Mechanism of Low Density Lipoprotein (LDL) Release in the Endosome

IMPLICATIONS OF THE STABILITY AND Ca²⁺ AFFINITY OF THE FIFTH BINDING MODULE OF THE LDL RECEPTOR*

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Uptake of low density lipoproteins (LDL) by their receptor, LDLR, is the primary mechanism by which cells incorporate cholesterol from plasma. Mutations in LDLR lead to familial hypercholesterolemia, a common disease affecting 1 in 500 of the human population. LDLR is a modular protein that uses several small repeats to bind LDL. The repeats contain around 40 residues, including three disulfide bonds and a calcium ion. Repeat 5 (LR5) is critical for LDL and β-migrating very low density lipoprotein binding. Based on the crystal structure of LDLR at endosomal pH (but close to extracellular calcium concentration), LR5 has been proposed to bind to the epidermal growth factor (EGF) precursor domain of LDLR in the endosome, thus releasing the LDL particles previously bound in extracellular conditions. We report here the conformational stability of LR5 as a function of temperature and calcium concentration under both extracellular and endosomal pH conditions. The repeat was very stable when it bore a bound calcium ion but was severely destabilized in the absence of calcium and even further destabilized at acidic versus neutral pH. The temperature and calcium concentration dependence of LR5 stability clearly indicate that under endosomal conditions the unfolded conformation of the repeat is largely dominant. We thus propose a new mechanism for LDL release in the endosome in which calcium depletion and decreased stability at acidic pH drives LR5 unfolding, which triggers LDL release from the receptor. Subsequent binding of LR5 to the EGF precursor domain, if it takes place at low calcium concentrations, would contribute to a further shifting of the equilibrium toward dissociation.

Most plasma cholesterol in humans is transported by low density lipoprotein (LDL) (1). LDL receptors (LDLR) in cell membranes bind LDL, and the complexes enter the cell by endocytosis (2–5). At the acidic pH of the endosome, LDL is released allowing LDLR recycling to the membrane (1). Although LDLR was initially thought to play the single role of helping to achieve cholesterol homeostasis, its expression in neurons suggests that it may also perform other roles (6, 7). LDLR gives its name to the LDL receptor family of transmembrane receptors that additionally comprises the low density lipoprotein receptor-related protein and the very low density lipoprotein receptor among others (8). This family of receptors is involved in a variety of important biological functions related to cell uptake and signal transduction (9, 10).

LDLR (11–13) is a transmembrane protein containing 839 amino acid residues organized in five domains: ligand binding, EGF precursor homologous, glycosylated, transmembrane, and cytoplasmatic (1, 14–16). The ligand-binding domain, consisting of seven tandem, structurally-homologous repeats, is responsible for binding lipoproteins (17–21); the EGF precursor domain participates in receptor recycling to the cell surface and, together with the binding domain, in lipoprotein release in the endosome (18, 22–27). Of the seven repeats of the binding domain, repeat 5 (LR5) is particularly important for LDL binding (17, 18) and release (24, 28, 29). LR5 contains 40 residues, including six cysteines that form three disulfide bridges, and four acidic residues, Asp-196, Asp-200, Asp-206, and Glu-207, involved in the binding of a structural calcium ion (20, 30–32). A β-hairpin at the N terminus is the only element of secondary structure in LR5, which additionally contains a small hydrophobic core formed by one isoleucine and one phenylalanine residue. The isolated LR5 repeat exhibiting the correct disulfide pattern is stable in the presence of Ca²⁺ (33), but when the Ca²⁺ ion is removed, it adopts a non-native apoLR5 conformation (33). The structural and functional importance of the Ca²⁺ ion is further stressed by the fact that recombinant LR5 cannot form native disulfide bonds in the absence of Ca²⁺ (33) and that

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4 The abbreviations used are: LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; LR5, fifth repeat of the ligand-binding domain from the LDL receptor; EGF, epidermal growth factor; FH, familial hypercholesterolemia; apoLRS, fifth repeat of the ligand-binding domain of the LDL receptor deprived of calcium; RP-HPLC, reversed-phase high performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; GdnSCN, guanidine thiocyanate; PIPES, piperazine-N,N′-bis(ethanesulfonic acid); MES, 2(N-morpholino)ethanesulfonic acid; ITC, isothermal titration calorimetry, MOPS, 4-morpholinepropanesulfonic acid; DSC, differential scanning calorimetry; LR1, the first repeat of the ligand-binding domain from the LDL receptor.
LDL binding is impaired at low Ca\(^{2+}\) concentrations (34). Many mutations of the LDLR gene have been described that lead to FH, an autosomal, dominant disease (35). Although FH homozygotes are very unusual and die at an early age, FH heterozygotes abound, representing 0.2% of the human population (36). It is well established that many LDLR mutations observed in patients with FH cause a local unfolding of the receptor, which compromises its correct functioning (3, 37, 38). Understanding LDLR function at the molecular level may help fight FH.

