparative C4
column (Vydac Grace) using a linear 15–35% gradient of 0.1% trifluoroacetic acid in acetonitrile over 50 min with a flow rate of 0.75 ml/min. The protein was kept in lyophilized aliquots. The purity and identity of LA5 were determined, respectively, by SDS-PAGE gel stained with silver and MALDI-TOF MS. A theoretical extinction coefficient at 280 nm ($\epsilon_{280} = 6050 \text{ M}^{-1}\text{cm}^{-1}$) was used to calculate the concentration of LA5.

Mutagenesis and Expression of LA5 Variants—The five mutants designed for the present work (S14A, E16K, D29G, D32A, and D35E) were generated using the QuikChange Site-directed Mutagenesis kit (Stratagene) according to the instructions of the manufacturer and verified by DNA sequencing. The expression and purification of the mutants were performed as described under “Expression and Purification of LA5” for the wild type (WT) form.

Oxidative Folding of WT and Mutant LA5—Native LA5 variants (2 mg) were reduced and denatured in 0.1 M Tris-HCl buffer, pH 8.4, containing 8 M guanidine hydrochloride (GdnHCl) and 150 mM dithiothreitol (DTT) at 23 °C for at least 2 h. To initiate folding, the sample was passed through a PD-10 column (Sephadex-25; GE Healthcare) previously equilibrated with 0.1 M Tris-HCl buffer, pH 7.3 or 8.4, including 0.1 mM EDTA or 10 mM CaCl$_2$ for experiments with the absence (apo) or presence (holo) of calcium, respectively. The protein was recovered in 1.2 ml and immediately diluted to a final protein concentration of 0.5 mg/ml in the same buffer, both in the absence (Control −) and presence of redox agents: 0.25 mM 2-mercaptoethanol (Control +), 0.5 mM oxidized glutathione (GSSG), or 0.5 mM/1 mM oxidized/reduced glutathione (GSSG/GSH). For some experiments, selected concentrations of denaturants (1–2 M urea, 0.5–4 M GdnHCl, or 4 M guanidine thiocyanate (GdnSCN)), NaCl (0.1 M) or protein disulfide isomerase (Sigma) (2 μM) were added to the reaction. To monitor the folding reaction, aliquots were removed in a time course manner, trapped by acidification with 4% aqueous trifluoroacetic acid, and analyzed by RP-HPLC as follows. A linear 15–35% gradient of 0.1% trifluoroacetic acid in acetonitrile was applied during 50 min into a 4.6-mm Jupiter C4 column (Phenomenex) at a flow rate of 0.75 ml/min. Alternatively, folding intermediates were trapped by derivatization with 0.1 M 2-aminoethylmethylythiosulfonate (AEMTS; Antracite) in 0.1 M Tris-HCl buffer, pH 8.4, at 23 °C for 15 min, further diluted in 10 volumes of 0.1% aqueous trifluoroacetic acid, and analyzed by RP-HPLC as follows. A linear 15–35% gradient of 0.1% trifluoroacetic acid in acetonitrile was applied during 50 min into a 4.6-mm Jupiter C4 column (Phenomenex) at a flow rate of 0.75 ml/min. Alternatively, folding intermediates were trapped by derivatization with 0.1 M 2-aminoethylmethylythiosulfonate (AEMTS; Antracite) in 0.1 M Tris-HCl buffer, pH 8.4, at 23 °C for 15 min, further diluted in 10 volumes of 0.1% aqueous trifluoroacetic acid, and analyzed by RP-HPLC as follows. A linear 15–35% gradient of 0.1% trifluoroacetic acid in acetonitrile was applied during 50 min into a 4.6-mm Jupiter C4 column (Phenomenex) at a flow rate of 0.75 ml/min. Alternatively, folding intermediates were similarly trapped by acidification and analyzed by RP-HPLC.

Stop/Go Folding of the Major LA5 Scrambled Isomer—Acid-trapped Xa scrambled isomer was isolated by RP-HPLC as described under “Oxidative Folding of WT and Mutant LA5,” freeze-dried, and allowed to re-initiate the folding at 23 °C by dissolving the sample (0.5 mg/ml) in 0.1 M Tris-HCl buffer, pH 8.4, both in the absence (Control −) and presence (Control +) of 0.25 mM 2-mercaptoethanol with and without 10 mM CaCl$_2$. For apo-Ca$_{2}^{2+}$ experiments, 0.1 mM EDTA was added to the reaction. Folding intermediates were similarly trapped by acidification and analyzed by RP-HPLC.

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Retractive Unfolding of WT LA5—Native LA5 (0.5 mg/ml) was dissolved at 23 °C in either 0.1 M Tris-HCl, pH 8.4, or 0.1 M acetate buffer, pH 4.5, containing different concentrations (0.5–200 mM) of DTT or Tris(2-carboxyethyl)phosphine (TCEP; Sigma), respectively. The reaction mixtures also included the presence of 0.1 mM EDTA or 10 mM CaCl$_2$. To monitor the refolding reaction, aliquots were removed at various time intervals, quenched with 4% aqueous trifluoroacetic acid, and analyzed by RP-HPLC as detailed under “Oxidative Folding of WT and Mutant LA5.”

