Evidence for Isomerization during Binding of Apolipoprotein-B100 to Low Density Lipoprotein Receptors*

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To determine the kinetics of human low density lipoproteins (LDL) interacting with LDL receptors, 125I-LDL binding to cultured human fibroblasts at 4 °C was studied. Apparent association rate constants did not increase linearly as 125I-LDL concentrations were increased. Instead, they began to plateau which suggested that formation of initial receptor-ligand complexes is followed by slower rearrangement or isomerization to complexes with higher affinity. To test this, 125I-LDL were allowed to associate for 2, 15, or 120 min, then dissociation was followed. The dissociation was biphasic with the initial phase being 64-110-fold faster than the terminal phase. After binding for 2 min, a greater percentage of 125I-LDL dissociated rapidly (36%) than after association for 15 min (24%) or 120 min (11%). Neither the rate constants nor the relative amplitudes of the two phases were dependent on the degree of receptor occupancy. Thus, the duration of association, but not the degree of receptor occupancy affected 125I-LDL dissociation. To determine if binding by large LDL, which is predominantly via apolipoprotein (apo) E, also occurs by an isomerization mechanism, the d = 1.006-1.05 g/ml lipoproteins were fractionated by ultracentrifugation. In contrast to small LDL which bound via apoB-100 and whose dissociation was similar to that of unfractionated LDL, large LDL dissociation after 2, 15, or 120 min of binding did not show isomerization to a higher affinity. This suggests that large and small LDL bind by different mechanisms as a result of different modes of interaction of apoE and apoB-100 with LDL receptors.

Cholesterol uptake via low density lipoprotein (LDL) receptors has major regulatory effects on cellular cholesterol homeostasis and plasma LDL levels (1). The binding and uptake of LDL by LDL receptors is specific and rapid, occurring within minutes in vitro. Based on linear Scatchard plots and simple, first-order kinetics, LDL appears to bind by independent interactions of single LDL particles with single receptors (1-3). However, because the receptors are clustered, receptor-bound LDL particles (M, 2.5 × 109) may be large enough to sterically hinder binding to adjacent LDL receptors (M, 115,000) (1, 4, 5). Consequently, each binding event may not be independent.

Our previous studies suggest that the binding of LDL is subject to steric constraints resulting from crowding of these relatively large ligands on lattices of closely spaced LDL receptors (5). Scatchard plots of LDL binding are concave-upward, and both the higher and lower affinity components of LDL binding are regulated by lovastatin. However, neither binding component is present on null cells that are incapable of expressing LDL receptors. Scatchard plots of large LDL binding show fewer higher affinity sites than do plots of small LDL binding. If the potential for receptor cross-linking via both apoE and apoB-100 is prevented by the presence of an apoE-specific monoclonal antibody (1D7), the difference between the apparent number of higher affinity sites bound by large versus small LDL is exaggerated (5). Thus, receptor cross-linking cannot explain the reduced estimate of high affinity sites seen with large LDL binding.

Taken together, these results support a lattice model for LDL binding. Lattice models may apply to any interaction between relatively large ligands and smaller, closely spaced receptors (6-8). Both one- and two-dimensional receptor lattices have been discovered. In either, ligand binding sterically hinders nearby sites causing the apparent affinity to decrease near saturation. As predicted by a lattice model, binding of large as compared to small LDL excludes binding to more receptors causing fewer high affinity sites (5). Lattice formation by LDL receptors has important effects on LDL metabolism. In metabolically active cells at 37 °C, steric hindrance allows fewer large than small LDL to be bound, internalized, and degraded (5).

Many ligand-receptor interactions in vivo do not occur at equilibrium. This probably applies to the LDL receptor pathway because LDL is rapidly internalized before binding equilibrium can be reached (1, 5). Therefore, kinetic studies are necessary to provide information about early binding events that is not available by studying equilibrium binding. In addition, kinetic studies permit testing of alternative binding models that may not be possible to test at equilibrium. The current studies were performed on cultured human fibroblasts kept at 4 °C so that surface-bound ligands were not internalized. Surprisingly, we found isomerization to a higher affinity during binding by apoB-100 in unfractionated and small LDL. In contrast, isomerization was not apparent during binding by large LDL which bound predominantly via apoE.