LDL release is essential for receptor recycling; a release mechanism has been proposed that is based on the x-ray structure of the receptor at endosomal pH (29). According to this mechanism, the EGF precursor domain of LDLR becomes, at acidic endosomal pH, a better ligand of the N-terminal binding domain than LDL, thus enforcing the release of the lipoprotein particle from the receptor (24, 29). It is, however, unclear at present whether the EGF precursor domain participates actively in the mechanism of LDL release or rather sequesters the N-terminal binding domain once LDL is released from the receptor. It should be mentioned that, despite the wealth of structural and functional information available on LR5 (20, 31–33), the conformational stability of this repeat has not yet been quantitated. There are good reasons for doing so because, protein stability being generally low, protein function is quite sensitive to point mutations, pH or temperature modifications, or ligand concentration. In this respect, the changes in pH and Ca\(^{2+}\) concentration experienced by the endosome relative to the initial extracellular conditions that allow LDL binding to LDLR could significantly modify the stability of LR5 or of other binding repeats in the receptor so as to influence LDL release by simply reducing the stability of the LDL:LDLR complex.

We have recently reported the oxidative folding pathway of LR5 (39). Using a combination of calorimetric and spectroscopic techniques, we report here the conformational stability of LR5 as a function of Ca\(^{2+}\) concentration and temperature at both extracellular and endosomal pH, which sheds light on the mechanisms involved in receptor recycling. The stability phase diagrams of LR5 in endosomal and extracellular conditions indicate that the lower stability of LR5 at low pH and low Ca\(^{2+}\) concentrations triggers its unfolding in the endosome, thus providing a mechanism for LDL release from the receptor.

**EXPERIMENTAL PROCEDURES**

**Cloning and Expression**—The plasmid pLDLR2, containing the full-length cDNA of LDLR was used as template to PCR-amplify the fragment corresponding to LR5, using the Expand high fidelity PCR Kit (Roche Applied Science) and to clone it into a pGEM-T plasmid (Promega). The following primers, which introduce flanking XhoI and BamHI restriction sites, were used: 5'-GGATCCACAGGGCGGTGCTCGCCT-3' and 5'-CTCGAGCTAAAGCGCATTTTCTTCGTC-3'. After cloning, the sequence of LR5 was confirmed, and the fragment was cloned into the expression vector pGEX-4T-3 (Amersham Biosciences) from which LR5 can be expressed as a protein fused with glutathione-sulfhydryl transferase from *Schistosoma japonicum*. To that end, the purified pGEX-4T-3 plasmid, containing the LR5 sequence, was introduced into competent *Escherichia coli* BL21 cells, and transformed colonies were selected in agar plates containing 100 μg/ml ampicillin. Typically, 11-liter cultures in LB medium were prepared in Erlenmeyer flasks, and 0.5 mM isopropyl-1-thio-β-D-galactopyranoside was used to induce expression of the fusion protein.

**LR5 Purification**—*E. coli* BL21 cells were resuspended in 50 mM Tris, pH 8, 150 mM NaCl, and 2 mM CaCl\(_2\) and sonicated. After centrifugation, the supernatant was loaded onto an affinity column (GSTPrep FF16/10, Amersham Biosciences) from which the fusion protein was purified with high yield. To release LR5, the fusion protein was cleaved with bovine thrombin from Sigma (10 units of thrombin/mg of GSTLR5). The N-terminal residue of the LR5 thus released is glycine instead of the wild-type aspartate. After cleavage, LR5 was refolded under conditions permitting disulfide exchange by exhaustive dialysis at 4 °C against 50 mM Tris buffer, pH 8, containing 10 mM CaCl\(_2\), 2 mM cysteine, and 0.5 mM cystine. LR5 was finally purified by RP-HPLC on a C18 preparative column (Symmetry columns) using a 0–100% acetonitrile gradient in 0.1% aqueous orthophosphoric acid. Protein elution was monitored by absorbance at 280 nm, and the purity of the repeat was determined by electrophoresis (PhastGel System, GE Healthcare) with silver staining (Amersham Biosciences). A single, strong band corresponding to LR5 was obtained, indicating that the repeat was pure (99%). MALDI-TOF MS using a Bruker Ultraflex confirmed the purity of the isolated protein. To determine the uniqueness of the disulfide pattern, analytical RP-HPLC on a C18 column (Vydac) was performed using a 15–35% acetonitrile gradient in 0.1% aqueous orthophosphoric acid. Protein elution was monitored by absorbance at 280 nm, and the purity of the repeat was determined by electrophoresis (PhastGel System, GE Healthcare) with silver staining (Amersham Biosciences). A single, strong band corresponding to LR5 was obtained, indicating that the repeat was pure (99%). MALDI-TOF MS using a Bruker Ultraflex confirmed the purity of the isolated protein. To determine the uniqueness of the disulfide pattern, analytical RP-HPLC on a C18 column (Vydac) was performed using a 15–35% acetonitrile gradient in 0.1% aqueous orthophosphoric acid. Under these conditions a single peak was obtained, which was verified by MALDI-TOF MS (39) to contain three disulfide bridges. The concentration of LR5 was determined using the theoretical extinction coefficient (40) ε\(_{280}\) = 6050 M\(^{-1}\) cm\(^{-1}\).