Disulfide Scrambling of WT LA5—Native LA5 was dissolved to a final protein concentration of 0.5 mg/ml in 0.1 M Tris-HCl buffer, pH 8.4, containing 0.25 mM 2-mercaptoethanol as thiol initiator, different concentrations of denaturants (0–8 M urea, 0–8 M GdnHCl, or 0–6 M GdnSCN), and either 0.1 mM EDTA or 10 mM CaCl$_2$. The reaction was allowed to reach equilibrium for 20 h at 25, 50, 70, and 90 °C. The samples were then quenched by acidification and analyzed by RP-HPLC as detailed in previous sections.

Mass Spectrometry and One-dimensional NMR Spectroscopy—The molecular masses of LA5 variants and AEMTS-derivatized intermediates (+76 Da for each free cysteine) were determined by MALDI-TOF MS using a Bruker Ultraflex spectrometer operating in linear mode geometry under 20 kV. Samples were prepared by mixing equal volumes of the protein and matrix solutions (10 mg/ml of 2,6-dihydroxyacetophenone (Sigma) dissolved in 30% acetonitrile containing 20 mM ammonium citrate, pH 5.5), and 0.5 μl of the mixture were spotted on the MALDI plate. A mixture of proteins from Bruker (protein calibration standard I; mass range of 3000 to 25000 Da) was used as mass calibration standard. For mono-dimensional NMR experiments, lyophilized proteins were dissolved to a final concentration of 1 mM in 10 mM Tris-HCl buffer, pH 7.3, containing H$_2$O/D$_2$O (9:1 ratio by volume) or 99.9% D$_2$O and 0.1 mM EDTA or 10 mM CaCl$_2$. NMR spectra were acquired at different temperatures (25–90 °C) on a Bruker AVANCE 600-MHz spectrometer using solvent suppression WATERGATE techniques. The collected spectra were processed and analyzed using the TopSpin1.3 software packages from Bruker Biospin.

RESULTS

Oxidative Folding of LA5—Oxidative folding of fully reduced and denatured LA5 (R) was carried out in Tris-HCl buffer at pH 8.4 both in the absence (Control −) and presence of selected redox agents, namely, 2-mercaptoethanol (Control +), GSSG, or a mixture of GSSG and GSH. The intermediates that arise along the folding reaction were trapped by acidification with trifluoroacetic acid and analyzed in a time course manner by RP-HPLC as detailed under “Experimental Procedures.” A high number of intermediates populate the early stages of the refolding process (up to 24 h) with almost identical RP-HPLC profiles regardless of the presence of thiol catalyst (see Fig. 2a). However, the addition of an oxidizing agent (GSSG) strongly accelerates the folding rates, leading to equilibrium in less than 4 h and without significantly altering the overall chromatographic pattern (Fig. 2b). Interestingly, after 72 h all of the four reaction conditions where calcium was not present reached the same
equilibrium, with an outstanding accumulation of a fraction hereafter named Xa. To verify the effect of calcium over the folding of LA5, its oxidative folding was repeated including an excess of CaCl₂ in the reaction (Fig. 2, a and b). The presence of calcium significantly accelerates the formation of intermediates with chromatographic profiles that resemble those obtained previously in the absence of this ion. Only at the final stages of folding (8–24 h) is the RP-HPLC pattern altered with a small occurrence of Xa and an absolute predominance of the native form (N) (see below). Again, the presence of GSSG strongly increases the rate of folding, while the addition of thiol catalyst, either 2-mercaptoethanol or GSH, slightly accelerates the reaction.

Samples of the different refolding reactions were quenched at selected time points by alkylation of thiol groups with AEMTS (22) and subsequently analyzed by MS to evaluate the disulfide bond content of the intermediates (supplemental Fig. S1). The disulfide bond nature of the different fractions of intermediates arising along the folding reaction was also precisely determined by RP-HPLC purification, derivatization with AEMTS, and final MS analysis (supplemental Fig. S2). The results show a progressive accumulation of 1- and 2-disulfide species (1S and 2S ensemble) that finally leads to the formation of the 3S ensemble. The absence of calcium precludes the acquisition of N and therefore leads to the complete accumulation of non-native 3-disulfide (scrambled) forms, with Xa representing up to 50% of the total protein in Control− and Control+ and more than 65% in the oxidizing conditions. By contrast, in the presence of calcium the amount of 3-disulfide species detected by MS approximates that of native protein (N) found by RP-HPLC, with a recovery of more than 95% of N after 36 h in Control− and Control+ and after 3 h in the conditions with GSSG. The low amount of scrambled iso-mers occurring at the same time as N (see 8 h in Fig. 2a) suggests a direct formation of native LA5 from the 2S ensemble probably caused by the action of Ca²⁺. This is further confirmed by the minor effect of GSH compared with the reactions including GSSG.

