Molecular Studies of pH-Dependent Ligand Interactions with the Low-Density Lipoprotein Receptor†

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ABSTRACT: The release of ligand from the low-density lipoprotein receptor (LDLR) has been postulated to involve a "histidine switch"-induced intramolecular rearrangement that discharges bound ligand. A recombinant soluble low-density lipoprotein receptor (sLDLR) was employed in ligand binding experiments with a fluorescently tagged variant apolipoprotein E N-terminal domain (apoE-NT). Binding was monitored as a function of fluorescence resonance energy transfer (FRET) from excited Trp residues in sLDLR to an extrinsic fluorophore covalently attached to Trp-null apoE3-NT. In binding experiments with wild-type (WT) sLDLR, FRET-dependent AEDANS fluorescence decreased as the pH was lowered. To investigate the role of His190, His562, and His586 in sLDLR in pH-dependent ligand binding and discharge, site-directed mutagenesis studies were performed. Compared to WT sLDLR, triple His → Ala mutant sLDLR displayed attenuated pH-dependent ligand binding and a decreased level of ligand release as a function of low pH. When these His residues were substituted for Lys, the positively charged side chain of which does not ionize over this pH range, ligand binding was nearly abolished at all pH values. When sequential His to Lys mutants were examined, the evidence suggested that His562 and His586 function cooperatively. Whereas the sedimentation coefficient for WT sLDLR increased when the pH was reduced from 7 to 5, no such change occurred in the case of the triple Lys mutant receptor or a His562Lys/His586Lys double mutant receptor. The data support the existence of a cryptic, histidine side chain ionization-dependent alternative ligand that modulates ligand discharge via conformational reorganization.

An important feature of the delivery of cholesterol to cells via the low-density lipoprotein receptor (LDLR) pathway relates to the ability of the receptor to recycle to the plasma membrane after releasing its cargo. During the internalization process, endocytic vesicles are converted to endosomes and these enclosed vesicular structures are acidified through the action of transmembrane proton pumps (*1*). The resulting decrease in endosomal pH is considered a key step in the release of ligand from the receptor. This separation event is critical to both receptor recycling and delivery of lipoprotein cargo to lysosomes for liberation of their cholesterol load. On the basis of the assumption that receptor−ligand interactions are electrostatic in nature, it is presumed that lowering the pH within the endosome will lead to an alteration in ionizable amino acid side chains that decrease the affinity of ligand for receptor. Indeed, the X-ray crystal structure of the soluble portion of the LDLR (*2*) provided a plausible molecular explanation for this process. This structure was determined at pH 5.3, a condition at which ligand binding interactions do not occur. Remarkably, the structure revealed that the β-propeller segment forms contacts with cysteine-rich LDLR type A (LA) ligand binding repeats 4 and 5 under these conditions, thereby blocking access to potential ligands. The model suggests that, at low pH, an intramolecular rearrangement occurs in which the β-propeller segment displaces ligand from ligand binding modules, thereby serving as an alternative ligand. While this cryptic, pH-dependent process emerged from X-ray structure analysis, it is entirely consistent with previous mutagenesis studies. Russell et al. (*3*) showed that deletion of the fifth of the seven LA repeats in LDLR abolishes binding activity toward apoE containing lipoprotein particles, while deletion of the β-propeller segment produces a mutant receptor that is capable of binding LDL but fails to release ligand at low pH (*4*).

In this study, we focus on His residues located in LA repeat 5 or the β-propeller segment (*2, 5*). Two of these His residues (at positions 190 and 562) are mutated to Tyr in familial hypercholesterolemia (*6*). In other proteins, key His residues have been implicated in regulating ligand release upon endocytosis (*7*−*9*) as well as in interactions of a chaperone with an LDLR family member in the ER and Golgi (*10*). In a cell-based assay employing flow cytometry to investigate binding of LDL to LDLR, Beglova et al. (*11*) reported evidence that His residues at positions 190, 562, and 586 function in the release of ligand from the receptor. To investigate this further, we sequentially replaced these His residues with either Ala or Lys. Results from in vitro

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binding experiments are consistent with a model in which cooperative electrostatic interactions between the LA repeats and the β-propeller domain contribute to pH-dependent structural alterations that not only prevent ligand binding but also lead to discharge of bound ligand.