**Spectroscopic Characterization of LR5 under a Variety of Solution Conditions**—Fluorescence and circular dichroism (CD) spectra were acquired to search for spectroscopic differences between the folded, thermally unfolded (80 °C), and GdnSCN (3 M) unfolded apoLR5 in the presence and absence of calcium. To mimic extracellular conditions, a 10 mM PIPES buffer with 150 mM NaCl was used, and for endosomal conditions, 10 mM MES, pH 5.5, with 150 mM NaCl was used. Typically, apoLR5 was obtained by adding 1.5 mM EDTA to the protein solution, and the calcium-bound form was obtained by adding 10 mM CaCl\(_2\).

Fluorescence emission spectra (300–450 nm, with excitation at 280 nm) were acquired in a thermostated Amino-Bowman series 2 spectrophotometer (Spectronic Instruments) using 2 μM LR5 samples. Far-UV CD spectra were acquired from 260 to 195 nm in a 1-mm-path length cuvette with 60 μM LR5, and near-UV CD spectra were recorded from 325 to 245 nm in a 40-mm-path length cuvette using 115 μM LR5. All CD spectra were recorded in a thermostated Chirascan (Applied Photophysics).

**GdnSCN Denaturation**—Chemical denaturation curves of apoLR5 were obtained using GdnSCN (Fluka) as the denaturant, and these were followed by tryptophan fluorescence emission. Typically, 1.5 μM LR5 samples in 10 mM PIPES, 150 mM NaCl, 1.5 mM EDTA, pH 7.0, plus different GdnSCN concen-
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trations from 0 to 6 M were prepared, and their fluorescence was measured at 9.5, 15.5, and 25.0 °C. The unfolded curves were fitted to a two-state equation (Equation 1) (41, 42),

\[ F = F_N + m_N D + (F_D + m_D D)e^{-\Delta G/RT} \]

where \( F \) represents the observed fluorescence signal, \( D \) is the molar concentration of GdmSCN, and \( F_N \) and \( F_D \) refer, respectively, to the fluorescence signals of native and denatured LR5, which are assumed to vary linearly with denaturant concentration with slopes \( m_N \) and \( m_D \). In Equation 1, the free energy is given by

\[ \Delta G = \Delta G_w - mD \]  

(Eq. 2)

where \( \Delta G_w \) is the free energy of unfolding in the experimental buffer without denaturant.

Thermal Denaturation Followed by Spectroscopic Characterization—Thermal denaturation curves of apoLR5 at pH 7.0 and 5.5 (in the buffers described above, with 1.5 mM EDTA) were followed by far-UV CD, near-UV CD, and tryptophan fluorescence emission. The curves were recorded from 5 to 90 °C at a scan rate of 1.5–2.0 °C/min. LR5 concentrations, wavelengths, and path lengths were 60 μM, 227.5 nm, and 1 mm (far-UV CD); 115 μM, 267.5 nm, and 0.4 cm (near-UV CD); and 2 μM, 350 nm (excitation at 280 nm), and 1 cm (fluorescence emission). Unfolding curves were fitted (43, 44) to a two-state equation (Equation 3),

\[ F = F_N + m_N T + (F_D + m_D T)e^{-\Delta G/RT} \]

(Eq. 3)

where \( F \) is the spectroscopic signal, \( T \) is absolute temperature, and \( F_N \) and \( F_D \) refer, respectively, to the spectroscopic signals of native and denatured LR5 at a reference temperature \( T_0 \) and are assumed to vary linearly with temperature with slopes \( m_N \) and \( m_D \).

In Equation 3, \( \Delta G \), free energy of the unfolding, is given by

\[ \Delta G = \Delta H(1 - T/T_m) + \Delta C_p (T - T_m) - T \ln(T/T_m) \]

(Eq. 4)

where \( T_m \) is the melting temperature, and \( \Delta C_p \) and \( \Delta H \) are the heat capacity and enthalpy changes, respectively, at the melting temperature.