**Evolution of Scrambled Isomers in the Oxidative Folding of LA5**—To assess the importance of the 3S population in the oxidative folding of LA5, the predominant species Xa was isolated by RP-HPLC and allowed to resume its folding in Tris-HCl buffer at pH 8.4 both in the absence and presence of 2-mercaptoethanol and calcium (Fig. 3). As could be expected from the results reported under “Oxidative Folding of LA5,” the absence of calcium completely precludes the formation of native form, even in the presence of thiol catalyst, which allows the rearrangement of the Xa disulfide bonds and gives rise to equilibrium with other scrambled isomers already observed during the oxidative folding reactions. The same result is obtained in the presence of calcium and absence of 2-mercaptoethanol. However, the simultaneous presence of both compounds allows the disul-
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FIGURE 3. Stop/Go folding of the Xa scrambled isomer of LA5. The major 3-disulfide species occurring in the oxidative folding of LA5 was purified by RP-HPLC, lyophilized, and allowed to re-initiate its folding in Tris-HCl buffer, pH 8.4, in the absence (Control−) and presence (Control+) of 0.25 mM 2-mercaptoethanol with and without 10 mM CaCl2. The reactions were trapped by acidification in a time course manner and subsequently analyzed by RP-HPLC. N and Xa stand for the native and scrambled forms of LA5. 3S is an ensemble of molecules comprising three disulfide bonds.

Oxidative Folding of LA5 under Physiological and Denaturing Conditions—To mimic the conditions inside the cell, the oxidative folding pathway of LA5 was also investigated at pH 7.3 in the presence of selected concentrations of NaCl and of the protein folding catalyst, protein disulfide isomerase. Because reactions without calcium are unable to render the native form, these experiments were always performed in the presence of CaCl2. Lowering the pH from 8.4 to 7.3 significantly increased the folding rate at the first stages of the process (Control− and Control+ in supplemental Fig. S3a compared with Fig. 2a) by reducing the rearrangement degree of 1- and 2-disulfide intermediates that initially funnels the folding reaction. The addition of physiological concentrations of NaCl also favors the rate of formation of disulfide intermediates, possibly by competing with non-native ionic interactions and/or by reducing electrostatic repulsions, but at the same time it seems to stabilize the Xa species, which strongly accumulates in the absence of reducing agent (supplemental Fig. S3a). In the absence of redox agents, protein disulfide isomerase is unable to function properly and the rate and efficiency of the folding reaction diminishes. This is circumvented by the addition of selected concentrations of GSSG and GSH, which promote the action of this enzyme, leading to an improved folding reaction (supplemental Fig. S3b). However, in contrast to its dramatic effect on the folding kinetics of other disulfide-rich proteins (23, 24), its influence here is rather moderate due to the calcium-promoted strong bias toward the native linkage making protein disulfide isomerase-catalyzed reshuffling reactions difficult.

Wild type LA5 was also refolded at pH 8.4 in the presence of selected concentrations of denaturant and calcium to investigate the role of non-covalent contacts on its oxidative folding. As in the folding carried out in the presence of NaCl, mild denaturing conditions (e.g. 1 M urea or 0.5 M GdnHCl) strongly accelerate the initial stages of the folding reaction (supplemental Fig. S4). This effect may be exerted thorough destabilization of non-native interactions that may hamper the adoption of the native conformation. Similarly, the presence of elevated concentrations of denaturant strongly accelerates the first steps of folding but negatively affects the final attainment of the native state by stabilizing the scrambled population. Actually, the condition including 4 M GdnSCN closely resembles the folding of the mutants that are unable to reach a native-like conformation (see next paragraph).

Oxidative Folding of LA5 Mutants—Five mutants affecting either a hydrogen-bonding network that holds the two lobes of the module together (S14A, E16K, and D32A) or the calcium binding motif (D29G and D35E) of LA5 were constructed and characterized in terms of oxidative folding. The folding experiments were always conducted at physiological pH in the presence of calcium excess. In the absence of redox agents (Control−), the RP-HPLC profiles of the five mutants are similar at the initial stages of the folding process, resembling those of the wild type form (Fig. 4). As in the case of the WT LA5, the addition of thiol catalyst (Control+) only accelerates the final stages of folding, whereas the presence of oxidizing agent strongly increases the rate of the overall folding process. Among the five mutants, S14A, E16K, and D29G seem to attain a native-like conformation (designated as N⁎). However, their folding reactions display a very low efficiency compared with the wild type, indicated by the fact that less than 60% of the protein is recovered as N⁎ after 96 h of reaction (see Control+ in Fig. 4). The rest of the protein remains trapped as a mixture of scrambled isomers that need the presence of redox agents to reshuffle their disulfide bonds and reach the native-like state (see supplemental Fig. S5). Altogether, the folding of D32A and D35E resembles that of wild type LA5 performed in the absence of calcium. None of these mutants folds into a predominant native-like conformation, even in the presence of GSSG/GSH (supplemental Fig. S5). The protein remains frozen in a mixture of scrambled isomers that mainly occur in one RP-HPLC fraction reminiscent of Xa (termed Xa⁎). Interestingly, excepting S14A, the other mutants show an increased folding rate at the beginning of the reaction as compared with that of the WT form.