EXPERIMENTAL PROCEDURES

Human Subjects—Lipoproteins were studied from nine normolipidemic human subjects. These subjects are described in a preceding paper (6). Since apoE was involved in binding of large LDL to receptors, we determined apoE phenotypes (9). All subjects had the most common phenotype (E3/3). All subjects gave written informed consent.

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The abbreviations used are: LDL, low density lipoproteins; apo, apolipoprotein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LRP, LDL receptor-related protein.
consent for the study which was approved by the Human Research Committee at The University of Iowa.

Lipoprotein Preparation—Subjects fasted for 14 h before blood sampling. Samples were adjusted to contain 1 mg of EDTA (sodium salt)/ml and several preservatives as previously described (5). The LDL (d = 1.02–1.05 g/ml) were isolated by ultracentrifugation (5). d = 1.006–1.05 g/ml lipoproteins were fractionated in an SW-41 rotor (Beckman Instruments, Palo Alto, CA) to isolate seven LDL fractions (5). Lipoproteins were iodinated (specific activity, 300–600 cpm/ng) by the iodomonochloride method (10). Lipid and apolipoprotein composition and particle diameters of lipoproteins were described previously (5, 11).

Fibroblast Binding Assays—Human foreskin fibroblasts were cultured from four normal infants. Mutant fibroblasts (null cells) that are incapable of expressing LDL receptors due to homozygosity for null alleles (12) were purchased from the Mutant Cell Repository (GM00486A1, Camden, NJ. Fibroblasts were grown to confluency (13), and LDL receptors were up-regulated by incubation with cholesterol-depleted media (containing 10% lipoprotein-deficient serum) for 48 h prior to the binding assays (13). To further up-regulate LDL receptors, in some cases, lovastatin (provided by Merck and Co., Inc., Rahway, NJ) was added to the media (1 μg/ml) for 24 h prior to the binding assay (14).

The surface binding of 125I-LDL to metabolically inactive (4°C) fibroblasts was determined in duplicate wells with minor modifications of the standard procedure (13). After removal of the growth medium, fibroblasts were preincubated for 20 min at 4°C in Eagle’s minimal essential medium containing 2.5 mM sodium HEPES, 42 mM NaHCO3, and 4% bovine serum albumin (final pH 7.4); then, 125I-LDL was added to each well, and aliquots from each well were taken to calculate ligand concentrations. We found that early during association, nonspecific binding was minimized by this approach.

Since divergent cations are required for LDL binding (1, 13), 1.8 mM CaCl2, 0.8 mM MgSO4, and 5 mM sodium HEPES (final pH 7.4) were included in the buffer used to wash wells after ligand binding. Because it was found that a significant percentage of 125I-LDL dissociated in less than 30 min, we washed wells five times in less than 5 min and excluded the two 10-min washes that are recommended (13). Care was taken to complete washing of each well in the same duration of time. This washing protocol, which is necessary for kinetic studies, caused a small increase in the amount of total, specific, and nonspecific binding at equilibrium (not shown). Nonspecific binding of 125I-LDL was defined as that bound in the presence of a 50-fold excess of unlabelled LDL or resistance to heparin displacement (13).

Kinetics Analysis—Because equations describing the kinetics of LDL binding to a lattice were not available, kinetic data were empirically fitted to simpler models as described below. The dissociation and association rates for specific binding of 125I-LDL were determined by nonlinear, least-squares fitting (NONLIN, C. M. Metzler, The Upjohn Co., Kalamazoo, MI). The goodness-of-fit for the various models used was assessed by comparing the estimated standard error for each model and by calculation of the F statistic (15, 16). For the more complex model to be justified, the estimated standard error decreased by at least a factor of two, and the F statistic exceeded the critical value for p < 0.05.

Association binding data were fitted to models that included one or two independent, saturable sites as described by Equations 1 or 2, respectively, as follows.

\[ B_i = B_{max}(1 - e^{-kt}) \]
\[ B_i = B_{max}(1 - e^{-kt}) + B_{max}e^{-kt} \]

In these equations, B is the amount of 125I-LDL bound at time (t), B is the amount bound at t = 0 in the one saturable-site model, and B and B are the amounts bound at t = 0 to the more rapidly or slowly dissociating sites, respectively. The dissociation rate constants are k and k.