MATERIALS AND METHODS

Recombinant Apolipoprotein E3 N-Terminal Domain and Soluble LDLR. Recombinant Trp-null apoE3-NT was produced and isolated from *Escherichia coli* as described by Fisher et al. (*12, 13*). WT[1] and mutant soluble LDLR (sLDLR, N-terminal residues 1−699) were isolated from conditioned media of stably transfected HEK 293 cells as described previously (*14*). Site-directed mutagenesis was performed using the QuikChange XL kit from Stratagene according to the manufacturer's instructions. All mutations were verified by dideoxy automated DNA sequencing. All variant sLDLRs that were generated were analyzed by SDS−PAGE under reducing and nonreducing conditions as a measure of native protein folding and disulfide bond formation (*15*).

ApoE3-NT•Phospholipid Complexes. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids (Pelham, AL). The phospholipid was dissolved in chloroform and methanol (3:1, v/v) and dried into a thin film in a glass tube. Following dispersion of the lipid in 50 mM sodium phosphate (pH 7.0) and 150 mM NaCl, apoE3-NT was added. This mixture was subject to bath sonication at 24 °C until it became clear (*16, 17*).

LDLR Binding Assay. Two micrograms of sLDLR (in 10 mM citric acid, 2 mM $CaCl_2$, and 90 mM NaCl; adjusted to specified pH values) was incubated with 1 μg of a Trp-null apoE3-NT previously labeled on Cys112 with the fluorescent probe *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine (AEDANS) and complexed with DMPC. Interaction between the AEDANS-Trp-null apoE3-NT•DMPC complex and sLDLR is detected by fluorescence resonance energy transfer (FRET) between excited Trp residues in sLDLR and the AEDANS moiety covalently attached to Trp-null apoE3-NT•DMPC (*14*). Following incubation, samples were excited at 280 nm and fluorescence emission intensity at 470 nm was determined (slit width of 5.0 nm) on a Perkin-Elmer model LS 50B luminescence spectrometer.

Analytical Ultracentrifugation. Sedimentation velocity experiments were conducted at 22 °C and 35000 rpm using a Beckman XL-I analytical ultracentrifuge and absorbance optics following the procedures outlined in the instruction manual published in 1997 by the Spinco Business Center of Beckman Instruments, Inc. (Palo Alto, CA). Runs were performed for 5 h during which a maximum of 75 scans were taken. The sedimentation velocity data were analyzed using the Transport Method contained in the Beckman Analysis Program (Optima XL-A/XL-I Data Analysis Software, version 4.0, 1997). Svedberg Program, version 6.39, was used to calculate the sedimentation constant, $S_{20,w}$, for each experiment by analyzing 12 sets of data selected between the 28th and 56th scan. Sednterp (Sedimentation Interpretation Program, version 1.01) was used to calculate the intrinsic sedimentation constant, $S^0_{20,w}$, and the axial ratio, *a*/*b*. Sednterp calculates the partial specific volume and degree of hydration from the amino acid composition of the protein using the methods of Cohn and Edsall (*18*) and Kuntz (*19*) and also calculates the solvent density and viscosity using known values from physical tables.

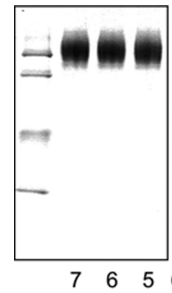

FIGURE 1: Native PAGE analysis of apoE3-NT DMPC complexes. AEDANS-apoE3-NT•DMPC disks were incubated in buffer at the indicated pH values for 1 h at 37 °C. Following this, the samples were electrophoresed on a 4 to 20% acrylamide gradient slab gel under nondenaturing conditions and stained with Coomassie Blue. The relative mobility of size standards is shown at the left. From top to bottom: 17.0, 12.2, 9.2, 8.2, and 7.1 nm diameter, respectively.