Reductive Unfolding of LA5—Reductive unfolding of native LA5 (N) was examined in Tris-HCl buffer, pH 8.4, and acetate buffer, pH 4.5, using DTT and TCEP, respectively, as reducing agents (25, 26). The intermediates that arose along the unfolding reaction were trapped by acidification at various time points and subsequently analyzed by RP-HPLC. Low concentrations of DTT (0.5–1 mM) were able to reduce the native protein into a mixture of 2- and 1-disulfide species with chromatographic elution time equivalent to those of the intermediates occurring during oxidative folding (Fig. 5a). A minor accumulation of Xa was also observed due to the disulfide reshuffling that takes place at the working pH. In the presence of calcium, LA5 undergoes an almost all-or-none mechanism of unfolding with a very
low accumulation of intermediates. In addition, the amount of DTT needed to break the disulfide bonds of this protein increases enormously (>100 times) upon coordination of the calcium ion to LA5. The use of TCEP as reductant (pH 4.5) completely alters the pattern of intermediates owing to the prevalence of reductive reactions over disulfide reshuffling. The minor intermediates occurring during folding and unfolding at pH 8.4 accumulate now to a much greater extent (see supplemental Fig. S2) with similar RP-HPLC profiles observed in the absence and presence of calcium (Fig. 5b).

Unlike with the use of DTT, the degree of accumulation of intermediates increases notably with the addition of calcium. Again, the presence of this ion strongly increases the amount of TCEP required to reduce the native protein: ~10 times versus the 100 times needed in the case of DTT. This difference could be explained by the lower affinity for calcium of LA5 at acid pH (27). Interestingly, the presence of calcium also increases the accumulation of a 2S fraction eluting close to N.

Conformational Stability of LA5—The conformational stability of LA5 was assessed at pH 8.4 by disulfide scrambling (28) using 0.25 mM 2-mercaptoethanol as thiol initiator and increasing concentrations of denaturants (urea, GdnHCl, or GdnSCN). In this approach, the unfolding promoted by the denaturant is reflected by the extent of conversion of the native protein into scrambled isomers. After reaching equilibrium for 20 h the unfolding reactions were trapped by acidification and analyzed by RP-HPLC (supplemental Fig. S6a). In the presence of thiol catalyst and absence of calcium, native LA5 reshuffles to a mixture of scrambled isomers. Surprisingly, the addition of denaturing agents does not unfold the protein to a high extent; <50% of the protein is converted into scrambled forms even when using 6 M GdnSCN. This low susceptibility against denaturants sharply contrasts with the low intrinsic stability displayed by apoLA5 when measured by conventional methods (midpoint of the unfolding transition at 0.7 M GdnSCN). Disparity in stability between the two methods has been previously reported for human EGF, ribonuclease, or phospholipase A2 (29, 30). A likely explanation for this observation is that whereas conventional methods report on global unfolding, disulfide scrambling might be highly influenced by the local conformation and chemical stability of the native disulfide bonds. The presence of calcium avoids any detectable disulfide reshuffling. Overall, LA5 turns out to be one of the most stable proteins ever studied by disulfide scrambling (23). The incubation of the unfolding reactions at increasing temperatures (up to 70 °C) helps to denature the native protein into scrambled isomers that predominate in the RP-HPLC profile in the presence of 8 M GdnHCl or 6 M GdnSCN and in the absence of calcium (supplemental Fig. S6b).

A preliminary analysis of the conformation and calcium binding properties of Xa compared with those of the native form of LA5 was done by one-dimensional NMR spectroscopy. The spectra of either form in the absence of Ca2+ exhibit a clear band broadening and peak collapse at the amide region, indicative of at least partial unfolding (Fig. 6). However, whereas the spectrum of the native form in the presence of the ion displays a good signal dispersion and peak sharpness, that of Xa remains unmodified, which suggests that the scrambled isomer does not bind calcium. The presence of a structural core in the native form bound to calcium is verified by the permanence of protected residues after exchange with the solvent deuterons. The high resistance of this form against temperature is additionally

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7 X. Arias-Moreno, A. Velazquez-Campoy, J. C. Rodriguez, M. Pocovi, and J. Sancho, unpublished data.