The association data were obtained under pseudo-first-order conditions. Measurement of radioactivity in the media was done at the beginning and end of association to ensure that ligand concentrations did not change. Data were fitted to a one-saturable-site model or a model including one saturable and one nonsaturable, independent sites as described by Equations 3 or 4, respectively. Equation 4, which was developed empirically, is the simplest model that accurately described association data.

\[ B_i = B_{max}(1 - e^{-kt}) \]
\[ B_i = B_{max}(1 - e^{-kt}) + k \]

In Equations 3 and 4, B is the amount bound at time (t), B is the amount bound at t = infinity, and k are pseudo-first-order rate constants, and k is a zero-order rate constant. The pseudo-first-order rate constants did not increase linearly with the ligand concentrations, but their relationship could be described by the following scheme.

\[ k_1 < k_2 \]
\[ k_1 < k_2 < k_3 \]

LDL = k1[LDL - R] + k2[LDL - R] + k3[LDL - R]

SD SCHEME

If LDL and its receptor (R) rapidly form a complex [LDL-R] which slowly isomerizes to a complex with higher affinity [LDL-R]*, then Scheme 1 is described by Equation 5 (17). In Equation 5, k is the observed rate constant of association (k is k from Equation 4), K is the equilibrium dissociation constant for the first step, and [LDL-R] is the concentration of [LDL-R] at which the rate constant was observed. Equation 5 is valid if [LDL] >> [R], k >> k, and [LDL-R]* is measured experimentally. It is assumed that the intermediate, [LDL-R], is not measured. The overall equilibrium dissociation constant (K) for both steps was calculated using Equation 6 (17). It is apparent from Equation 5 that at LDL goes to infinity, k will plateau at a maximum value of k + k.

\[ k_{obs} = k_1 + k_2 + k_3(1 + K/[LDL]) \]

\[ K = K/k_1(k_2 + k_3) \]

RESULTS

Association Rates—The association of 125I-LDL to receptors was determined under pseudo-first-order conditions, i.e. ligand concentrations, which were in excess, did not change during the course of the experiment. Specific binding began to plateau by 60 min, but did not reach equilibrium, even at 360 min (Fig. 1A). A similar binding pattern was seen whether or not LDL receptors were further up-regulated using lovastatin, a cholesterol synthesis inhibitor (not shown). Interestingly, by Scatchard analysis of the data in Fig. 1A, the apparent binding affinity and receptor number estimate, based on the initial slopes, continued to increase gradually throughout the duration of association (Fig. 1B).

In Fig. 1, nonspecific binding was defined as radioactivity bound in the presence of a 50-fold excess of unlabelled LDL. When nonspecific binding was defined as resistance to heparin displacement, specific (heparin releasable) binding at 125I-LDL concentrations of less than 4.1 μg of protein/ml began to plateau after 60 min of association but failed to reach equilibrium by 300 min (Fig. 2A). At higher 125I-LDL concentrations, heparin releasable binding reached equilibrium (Fig. 2A), unlike corresponding data shown in Fig. 1. This difference was probably caused by a higher estimate of nonspecific binding using heparin displacement (see also Fig. 8). Scatchard analysis of the data in Fig. 2A showed that the apparent affinity (based on the initial slopes) increased with increasing association time (Fig. 2B). Thus, by two different estimates of specific binding, true equilibrium was not reached during 5–6 h of association (Figs. 1 and 2).

We were interested that the gradual increase in the amount of 125I-LDL bound after 60 min (Figs. 1A and 2A) resulted from binding to a another receptor, such as the LDL receptor-related protein (LRP) (18). However, studies of null fibroblasts, which express LRP normally but are incapable of expressing LDL receptors (19), showed that binding to other sites such as LRP was not an important factor. Specific
FIG. 1. Association of $^{125}$I-LDL to normal human fibroblasts at 4 °C. Cells that had grown to confluency in 35-mm plastic wells were incubated with cholesterol-depleted medium for 48 h to up-regulate LDL receptors prior to the assay. Cells in duplicate wells were incubated with media containing $^{125}$I-LDL ($d = 1.02-1.05$ g/ml) at concentrations from 0.19 to 25.4 μg of protein/ml, as indicated. After association, each well was washed rapidly five times to remove unbound radioactivity as described in “Experimental Procedures.” Nonspecific binding was defined as $^{125}$I-LDL bound in the presence of a 50-fold excess of unlabeled LDL. A, progress of specific $^{125}$I-LDL binding with time. B, Scatchard plots of the data in A taken after 20–360 min of association, as indicated.