RESULTS

Design and Rationale. A model describing a pH-dependent ligand displacement mechanism for discharge of bound ligand from sLDLR has been proposed on the basis of X-ray crystallography and site-directed mutagenesis (*2, 5, 20*). In this study, a known LDLR ligand, apoE3-NT, was employed in assays of ligand binding and release. When AEDANS-labeled Trp-null apoE3-NT binds to the receptor, a stable fluorescence signal is established via intermolecular FRET from excited Trp residues in the receptor to the AEDANS moiety covalently bound to apoE. A corresponding decrease in AEDANS fluorescence intensity upon dissociation of the ligand can be monitored as a real-time measure of ligand release (*14*). This concept provides a general experimental design for the investigation of structural requirements for ligand binding and may be adaptable to other LDLR family members and their ligand interactions.

pH-Dependent Binding of apoE3-NT•DMPC Disks to sLDLR. To establish that interaction between WT sLDLR and apoE3-NT•DMPC in the assay system described here will display pH dependence, FRET between excited Trp in sLDLR and AEDANS bound to Trp-null apoE3-NT was monitored. Initially, ligand stability as a function of solution pH was evaluated by nondenaturing PAGE analysis (Figure 1). The data reveal that incubation of fluorescently labeled apoE3-NT•DMPC disk complexes in different pH buffers had no effect on particle migration behavior or band intensity. Thus, we conclude that the ligand employed in these studies is stable to the pH variation that was examined. In binding experiments with sLDLR at pH 7, the expected enhancement in AEDANS fluorescence intensity was observed (Figure 2). As the solution pH was decreased from 7 to <5, however, there was a corresponding decrease in AEDANS fluorescence intensity. At pH 5.9, the enhancement of AEDANS fluorescence intensity upon introduction of sLDLR was 21% of the

[1] Abbreviations: AEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl) ethylenediamine; apo, apolipoprotein; DMPC, dimyristoylphosphatidylcholine; FRET, fluorescence resonance energy transfer; NT, amino-terminal; sLDLR, soluble low-density lipoprotein receptor; WT, wild type.

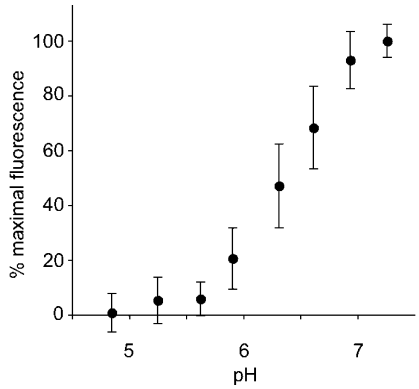

FIGURE 2: Effect of solution pH on ligand binding to sLDLR. Two micrograms of AEDANS-Trp-null apoE3-NT•DMPC and 1 μg of sLDLR were incubated in 10 mM citric acid, 2 mM $CaCl_2$, and 90 mM NaCl, adjusted to the indicated pH values. Samples (300 μL final volume) were excited at 280 nm, and the fluorescence emission intensity at 470 nm was determined. Values reported are means ± the standard deviation ($n = 3$).

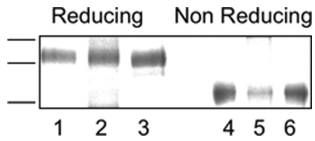

FIGURE 3: Characterization of sLDLR variants. WT and variant sLDLR were subjected to SDS−PAGE under reducing (lanes 1−3) and nonreducing (lanes 4−6) conditions: lanes 1 and 4, WT sLDLR; lanes 2 and 5, triple His → Ala mutant; and lanes 3 and 6, triple His → Lys mutant. Samples were separated on a 4 to 20% acrylamide gradient slab gel electrophoresed at a constant current of 30 mA and stained with Coomassie Blue.

value observed at pH 7.4. Control experiments showed that AEDANS fluorescence intensity via direct excitation at 349 nm was unaffected by pH over this range. Given the ionization behavior of His side chains over this pH range together with the X-ray crystal structure of sLDLR, these data are consistent with a "histidine switch" mechanism (*21*) in which a conformational change in the receptor facilitated by protonation of key His side chains prevents access of the apoE3-NT•DMPC ligand to LA repeats in LDLR (*2*).