Thermal Denaturation Followed by Differential Scanning Microcalorimetry—The heat capacity of LR5 was measured as a function of temperature at pH 7 and 5.5 in a differential scanning VP-DSC MicroCalorimeter (Microcal LLC, Northampton, MA). Protein samples and reference solutions were degassed and carefully loaded into the cells to avoid bubble formation. The base line of the instrument was routinely recorded with both cells filled with buffer before the experiments were performed. To reach the conditions of the experiments, LR5 samples were lyophilized, resuspended, and passed through a PD-10 column (Amersham Biosciences). Thermal denaturation scans were performed with 400 μM protein solutions in either pH 7.0 or 5.5, with 150 mM EDTA or 1.5 mM CaCl₂ when required. Experiments were carried out at a scanning rate of 1 °C/min from 10 to 100 °C. The reversibility of unfolding, as checked by sample reheating after cooling inside the calorimetric cell, was higher than 80%. LR5 denaturation in the absence of calcium was analyzed assuming a two-state transition model. The excess average unfolding enthalpy is given by

\[ \langle \Delta H(T) \rangle = F_0(T)(\Delta H(T) + F_0 \Delta H_0(T)) \]

(Eq. 5)

where \( \Delta H(T) \) is the unfolding enthalpy at temperature \( T \), and \( F_0(T) \) is the fraction of unfolded protein molecules, which is governed by Equation 4. The temperature derivative of the excess average unfolding enthalpy equals the excess heat capacity, which is the direct observable when measured with the calorimeter.

LR5 denaturation in the presence of calcium was analyzed assuming a two-state transition coupled to calcium dissociation. Total protein and ligand concentrations were

\[ [P] = [N] + [U] + [NL] = (1 + K) [N] + K_L [N][L] \]  

(Eq. 6)

\[ [L] = [L] + [NL] = [N] + K_L [N][L] \]  

(Eq. 7)

where \([N],[NL],\) and \([U]\) are the concentrations of free native, bound native, and unfolded protein, \([L] \) is the concentration of free ligand, \( K \) is the unfolding equilibrium constant, and \( K_L \) is the ligand binding equilibrium constant, which is temperature-dependent according to

\[ K_L(T) = K_L(T_0) \exp \left( \frac{-\Delta H_L(T_0)}{RT} \left( 1 - \frac{T}{T_0} \right) \right) \]

(Eq. 8)

where \( K_L(T_0) \) and \( \Delta H_L(T_0) \) are the binding constant and the binding enthalpy, respectively, at reference temperature \( T_0 \) (usually 25 °C) and \( \Delta C_{p_L} \) is the change in heat capacity upon binding. The concentration of any species at any temperature is calculated for any total concentration of protein and ligand from the temperature dependence of the equilibrium constants for unfolding and binding, \( K_L(T) \), by solving Equations 6 and 7.

On the other hand, the excess average enthalpy in the presence of ligand is as follows (45),

\[ \langle \Delta H(T) \rangle = F_0(T)(\Delta H(T) + F_0 \Delta H_0(T)) \]

(Eq. 9)

where \( F_0 \) is the fraction of protein molecules bound to ligand. The temperature derivative of the excess average enthalpy equals the excess heat capacity, which is the direct observable when measured with the calorimeter.

Calcium Binding to LR5 Followed by Isothermal Titration Calorimetry—Isothermal calorimetry experiments (ITC) were performed in a VP-ITC MicroCalorimeter (Microcal) in the presence of a slight excess of EDTA (46). Purified LR5 solutions typically carry free calcium coming from impurities in the buffer solution. Complete removal of calcium from the protein solution using a Chelex resin was attempted, but it was not successful and led to protein aggregation. Therefore, titration experiments were performed in the presence of a chelator (EDTA) (46) because the affinity of EDTA for calcium is higher than that of LR5, in a typical ITC experiment with excess EDTA, injected calcium binds first to EDTA, and once EDTA is saturated, titration of apoLR5 begins. As described (47), cal-
cium binding to EDTA is coupled to the release of one proton, and, consequently, the binding affinity is highly dependent on pH. In addition, the binding enthalpy is dependent on pH and on the buffer ionization enthalpy. A similar behavior was expected for the binding of calcium to LR5. Because the purpose here was to determine the calcium binding affinities at extracellular and endosomal pH values, the enthalpy values reported are the observed buffer-dependent enthalpies of binding under the conditions assayed, and determination of buffer-independent binding enthalpies will be left for future work.

After degassing all solutions, the experiments were carried out with 15 μM LR5 in a 10 mM solution of the appropriate buffer containing 150 mM NaCl and EDTA. The buffers used were: at pH 7, PIPES (ionization enthalpy: 2.74 kcal/mol); and at pH 5.5, sodium acetate (ionization enthalpy: 0.12 kcal/mol). To estimate the heat capacity change upon binding, experiments were carried out at different temperatures. To check the performance of the method, thermodynamic parameters for the binding of calcium to EDTA were determined under the same conditions. They were in agreement with data described previously (47) and were used in the analysis performed to determine the parameters for the binding of calcium to LR5.