FIGURE 4. Oxidative folding of LA5 mutants. RP-HPLC traces of the acid-trapped intermediates that accumulate along the folding of wild type (WT), S14A, E16K, D29G, D32A, and D35E LA5. The reactions were performed in Tris-HCl buffer, pH 7.3, 10 mM CaCl2, in the absence (Control−) and presence (Control+) of 0.25 mM 2-mercaptoethanol. N, N*, and R indicate the elution positions of the native, native-like, and fully reduced/denatured forms of the LA5 variants, respectively. Xa and Xa* stand for major 3-disulfide scrambled isomers of wild type and mutant LA5, respectively.
demonstrated by monitoring the one-dimensional spectra at increasing temperatures (up to 90 °C). Similar spectral patterns are observed at both 25 and 70 °C.

**DISCUSSION**

**Nonspecific Packing at the Initial Phase of LA5 Oxidative Folding**—Oxidative folding involves two major chemical events: disulfide formation (oxidation) and disulfide reshuffling (isomerization). Under typical oxidative folding conditions (Control+/H11001), the disulfide species interconvert rapidly within each disulfide ensemble by disulfide reshuffling, whereas oxidation between disulfide ensembles is relatively slow. Each disulfide ensemble can be considered to be in thermodynamic equilibrium; therefore, the ratios of disulfide species within the ensemble reflect their relative stability (31). Under these conditions, leaving entropic factors aside, non-covalent interactions shape the distribution of disulfide species in each particular ensemble. In the absence of free thiols (Control−), isomerization reactions are strongly restricted and an entropically determined quasi-stochastic distribution of disulfides is expected at the early stages of folding. The fact that the disulfide profiles of LA5 with and without free thiols are similar argues that non-covalent interactions are not important at its initial phase of folding.

In the presence of calcium, denatured and reduced LA5 folds to the native structure through a sequential oxidation of cysteine residues that give rise to equilibrated populations of 1-, 2-, and 3-disulfide species (Fig. 2 and supplemental Fig. S2). Among the 3-disulfide ensemble, the native form dominates the reaction at any time point. The presence of free thiols does not accelerate significantly the flow between the reduced/unfolded form and the 2-disulfide intermediates or change the apparent composition of the 1- and 2-disulfide ensembles. Also, no predominant population of a particular meta-stable intermediate is detected. This confirms that not even in the presence of calcium do specific non-covalent interactions actively participate in guiding LA5 folding during the early phase of packing and suggests instead that this stage is dominated by non-specific disulfide pairings that account for the absence of a predominant folding route. In agreement with this scenario, the effective concentration of individual LA5 native disulfides under physiological conditions has been shown not to differ from those found in highly denaturant environments, where non-covalent interactions are weakened or eliminated (32). In addition, LA5 variants with 49% of their side chains truncated to alanine still fold into a preferred disulfide isomer (33). Therefore, the folding landscape of LA5 at the packing stage can be envisaged as a wide and smooth funnel (Fig. 7).

**Calcium Shapes Consolidation at the Final Phase of LA5 Oxidative Folding**—The same 1-disulfide species could be detected both in the presence and absence of calcium, indicating that the ion does not govern the early stages of LA5 folding (Fig. 2 and supplemental Fig. S1). It has been previously shown that calcium does not bind to 1-disulfide natively bonded species or have any detectable impact on their conformation (32). Also, no

![FIGURE 5. Reductive unfolding of LA5.](image-url)
 significant reduction in the heterogeneity of the 2-disulfide ensemble could be observed in the presence of the ion (Fig. 2 and supplemental Fig. S1). This refutes a calcium-promoted preferential oxidation of 1-disulfide species toward the formation of native disulfides. It is known that 2-disulfide species containing the first and the second or the first and the third native disulfide bond do not bind calcium and coordination of the ion only occurs when both C-terminal disulfide bonds (C12–C30 and C24–C39) are formed (32).

In the native structure of the holoprotein, the conserved Asp-25, Asp-29, Asp-35, and Glu-36 acidic side chains of the calcium coordination site are highly buried, with solvent exposures below 30% in all cases. Burial of these residues is opposed by their polar nature and the large electrostatic repulsion that their proximity would cause. Thus, definition of the LA5 calcium-binding site involves a high kinetic barrier for the folding reaction, which probably explains why a double covalent linkage at the C-terminal lobe is required before the ion can bind the apoprotein. Formation of the two C-terminal disulfide bonds likely reduces the kinetic barrier and makes the free energy associated to calcium coordination more negative by compensating for the entropic cost of defining the calcium-binding site. Calcium coordination then funnels the folding reaction at the consolidation stage toward the native state, both thermodynamically and kinetically, by providing a high stability to the (C12–C30, C24–C39) species relative to any other 2-disulfide intermediate through the selective burial of these two disulfide bonds. In this way, formation of the last native disulfide, rather than disruption of the already stabilized disulfides, would become the most favorable fluctuation that would result in a narrow folding landscape during the final consolidation of LA5 toward the native global free energy minimum (Fig. 7).