SDS−PAGE Analysis of sLDLR and Variants. To evaluate the role of His190, His562, and His586 in pH-induced ligand release, recombinant sLDLRs in which each of these residues was converted to either Ala or Lys were generated. Prior to binding studies being performed, however, isolated variant sLDLRs were evaluated by SDS−PAGE. It is known that correctly folded LDLR family members display characteristic migration behavior in the presence and absence of β-mercaptoethanol (*15*). The pattern observed with WT sLDLR was similar to that reported by others, with slower migration into the gel under reducing conditions (Figure 3). By the same token, both variant sLDLRs (triple Ala mutant and triple Lys mutant) behaved in a similar manner, indicating they are correctly folded in solution.

Effect of Mutations in sLDLR on Ligand Binding. Whereas Ala substitution introduces a small, uncharged side chain in place of His, the Lys substitution will introduce a positively charged side chain that does not ionize over the pH range of 7.0−5.0. In control experiments with WT sLDLR, the expected decrease in AEDANS fluorescence intensity was observed as the pH was decreased from 7.0 to 6.0 or 5.0 (Figure 4). In the case of the triple His → Ala mutant sLDLR,

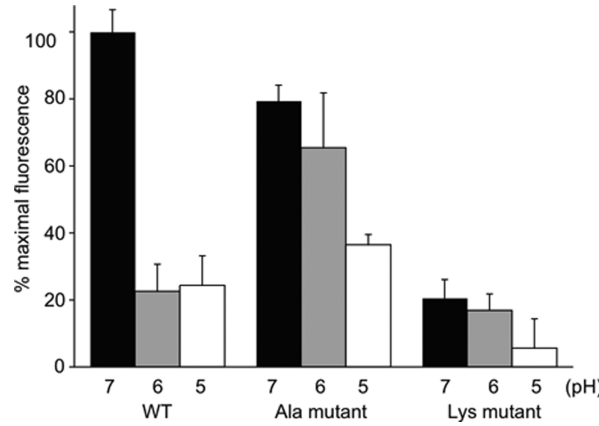

FIGURE 4: Effect of mutations in sLDLR on pH-dependent ligand binding. Two micrograms of AEDANS-Trp-null apoE3-NT•DMPC and 1 μg of a given sLDLR were incubated in 10 mM citric acid, 2 mM $CaCl_2$, and 90 mM NaCl, adjusted to pH 7.0, 6.0, or 5.0. Samples (300 μL final volume) were excited at 280 nm, and the fluorescence emission intensity at 470 nm was determined. Values are means ± the standard deviation ($n = 6$).

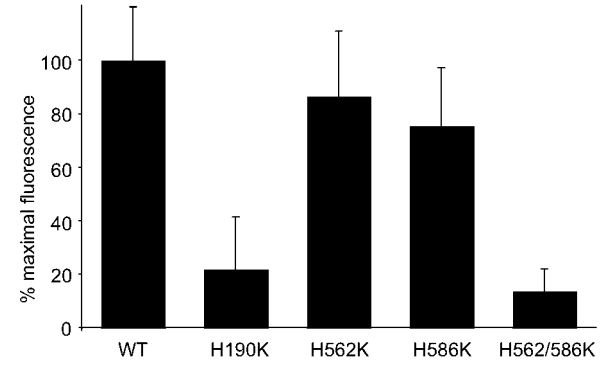

FIGURE 5: Effect of individual His mutations on binding of ligand to sLDLR at neutral pH. Two micrograms of AEDANS-Trp-null apoE3-NT•DMPC and 1 μg of a given sLDLR variant were incubated in 10 mM citric acid, 2 mM $CaCl_2$, and 90 mM NaCl, adjusted to pH 7.0. Samples (300 μL final volume) were excited at 280 nm, and the fluorescence emission intensity at 470 nm was determined. Values are means ± the standard deviation ($n = 6$).

binding at pH 7.0 was weaker than with WT sLDLR, and very little change in fluorescence intensity occurred when the pH was decreased to 6.0. At pH 5.0, the AEDANS fluorescence intensity was lower but remained above values observed for WT sLDLR at this pH. On the other hand, in the case of a mutant sLDLR in which each of the His residues was converted to Lys, little binding was detected at pH 7.0 and essentially no change in AEDANS fluorescence intensity was noted as the pH was decreased to 6.0 or 5.0.