In titration experiments of LR5 in the presence of EDTA, two coupled binding equilibria were considered,

$$M + L \leftrightarrow ML$$  \hspace{1cm} (Eq. 10)
$$Q + L \leftrightarrow QL$$  \hspace{1cm} (Eq. 11)

where M refers to the macromolecule (LR5), Q to the chelator (EDTA), and L to the ligand (calcium). From the mass balances of macromolecule, chelator and ligand, the total concentration of ligand can be written as

$$[L] = [L] + [M] \frac{K_m[L]}{1 + K_m[L]} + [Q] \frac{K_Q[L]}{1 + K_Q[L]}$$  \hspace{1cm} (Eq. 12)

where $K_m$ and $K_Q$ are the binding constants of ligand to the macromolecule and chelator, respectively. $K_m$ is the association constant $K_f$ from the differential scanning calorimetry (DSC) analysis. The last two terms in Equation 12 represent the concentration of ligand bound to the macromolecule and chelator, respectively. The equation can be solved numerically and allows the concentration of each complex to be calculated. Because the heat evolved per injection is proportional to the variation of $[ML]$ and $[QL]$ and the corresponding molar binding enthalpies, the observed heat effect, $q_i$, associated with any injection (i) is given by

$$q_i = V \left( \frac{\Delta H_m([ML]_{-i}) - (1 - v/V)[ML]_{-i}}{\Delta H_m([ML]_{-i})} - \frac{\Delta H_Q([QL]_{-i}) - (1 - v/V)[QL]_{-i}}{\Delta H_Q([QL]_{-i})} \right)$$  \hspace{1cm} (Eq. 13)

where $V$ is the volume of the calorimeter cell, $v$ is the injection volume, and $\Delta H_m$ and $\Delta H_Q$ are the enthalpies for the binding of L to M or to Q, respectively. The thermodynamic parameters for calcium binding to EDTA are: $K_Q = 1.3 \times 10^7$ M$^{-1}$, $\Delta H = -3.6$ kcal/mol for pH 7, and $K_Q = 8.8 \times 10^4$ M$^{-1}$, $\Delta H = 1.8$ kcal/mol for pH 5.5. These values were used in the nonlinear regression for analysis of the calcium-LR5 binding assays (Equations 12 and 13).

Calcium Binding to ApoLR5 Followed by Fluorescence Emission—Changes in fluorescence emission of the single tryptophan in LR5 (Trp-22) were followed after excitation at 280 nm in a thermostated Aminco-Bowman series 2 spectrophotometer (Spectronic Instruments) 2 μM LR5 solutions in buffer (10 mM PIPES, MOPS, or imidazole, pH 7.0, plus 150 mM NaCl; or 10 mM acetate or MES, pH 5.5, plus 150 mM NaCl) with excess EDTA at either pH were used. 5-μl injections of a 100 μM CaCl$_2$ solution were made. The changes observed in emission intensity after ligand injection were proportional to the concentration of the LR5-Ca$^{2+}$ complex according to

$$F = F_0 + \epsilon[ML]$$  \hspace{1cm} (Eq. 14)

where $F_0$ is the emission intensity at zero total ligand concentration and $\epsilon$ is the emission coefficient. In fluorescence titration experiments of LR5 in the presence of EDTA, two coupled binding equilibria (Equations 10 and 11) were considered.

RESULTS AND DISCUSSION

Spectroscopic Characterization of ApoLR5 and LR5-Ca$^{2+}$ under Native and Denaturing Conditions—Protein stability can be determined conveniently by exploiting the differences in spectroscopic properties of the protein conformations involved. The near-UV fluorescence emission signal of LR5 arises from its single tryptophan. The emission spectra of apoLR5 at pH 7.0 and 5.5 (Fig. 1, a and d, respectively) display maxima around 354 nm, consistent with a solvent-accessible environment for the tryptophan residue, as seen in the x-ray structure (20). Calcium binding to apoLR5 increases emission intensity at both pH 7.0 and 5.5 without changing the maxima. Similar effects have been reported for the binding of calcium to two complement-like repeats from LDLR-related protein (48).

In contrast, denaturation of apoLR5 at pH 7.0, by either increasing the temperature or adding GdnSCN, lowers emission intensity. At pH 5.5, temperature denaturation also lowers emission intensity, whereas GdnSCN denaturation produces no significant change. The far-UV CD emission spectra of apoLR5 at both pH 7.0 and 5.5 (Fig. 1, b and e) display maxima around 227 nm and minima around 202 nm. Upon calcium binding, the absolute signal of both peaks increases, but it is almost lost upon thermal denaturation. On the other hand, the near-UV CD emission spectra of apoLR5 at either pH show minima around 267 nm (Fig. 1, c and f), the intensity of which greatly increases upon Ca$^{2+}$ binding. Thermally and chemically denatured apoLR5 shows almost no near-UV CD signal at either pH. Overall, the effects of calcium in the far- and near-UV CD spectra are reminiscent of those reported for LR1 (21, 49).