This strong restriction in the conformational search to attain the native state should give rise to a significant increase in folding kinetics. Indeed, that calcium limits the number of intermediates and speeds up folding is clearly seen by comparison of the last stages of folding in the presence and absence of the ion (Fig. 2). Without calcium, the 3-disulfide ensemble is far more heterogeneous and no preferential population of native form is found at equilibrium. The effect of calcium in restricting the formation of 3-disulfide scrambled isomers was already noted for the LA1 module (34). The folding of LA5 in the presence of calcium can thus be dissected in two stages: an initial stage of nonspecific packing, followed by a final stage of consolidation dominated by native-like conformations.

Scrambled Isomers Are Key Intermediates in LA5 Oxidative Folding—A minor subpopulation of the 2-disulfide ensemble overcomes the preferential stabilizing effect of calcium and oxi-
izes its third disulfide bond to render a transient population of scrambled isomers. This alternative pathway is more evident in the presence of an oxidizing agent (GSSG) able to accelerate disulfide bond formation and, therefore, the flow between ensembles of intermediates. One of these scrambled isomers is Xa, which in the absence of calcium becomes the predominant 3-disulfide species in all tested conditions (Fig. 2). Surprisingly, this scrambled form represents more than 60% of the protein population at equilibrium, ten times more abundant than any other isomer. Because the ratios of disulfide species at equilibrium within the 3-disulfide ensemble reflect their relative stabilities (see above), Xa constitutes the global free energy minimum in the LA5 folding carried out in the absence of calcium. Among the small, disulfide-rich proteins studied to date, LA5 is the only one showing such predominance of a particular scrambled form. A preliminary comparison of several spectroscopic properties of Xa and native LA5 indicates that while the native form can bind calcium with the concomitant modification of both intrinsic fluorescence emission and far-UV CD, as well as one-dimensional NMR (Fig. 6), Xa displays a poorly defined structure both in the absence and presence of calcium, which indicates that it cannot bind the ion. Nevertheless, Stop/Go experiments demonstrate that the Xa scrambled isomer is not a dead-end form because it can evolve into the native conformation (Fig. 3). In the absence of a thiol catalyst Xa is completely stable, independently of the presence of calcium, and does not shift into any other conformation, confirming that it is unable to coordinate the ion. In the presence of thiols and absence of calcium, the reshuffling of disulfides results in a rapid equilibrium in which the distribution of scrambled species is very similar to that obtained when the reaction begins from the reduced/unfolded protein. This indicates that the reshuffling reactions dominate the last stages of LA5 folding in the absence of calcium. In the presence of calcium and thiols Xa renders scrambled forms, whose initial distribution is very similar to that found in the absence of the ion except for the fact that small amounts of native LA5 are already detected. A progressive formation of native form occurs at the expense of Xa, confirming that reshuffling gives rise to a species with the native disulfide connectivity and able to coordinate calcium. However, the possibility that a scrambled isomer may bind calcium and reshuffle into the native protein, constituting an alternative folding route, cannot be excluded. The high stability of the holoprotein, relative to any other isomer unable to bind the ion, promotes its accumulation at equilibrium where it accounts for >98% of the total protein.

What is the biological significance, if any, of the remarkable accumulation of Xa in the absence of calcium and its almost complete conversion into the native form in its presence? Most disulfide-rich proteins studied so far consist of a single, globular domain. In the LDLR, however, the existence of seven LA modules and three EGF-like domains, each containing three disulfides, implies that the folding process toward the functional receptor must involve the formation of 30 native disulfide bonds (35). This means that, in principle, a myriad of non-native disulfide bonds between cysteines of different domains could be formed. It has been shown that during the synthesis of the receptor the LA1 repeat, which is translated first, apparently gains its native conformation in the last stages of folding (11). The formation of a stable scrambled form, like Xa, soon after the synthesis would be an efficient way to kidnap cysteines, thus avoiding wrong interactions with free cysteines.

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from other domains during folding. Supporting this view, it has been reported that the folding of the modules of LA pairs occurs independently, even if mutations in one of the partners prevent the coordination of calcium (12).

**Calcium Coordination Hinders LA5 Reductive Unfolding—** The stability of an individual disulfide bond against reduction is supported by its adjacent structure. An extremely high concentration of DTT is required to reduce LA5 in the presence of calcium, indicating that its coordination promotes an effective burial of cysteines that hinders the access of reducing agents (Fig. 5). Accordingly, in the native structure C5, C12, C17, C30, and C39 expose <25% of their surface to solvent. The high sensitivity of LA5 toward DTT in the absence of calcium confirms that the native disulfide bonds are not sufficient to maintain a compact, globular structure, in agreement with previous computer simulations (36) and NMR experiments (Fig. 6). At acid pH, reductive reactions prevail over disulfide reshuffling, thus preventing the formation of intermediates containing non-native disulfide bonds. Under this condition a significant accumulation of highly resistant folding intermediates occurs, some of them with native-like mobility, especially in the presence of calcium (Fig. 5). This indicates that even if calcium does not drive the formation of specific disulfides, once the native structure is acquired it hampers their sequential reduction. This stabilizing effect on partially oxidized folding intermediates dissipates when intra-molecular reshuffling is allowed, i.e., at pH 8.4, because the coordination site geometry is lost, resulting in an almost all-or-none reduction process with little accumulation of intermediates.