To assess the role of individual His residues in binding of ligand to and release of ligand from sLDLR, the effect of single Lys mutations on FRET-dependent AEDANS fluorescence was examined. Using WT sLDLR as a control, the effect of mutations of His190, His562, and His586 to Lys were studied at pH 7 (Figure 5). In each case, the strength of binding of ligand to the single mutant sLDLR was intermediate between the maximal strength of binding observed for WT sLDLR and that for the triple His → Lys mutant. Whereas ligand binding to His190Lys sLDLR was poor, the binding activities of His562Lys or His586Lys sLDLR were only slightly lower than that of control WT

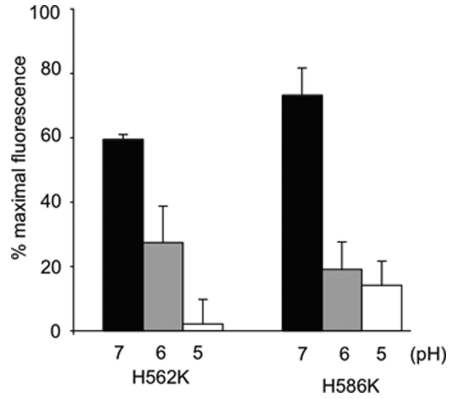

FIGURE 6: Effect of soution pH on ligand binding to single His mutant sLDLR. Effect of mutations in sLDLR on pH-dependent ligand binding. Two micrograms of AEDANS-Trp-null apoE3-NT·DMPC and 1 μg of a given sLDLR were incubated in 10 mM citric acid, 2 mM $CaCl_2$, and 90 mM NaCl, adjusted to pH 7.0, 6.0, or 5.0. Samples (300 μL final volume) were excited at 280 nm, and the fluorescence emission intensity at 470 nm was determined. Values are means ± the standard deviation ($n = 6$).

sLDLR. However, ligand binding activity at neutral pH was abolished when His562 and His586 were both converted to Lys.

Effect of Solution pH on the Binding Interaction of Single His Variant sLDLR. On the basis of the data presented in Figure 5, where the ligand binding ability of the H562K sLDLR and H586K sLDLR variants was generally similar to that of WT sLDLR, we sought to determine if these variants will display a pH dependence of ligand binding similar to that seen with WT sLDLR (Figure 6). The observed loss of binding ability as a function of decreasing pH for both single mutant variants is consistent with the concept that the His residues at these positions in WT sLDLR act in concert to induce pH-dependent release of ligand from the receptor.

Evaluation of Ligand Release. Whereas data presented above illustrate the effect of mutations in sLDLR on ligand binding, we sought to evaluate whether these mutations also influence ligand release. pH-dependent ligand release was monitored by FRET as a function of time following a transition from pH 7.0 to 6.0. In the case of WT sLDLR, a rapid decrease in AEDANS fluorescence intensity accompanied the shift in pH. Within seconds of the pH reduction, a >80% decline in AEDANS fluorescence was noted (Figure 7), consistent with discharge of bound ligand from the receptor. Similar to ligand binding data presented in Figure 4, in the case of the triple His → Ala mutant sLDLR, pH-induced changes in AEDANS fluorescence intensity were slower and the end point that was reached corresponded to approximately 50% of the maximal AEDANS fluorescence intensity (i.e., pH 7). Thus, His → Ala mutations affect both the kinetics and magnitude of pH-induced release of ligand from sLDLR.