Although the small size of LR5, its low secondary structure content, and the fact that it displays a single solvent-exposed tryptophan residue would not encourage a priori the use of simple spectroscopic techniques to investigate its conformational dynamics, it is clear from the analyses given above that both the unfolding and the calcium binding equilibria can be monitored using intrinsic fluorescence emission and circular dichroism (in both the near- and far-UV regions).

Low Intrinsic Stability of apoLR5 Further Decreased at Acidic pH—The thermal unfolding curves of apoLR5 followed by fluorescence and near-UV and far-UV CD at pH 7.0 and 5.5 are
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shown in Figs. 2 and 3, respectively, together with the calculated fractions of native protein. Heating fluorescence and near-UV CD curves at pH 7.0 coincide with the corresponding re-heating curves recorded after cooling (not shown), and no protein concentration dependence of the thermodynamic parameters is observed (i.e. same $T_m$ for fluorescence at 2 μM and DSC at 400 μM; Table 1). The thermal unfolding of apoLR5 is therefore fully reversible, and the repeat remains monomeric in the range of protein concentrations used.

For each pH (7.0 and 5.5), fluorescence and far-UV and near-UV CD thermal unfolding curves were individually and globally fitted to the two-state model. The $T_m$ and $\Delta H$ values obtained for each of the individual curves and those derived from their global fit are very similar (Table 1). In addition, the DSC thermograms shown in Fig. 4 were fitted to the two-state model, and the $T_m$ and $\Delta H$ values obtained also coincide with those determined spectroscopically (Table 1). The spectroscopic and calorimetric analyses together provide strong evidence that apoLR5 thermal unfolding follows a simple two-state mechanism, indicating that apoLR5 is more stable at extracellular pH ($T_m = 41 ^\circ C$) than at endosomal pH ($T_m = 37 ^\circ C$). The Gibbs energy of stabilization for apoLR5 at 25 °C is 0.84 and 0.5 kcal/mol, at pH 7 and 5.5, respectively; at 37 °C it is 0.24 and 0 kcal/mol, at pH 7 and 5.5, respectively.

The stability of apoLR5 has also been quantitated by chemical denaturation. Neither urea nor guanidine hydrochloride can denature apoLR5 or LR5-Ca$^{2+}$ completely. However, whole, fully reversible, unfolding transitions are observed using GdnSCN (Fig. 5). A global fit to the two-state model of apoLR5 GdnSCN-driven denaturation curves at pH 7.0 at three different temperatures (9.5, 15.5, and 25.0 °C), were performed considering the $m$ value in Equation 1 as being temperature-inde-
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It should be mentioned here that molecular crowding in vivo could, in principle, increase the stability of native protein conformations relative to those determined in dilute solutions (50, 51). We noticed, however, that crowding effects are related to volume differences between the conformations involved. Because of the small size of LR5 and the presence of three disulfide bonds, the difference in volume between native and unfolded LR5 is expected to be small, and thus crowding is unlikely to modify substantially the low intrinsic stability reported here.

Calcium Binding to ApoLR5 Greatly Increases Protein Stability—The intrinsic stability of apoLR5 is greatly increased by Ca"²⁺ binding. The stabilizing effect exerted by Ca"²⁺ is readily seen in the DSC thermograms and native fractions shown in Fig. 4, a and b, respectively. In fact, analysis of these thermal denaturation experiment performed in the presence and absence of calcium provides a preliminary set of values for the binding parameters of the LR5-Ca"²⁺ complex (see “Experimental Procedures”). The dissociation constant and the binding enthalpy thus obtained are 1.4 μM and -6.4 kcal/mol, respectively. More accurate values have been determined directly by ITC and fluorescence titration. To better control the Ca"²⁺ concentration in apoLR5 titration experiments, EDTA was added to the buffer (see “Experimental Procedures”). Calcium binding to free EDTA takes place initially, and as EDTA gets saturated, calcium binding to LR5 proceeds. As a consequence, a biphasic titration may be observed in ITC experiments if the enthalpies for calcium binding to EDTA and LR5 are significantly different. Titration at pH 7 (Fig. 6a) exhibits a biphasic behavior, which corresponds to initial titration of free EDTA with calcium and, as EDTA gets saturated, to calcium binding to LR5, which takes place with a more negative enthalpy. However titration at pH 5.5 (Fig. 6b) does not show such behavior because the enthalpies of calcium binding to EDTA and LR5 are very similar. The spectroscopic titration at pH 7.0 (Fig. 6c) shows the expected behavior: calcium initially binds to free EDTA and fluorescence emission intensity changes little. Then, as EDTA gets saturated, calcium binds to LR5 and the intensity increases sharply.