**FH-linked Mutations Reshape LA5 Oxidative Folding—** The mutants analyzed in this work can be classified in two groups: those resulting in elimination or misplacement of direct calcium ligands (D29G and D35E) and those promoting the removal of the hydrogen-bonding network connecting the N- and C-terminal lobes of LA5 (S14A, E16K, and D32A). In the LDLR mutation data base of the University College in London D29G appears annotated as displaying <2% LDLR activity, E16K as displaying 5–15% activity, and D32A and D35E as probably damaging. We have additionally expressed and characterized the mutation S14A because it modifies the hydrogen-bonding network in LA5, but, as far as we know, the arising of this mutation in humans has not been reported. Surprisingly, with the exception of S14A, all other changes promote an acceleration of the initial stages of folding, resulting in the rapid accumulation of folding intermediates (Fig. 4). Thus, folding defects are already apparent at the packing stage, before the binding of calcium. Most mutations imply the substitution of an acidic residue, suggesting that ionic interactions and/or electrostatic repulsion might play a relevant role during LA5 packing.

The D35E mutation at the calcium-binding site strongly influences the folding process, abolishing the formation of the native structure and leading to a predominant accumulation of a Xa-like scrambled isomer (Fig. 4 and supplemental Fig. S5). Unexpectedly, an apparently more drastic mutation of the Asp-29 calcium ligand to glycine allows the formation of a predominant native-like form, which suggests that this mutant may still be able to coordinate calcium. For this mutant, in the absence of thiols, a Xa-like isomer dominates the folding reaction at equilibrium, indicating that, even if the mutant can bind calcium, the affinity is probably low and the cation cannot completely funnel the folding reaction toward the native state. However, the native-like form of the mutant is still more stable than the Xa-like isomer, because when reshuffling is allowed the native-like structure becomes the most populated species.

Truncation of the non-coordinating but well conserved Asp-32 side chain to alanine completely abolishes the attainment of a native conformation and results in a predominant accumulation of a Xa-like form, confirming that Asp-32 is essential for LA5 calcium-dependent folding. The reason for this could simply be the structural importance of the integrity of the hydrogen-bonding network formed by Ser-14, Glu-16, and Asp-32 that connects the two lobes of LA5. An alternative explanation can be proposed based on a study of the related cellular receptor of Rous sarcoma virus that suggests that LA domains can use two slightly different calcium-binding sites (37). One of them would include the canonical side chains of Asp-25, Asp-29, Asp-35, and Glu-36, whereas in the other one Asp-32 would replace Asp-29 at the coordination site. We notice that this explanation is consistent with the observed behavior of the D29G mutant that appears to be able to attain a native-like structure and to bind calcium (see above). The capability of reshaping a mutated calcium-binding site using alternative ligands would obviously be advantageous. However, more work is needed to test this possibility.

Ser-14 and Glu-16 are a pair of residues also involved in the hydrogen-bonding network connecting the two lobes of LA5. They are not conserved among LA domains, and no significant co-variation has been identified in the SMART family alignment (data not shown). It has been reported that their simultaneous replacement by alanines gives rise to a folding-defective LA variant (33). We show here that their individual truncation suffices to significantly alter the folding reaction of the module by strongly modifying the distribution of LA5 species at equilibrium, so that when isomerization is impeded scrambled isoforms accumulate to a high extent. However, when isomerization is allowed, the native form clearly becomes the most stable/abundant species, which suggests that both mutants might be able to bind calcium, albeit with lower affinity than the wild type as judged by the remarkable accumulation of the Xa-like isomer. Work is in progress to determine the affinity of these mutants and of D29G for calcium.

Comparison of the folding reaction of wild type LA5 and FH-related mutants indicates that the latter display a reshaped folding landscape where reduced stability of the native state turns Xa-like scrambled isoforms into strong kinetic traps of the folding reaction (Fig. 7). Goldstein and co-workers (38) demonstrated that a misfolded LA5 module does not significantly affect LDLR processing, allowing the mutated receptor to be translocated to the cell surface with reasonable efficiency even if it then displays reduced lipoprotein binding activity. As discussed above, we propose that the formation of stable scrambled isoforms (Xa and alike) avoids the exposition of highly reactive cysteines in the misfolded module and could contribute to the proper processing and transport of mutated LDLRs by minimizing long-range effects of the mutations, thus reducing their
negative impact on the function of the modular LDLR. Overall, the results presented in this work provide valuable insights into the specific sequential and structural determinants shaping the folding pathway of LA domains. Because many FH mutations affect the folding of these modules, it is anticipated that chemical chaperones that selectively target these folding defects might become useful compounds for tackling this genetic disorder.