Hydrodynamic Studies. To evaluate the effect of mutations in sLDLR on the shape of the molecule at different pH values, sedimentation velocity experiments were performed in the analytical ultracentrifuge. As shown in Table 1, the sedimentation coefficient for WT sLDLR increased from 5.3 to 6.1 with a decrease in solution pH from 7 to 5. The increased sedimentation coefficient and the corresponding axial ratio value for WT sLDLR at pH 5 are consistent with

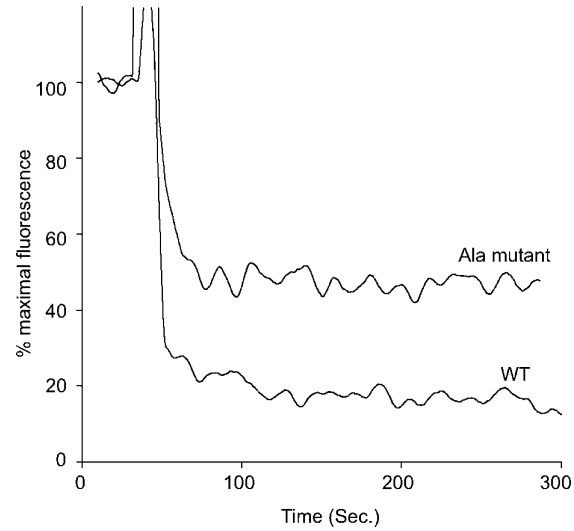

FIGURE 7: Effect of solution pH on release of ligand from sLDLR. One microgram of AEDANS-Trp-null apoE3-NT·DMPC and 2 μg of WT or His → Ala sLDLR were incubated in 10 mM citric acid, 2 mM $CaCl_2$, and 90 mM NaCl (pH 7.0). After 30 s, the solution pH was changed via addition of an aliquot (6 μL) of 2 M citric acid (pH 6.0). Samples (300 μL final volume) were excited at 280 nm, and the fluorescence emission intensity at 470 nm was monitored as a function of time.

Table 1: Hydrodynamic Studies of sLDLR Variants

sLDLR sample	pH	concn (mg/mL)	sedimentation coefficient ($S_{20,w}$)	axial ratio (a/b)
wild type	7	0.41	5.3	3.9
wild type	5	0.36	6.1	1.3
H562K/H586K	7	0.11	5.5	3.0
H562K/H586K	5	0.23	5.4	3.5
H190K/H562K/H586K	7	0.50	5.2	4.0
H190K/H562K/H586K	5	0.25	5.1	4.3

adoption of a closed conformation at low pH. In the case of the triple His → Lys mutant receptor, consistent with an inability to bind ligand at either pH (Figure 4), only minor differences in the sedimentation coefficient and axial ratio were observed. A similar result was obtained for an H562K/H586K double mutant sLDLR, revealing that mutations at sites outside the ligand binding domain not only can block ligand binding but also can prevent pH-dependent changes in molecular shape observed with WT sLDLR.

DISCUSSION

X-ray crystallography studies of sLDLR at endosomal pH provide the framework for a testable model of pH-dependent ligand release. The three-dimensional structure-derived model is consistent with previous mutagenesis studies that demonstrated ligand binding but no release when the β-propeller segment was deleted (*4*). Likewise, naturally occurring LDLR mutations (*22, 23*) and site-directed mutagenesis studies (*11*) have provided evidence that His residues within the β-propeller and LA repeat 5 are key elements of the pH-induced ligand release mechanism. Studies by Beglova et al. (*11*) using transfected ldlA-7 cells showed that His190Tyr or His562Tyr LDLR variants bound LDL at neutral pH and released the ligand in response to low pH, albeit with an efficiency lower than that of the native receptor. These authors subsequently changed all three His residues to Ala