Our analysis indicates that calcium binding to apoLR5 at extracellular pH, 150 mM NaCl, and 25 °C is characterized by a dissociation constant of 0.8 μM (from ITC) or 0.3 μM (from fluorescence titration) and by a binding enthalpy of -5.5 kcal/mol (Table 2). The values obtained for the dissociation constant are in the same range as those reported by North and Blacklow (31) (0.2 μM at pH 7.0 and 100 mM NaCl) and by Simonevic et al. (52) (0.5 μM at pH 7.4 and 100 mM NaCl). At endosomal pH, however, the affinity of apoLR5 for calcium is considerably lower (K_d = 30 μM), close to that obtained by Simonevic et al. (52) (13.1 μM at pH 5.0 and 100 mM NaCl), and the binding enthalpy is +1.8 kcal/mol. The difference in the binding enthalpies at pH 7 and 5.5 is not due to the influence of the buffer, because there is little proton exchange upon binding, if any, at pH 7 (not shown) and the ionization enthalpy of acetate is almost zero.

The folding and binding equilibria of LR5 thus involve no intermediates, and they can be summarized in the following scheme where apoLR5 represents a Ca"²⁺-depleted, yet some-
what compact conformation of the repeat that can experience further thermal and chemical unfolding and that remains Ca\(^{2+}\)/H\(_{11001}\) binding-competent.

\[ U \leftrightarrow \text{apoLDLR5} \leftrightarrow \text{LDLR5-Ca}^{2+} \]

\[ +\text{Ca}^{2+} \]

**SCHEME 1**

The information available from NMR studies (33) and Molecular Dynamics simulations (53) clearly indicates that the conformation of apoLR5 is quite different from the native conformation of the LR5-Ca\(^{2+}\) complex (20, 33).

**Unfolding of LR5 in the Endosome Can Trigger LDL Release**—It is well known that the correct folding of LR5 takes place only in the presence of calcium (33), similar to what has been reported for the structurally homologous LR1 (49). The structural instability of LR5 in the absence of Ca\(^{2+}\) has also been observed in short Molecular Dynamics simulations of wild type and FH-related mutants (53) indicating that the binding site is highly unstable in the Ca\(^{2+}\)-deprived native conformation. Our conformational analysis here shows that the affinity of the LR5-Ca\(^{2+}\) complex is markedly decreased at endosomal pH as compared with extracellular pH (Table 2). On the other hand, the conformational stability of the apo form, which is quite low even at pH 7 and 25 °C, is further decreased at both lower pH values and higher temperatures. The complex interplay of Ca\(^{2+}\) concentration, temperature, and pH on the stability of LR5 can best be visualized in pCa-temperature phase diagrams (54) such as those represented in Fig. 7, a and b, for extracellular and endosomal conditions, respectively. The diagrams define the regions where the unfolded conformation, folded apoLR5, or functional LR5-Ca\(^{2+}\) complex is the dominant species. Importantly, the diagrams clearly reveal that the stability realm of the
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![Diagram showing the mechanism of LDL release](image)

**FIGURE 8. Proposal for a new mechanism for the release of LDL particles in the endosome based on the instability of LR5 at endosomal low pH and low [Ca\(^{2+}\)]**

In this study, we propose a new mechanism for the release of LDL particles in the endosome. The instability of LR5 at endosomal conditions is shown in Fig. 8, where the lower pathway (adapted from Ref. 62) represents the currently accepted view, which is based on ample structural and biochemical evidence obtained at endosomal pH values, but typically at high [Ca\(^{2+}\)], known to occur in early endosomes before acidification takes place. The upper pathway is a new proposal based on the evidence gathered in this work, which shows that under low pH and low [Ca\(^{2+}\)] conditions such as those thought to arise in the endosome, LR5 is unable to bind Ca\(^{2+}\) and appears in an unfolded conformation not expected to bind LDL particles. The arrow with a question mark indicates speculation on the non-tested possibility that unfolded LR5 is still recognized (and folded upon binding) by the \(\beta\)-propeller domain of LDLR, thus further shifting the LDLLR-LDL equilibrium toward dissociation.

Under these conditions (black dot in Fig. 7b), insufficient affinity of LR5 for Ca\(^{2+}\) ions and reduced conformational stability of the apo form conspire to lower the fraction of active LR5-Ca\(^{2+}\) molecules to no more than 7%. As a consequence, the affinity of the receptor for LDL particles must be severely reduced and, thus, allow LDL release.

The additional energy of stabilization induced by a ligand binding to the native state is given by +RT\ln(1+\(K_{\text{s}}[L]\)), where \(K_{\text{s}}\) is the binding affinity and \([L]\) is the free ligand concentration. Thus, considering the \(K_{L}\) values at pH 7 and 5.5 and the concentration of calcium in the plasma and the endosome, the values of the additional energy of stabilization induced by calcium at 25 °C are 4.6 kcal/mol and 0.1 kcal/mol, respectively.