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REFERENCES
1. Beglova, N., and Blacklow, S. C. (2005) Trends Biochem. Sci 30, 309–317
2. Blacklow, S. C. (2007) Curr. Opin. Struct. Biol. 17, 419–426
3. Hobbs, H. H., Russell, D. W., Brown, M. S., and Goldstein, J. L. (1990) Annu. Rev. Genet. 24, 133–170
4. Gent, J., and Braakman, I. (2004) Cell Mol. Life Sci. 61, 2461–2470
5. Fass, D., Blacklow, S., Kim, P. S., and Berger, J. M. (1997) Nature 388, 691–693
6. North, C. L., and Blacklow, S. C. (2000) Biochemistry 39, 2564–2571
7. Atkins, A. R., Brereton, I. M., Kroon, P. A., Lee, H. T., and Smith, R. (1998) Biochemistry 37, 1662–1670
8. Atkins, A. R., Brereton, I. M., Kroon, P. A., Lee, H. T., and Smith, R. (1998) Biochemistry 37, 1662–1670
9. Koduri, V., and Blacklow, S. C. (2001) Biochemistry 40, 12801–12807
10. North, C. L., and Blacklow, S. C. (2000) Biochemistry 39, 2564–2571
11. Jansens, A., van Duijn, E., and Braakman, I. (2002) Science 298, 2401–2403
12. North, C. L., and Blacklow, S. C. (2000) Biochemistry 39, 13127–13135
13. Beglova, N., North, C. L., and Blacklow, S. C. (2001) Biochemistry 40, 2808–2815
14. North, C. L., and Blacklow, S. C. (1999) Biochemistry 38, 3926–3935
15. Blacklow, S. C., and Kim, P. S. (1996) Nat. Struct. Biol. 3, 758–762
16. Creighton, T. E. (1997) Biol. Chem. 378, 731–744
17. Wedemeyer, W. J., Welker, E., Narayan, M., and Scheraga, H. A. (2000) Biochemistry 39, 4207–4216
18. Arolas, J. L., Aviles, F. X., Chang, J. Y., and Ventura, S. (2006) Trends Biochem. Sci. 31, 292–301
19. Chang, J. Y. (2008) Antioxid. Redox Signal. 10, 171–178
20. Weissman, J. S., and Kim, P. S. (1991) Science 253, 1386–1393
21. Welker, E., Narayan, M., Wedemeyer, W. J., and Scheraga, H. A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2312–2316
22. Iwaoka, M., Juminaga, D., and Scheraga, H. A. (1998) Biochemistry 37, 4490–4501
23. Arolas, J. L., Bronsoms, S., Ventura, S., Aviles, F. X., and Calvete, J. J. (2006) J. Biol. Chem. 281, 22906–22916
24. van den Berg, B., Chung, E. W., Robinson, C. V., Mateo, P. L., and Dobson, C. M. (1999) EMBO J. 18, 4794–4803
25. Chang, J. Y., Li, L., and Bulychev, A. (2000) J. Biol. Chem. 275, 8287–8289
26. English, B. P., Welker, E., Narayan, M., and Scheraga, H. A. (2002) J. Am. Chem. Soc. 124, 4995–4999
27. Simonovic, M., Dolmer, K., Huang, W., Strickland, D. K., Volz, K., and Gettins, P. G. (2001) Biochemistry 40, 15127–15134
28. Chang, J. Y. (1997) J. Biol. Chem. 272, 69–75
29. Lu, B. Y., Jiang, C., and Chang, J. Y. (2005) Biochemistry 44, 15032–15041
30. Singh, R. R., and Chang, J. Y. (2004) Biochem. J. 377, Pt. 3, 685–692
31. Welker, E., Wedemeyer, W. I., Narayan, M., and Scheraga, H. A. (2001) Biochemistry 40, 9059–9064
32. Atkins, A. R., Brereton, I. M., Kroon, P. A., Lee, H. T., and Smith, R. (1998) Biochemistry 37, 1662–1670
33. Koduri, V., and Blacklow, S. C. (2001) Biochemistry 40, 12801–12807
34. Atkins, A. R., Brereton, I. M., Kroon, P. A., Lee, H. T., and Smith, R. (1998) Biochemistry 37, 1662–1670
35. Jeon, H., and Blacklow, S. C. (2005) Annu. Rev. Biochem. 74, 535–562
36. Cuesta-Lopez, S., Falo, F., and Sancho, J. (2007) Proteins 66, 87–95
37. Guo, Y., Yu, X., Rihani, K., Wang, Q. Y., and Rong, L. (2004) J. Biol. Chem. 279, 16629–16637
38. Russell, D. W., Brown, M. S., and Goldstein, J. L. (1989) J. Biol. Chem. 264, 21682–21688