or Tyr to assess the cooperativity of the process. Both triple mutants were capable of binding receptor but failed to release ligand at low pH, providing evidence that these His residues participate in concert to promote ligand release. In this study, we have confirmed and extended these findings using a cell-free, solution assay employing sLDLR and apoE·DMPC disk complexes as ligands. The data reveal subtle differences between WT sLDLR and a triple His → Ala mutant sLDLR, with the latter displaying an attenuated pH-dependent effect on ligand binding. On the other hand, the triple His → Lys mutant in which each of the three His residues was converted to Lys failed to bind ligand at any pH that was examined. Since the pK_a of the lysine side chain is ~10.5, unlike His, these Lys will retain a positive charge at pH 7. Consistent with the postulated role of electrostatics in modulating intramolecular rearrangement within the receptor, the data suggest a histidine switch mechanism is operative in this system. To further refine this system, His residues were singly substituted with Lys and the effect on ligand binding at pH 7.0 was determined. The data showed that mutation at His190 more effectively blocked ligand binding compared to His562 or His586. Interestingly, however, a double mutant in which both His562 and His586 were converted to Lys abolished ligand binding. Rudenko et al. (*2*) showed that, at endosomal pH, His190 interacts with Glu581 while both His562 and His586 interact with Asp149. Thus, it is conceivable that ionization of both these residues is required to stabilize the interaction with Asp149 and, thereby, interfere with ligand binding.

A key aspect of the model proposed by Rudenko et al. (*2*) is that LDLR adopts a closed conformation at endosomal pH that prevents access to the ligand binding site due to interactions with the β-propeller motif that are induced by side chain ionization of key His residues. Consistent with this model, sedimentation velocity experiments on WT sLDLR gave rise to an increase in the sedimentation coefficient as well as a decrease in the axial ratio as a function of the pH being lowered from 7 to 5. Furthermore, consistent with their inability to bind ligand at either pH, the triple His → Lys mutant sLDLR as well as the H562K/H586K mutant sLDLR did not undergo a corresponding pH-dependent change in sedimentation coefficient. Moreover, the calculated axial ratios for both these mutant receptors were largely unchanged at pH 7 and 5. The absence of a change in these parameters is consistent with the ionization properties of Lys versus His. On the basis of the lack of Lys side chain ionization over this pH range, one may predict that no conformational change will occur if the model is correct. The similar values observed for this mutant receptors at the two pH values are also consistent with their lack of binding activity at either pH. Whether the conformation adopted these mutant sLDLRs is the same as that adopted by WT receptor at pH 5, however, remains an open question since the axial ratios observed for both mutant sLDLRs that were studied were more similar to the WT receptor at pH 7. Given this, it appears that the mutant sLDLRs may achieve a unique conformation that results in ligand binding site blockage with retention of the more extended conformation. Further studies will be required to elucidate the precise nature of this conformation. Given the successful application of X-ray crystallography to determining the WT receptor structure, such studies should be feasible.

Whereas exploitation of His → Lys substitution mutations has not to our knowledge been used to illustrate effects on receptor−ligand interactions, this general approach has been employed in other systems. For example, Briand et al. (*24*) showed that replacement of Lys188 of trypsin with His creates a metal chelation site in the substrate binding pocket of this protease. In this case, the His mutation introduced a metal binding switch, capable of modulating enzyme activity as a function of pH. Likewise, Nyarko and co-workers (*25*) reported that the light chain subunit of dynein exists as a dimer at physiological pH but dissociates to a folded monomer at pH <4.8. By mutating His55 to Lys, these authors showed that pH-induced dimer dissociation is reversible and governed by the ionization state of His55. Indeed, mutagenesis of His55 to Lys resulted in a monomer in the pH range of 3−8, while mutation to Ala resulted in a dimer across the same pH range. In other work, Martinez and Bowler (*26*) investigated the kinetics of an alkaline conformational transition in cytochrome *c* using pH jump stopped-flow methods to probe the nature of an ionizable "trigger" group for this conformational change. These authors reported that a Lys73His mutation shifted the pK_a of the ligand replacing Met80 from ~10.5 to ~6.6, which supports the concept that ionization equilibria modulate protein folding in this system. Thus, alteration of the ionization potential of key residues in proteins via mutagenesis represents a useful and discriminating method for examining mechanism.

In this system, a key factor in experimental design is the availability of a high-resolution structure for the receptor at low pH together with a discriminating assay for probing ligand binding and release. Taken together, the data presented show that the solution-based FRET assay employed recapitulates findings using transfected cells in culture and, further, can be used to dissect the molecular basis of ligand binding and release. The results fit well with the model of pH-dependent ligand release proposed by Rudenko et al. (*2, 5*) and reveal the critical role of His side chain ionization in modulation of interactions of ligand with the LDLR.

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