Therefore, extrapolating at 37 °C, the presence of calcium in plasma at micromolar concentrations shifts the conformational equilibrium dramatically toward the LR5 native state, with 100% calcium-bound LR5 (NL in the phase diagram), whereas the presence of calcium at micromolar concentrations in endosomes does not have any influence on the LR5 conformational equilibrium, leaving it unchanged with just only 7% calcium-bound LR5.

The liberation of LDL particles in the endosome is generally thought to be related to competition between the EGF precursor domain in LDLR and the LDL particles for the binding repeats involved in LDL uptake, significantly for LR5 (57). This model has been inspired by the three-dimensional structure of LDLR at endosomal pH, which shows that the LR5 repeat binds back to the EGF precursor domain of the receptor (29). The model, which has been subsequently tested by mutational studies (24), proposes that a triad of histidine residues located in LR5 and in the EGF precursor domain modulates the relative affinity of LDL for LDLR and for the EGF precursor domain as a function of pH. The LDLR-LDL complex, which would be stable at extracellular pH, would dissociate as a consequence of increased affinity of LR5 for EGF precursor domain when the pH drops in the endosome (Fig. 8, lower pathway). We note, however, that both the tridimensional structure of the receptor at endosomal pH and the subsequent mutational analysis consistent with this mechanism have been carried out in buffers of Ca\(^{2+}\) concentrations (0.5 and 2 mM, respectively) that are close to extracellular conditions. According to the phase diagram at pH 5.5 (Figure...
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7b), at those Ca$^{2+}$ concentrations LR5 is well folded at the temperatures used for crystallization (29) and for the biochemical assays (24) (25 and 4 °C, respectively; white triangle and white square, respectively, in Fig. 7b) and carries its structural Ca$^{2+}$ ion, as it is observed in the reported x-ray structure at low pH (29). The currently accepted mechanism, which proposes that LDL release is linked to a protonation of three histidine residues triggering displacement of the bound LDL particle by the receptor EGF precursor domain, seems thus to be based on evidence applying to endosomal pH but extracellular Ca$^{2+}$ conditions.

The situation in the acidifying endosome is, however, more complex. In fact, 10 min after the endosome is formed, the concentration of Ca$^{2+}$ appears to drop to 5 μM (56). As explained above, LR5 no longer remains stable at this low Ca$^{2+}$ concentration (Fig. 7b). Instead, LR5 releases the bound Ca$^{2+}$ ion, and the arising apoLR5 conformation experiences a further expansion leading to the unfolded state. Thus, based on the observed pH and [Ca$^{2+}$]-dependent stability of LR5 we propose that, at the low pH and low Ca$^{2+}$ concentrations simultaneously arising in the endosome, a simpler LDL release mechanism takes place by which LR5 unfolding drives LDL dissociation (Fig. 8, upper pathway). It is possible that other LDLR binding repeats involved in LDL interactions become similarly destabilized upon acidification, which would contribute to further reduction of the affinity of the receptor for LDL. This mechanism does not necessarily exclude the possibility that the self-complex of LDLR observed by x-ray (29) may be formed in the endosome. Even though it is a long and well established fact that LDLR-LDL interaction is hampered at low Ca$^{2+}$ concentrations (34) (very likely because of the importance of the Ca$^{2+}$ ion for the native folding of the binding repeats, including LR5), the actual impact of LR5 unfolding at endosomal conditions on the affinity of this repeat for the EGF precursor domain remains to be tested. It is possible that LR5 unfolding in the endosome triggers the dissociation of the LDL particles without completely impairing LR5/EGF precursor domain interaction. Investigations of natively unfolded proteins have made clear that some disordered conformations may become folded upon binding to an appropriate receptor (58). If this were the case, the unfolding of LR5 would provide the pathway for LDL release, still allowing subsequent interaction of LR5 with the EGF precursor domain, which would further shift the LDL-LDLR equilibrium toward dissociation. Formation of an internal complex between LR5 and the EGF precursor domain might also be important for modulating the interaction of LDLR with proprotein convertase subtilisin/kexin type 9 (PCSK9), a protein involved in the degradation of LDLR (59). Whatever the case, note that the release mechanism that we have proposed does not take into account the possible influence of bound LDL particles on the pH-dependence of the stability and Ca$^{2+}$ affinity of the modules from the ligand-binding domain. Work is in progress to investigate these issues.

Conformational changes induced by low pH of the endosome have been widely described for other systems, such as viral release (60) and toxin entry into the cytoplasm (61). Recently, it has been shown that RAP (receptor-associated protein), a chaperon that escort receptors of the LDLR family from the endoplasmic reticulum to the Golgi, dissociates from the receptors by a mechanism involving partial unfolding of RAP triggered by histidine protonation at the acidic pH of the Golgi (10). It thus appears that acid-driven unfolding could be a general cellular mechanism used to induce dissociation of protein complexes in acidic organelles.

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