FIG. 2. Association of $^{125}$I-LDL to normal human fibroblasts at 4 °C. The binding assay was performed as described in the Fig. 1 legend except that after the indicated association times, medium containing heparin (10 mg/ml) was incubated with cells for 60 min at 4 °C. Then, radioactivity released into the medium or still associated with the cells was measured. Only the heparin-releasable radioactivity is shown. B, Scatchard plots of the data in A after 20–300 min of association, as indicated.

FIG. 3. Association of $^{125}$I-LDL to null fibroblasts at 4 °C. Null fibroblasts that are incapable of expressing LDL receptors (12) were prepared as in the Fig. 1 legend except that in B, cells were also treated with lovastatin (1 μg/ml) for 24 h prior to the assay. Cells were incubated with media containing $^{125}$I-LDL at concentrations ranging from 0.22 to 7.9 μg of protein/ml, as indicated. Specific binding only is shown. Nonspecific binding was defined as $^{125}$I-LDL bound in the presence of a 50-fold excess of unlabeled LDL. Total binding at all concentrations tested was less than 2.5 ng (not shown).
binding of $^{125}\text{I}-\text{LDL}$ to null cells was low (Fig. 3A) and was not up-regulated by lovastatin (Fig. 3B). At all concentrations tested, binding of $^{125}\text{I}-\text{LDL}$ tended to increase with time, but the maximum increase in specific binding during the 360 min association was less than 1.6 ng; total binding was less than 2.5 ng (not shown).

The possibility that ligand heterogeneity contributed to the association kinetics was evaluated by studying the binding of LDL fractions. Seven fractions of the $d = 1.006-1.05$ g/ml fraction were isolated by ultracentrifugation. These fractions and their binding to LDL receptors at equilibrium are described in detail in a preceding paper (5). Fraction 1 particles had an average diameter of 27 nm and bound to a greater extent via apoE than apoB-100, whereas fraction 7 particles had an average diameter of 20 nm and bound exclusively via apoB-100. Fractions 5–7 contained approximately 80% of the $d = 1.006-1.05$ g/ml protein. Therefore, unfraccionated LDL ($d = 1.02-1.05$ g/ml) was a mixture of smaller particles and contained very few larger particles that were present in fractions 1–3 (5). At all association times and ligand concentrations tested, fewer large (fraction 1) than small LDL (fraction 4) were bound (Fig. 4). This was consistent with the presence of steric hindrance during binding as described in the lattice model proposed previously (5). Fig. 4 also illustrates that the association of $^{125}\text{I}$-fractions failed to reach true equilibrium by 240 min. Since similar association curves were observed for both unfraccionated and fractionated LDL (Figs. 1, 2, and 4), ligand heterogeneity did not appear to be a confounding factor.

Due to the slow increase in the terminal phase of binding (Figs. 1, 2, and 4), association curves deviated from the predictions of a one-saturable-site model (Equation 3). Therefore, data were fitted to Equation 4 which has an additional nonsaturable site that empirically described the terminal, linear phase of binding. The observed rate constants for the nonsaturable site ($k_o$, Equation 4) were considerably smaller and less sensitive to changes in ligand concentrations than those for the saturable site ($k_s$, Equation 4). Because binding during the linear phase was much larger than binding to null cells under similar conditions (Fig. 3), it could not be attributed to nonspecific binding or binding to sites other than LDL receptors. Instead, it was probably due to slow parking and reparking of ligands on the lattice as saturation was approached (see “Discussion”).

Observed rate constants of association ($k_s$ in Equation 4 or $k_{obs}$ in Equation 5) did not increase linearly with increases in ligand concentrations. Fig. 5 shows that $k_{obs}$ began to plateau as unfraccionated $^{125}\text{I}-\text{LDL}$ concentrations were increased which is inconsistent with simple bimolecular binding. This surprising finding first suggested to us the isomerization mechanism in Scheme 1 which was tested by the dissociation experiments described below.

**Dissociation Rates**—The possibility that more than one class of binding sites contributed to LDL binding seems unlikely because of the inability of null cells to bind substantial amounts of LDL (Fig. 3). To test negative cooperativity and receptor cross-linking as mechanisms for LDL binding, dissociation kinetics were studied. A common type of negative cooperativity is a ligand-induced conformational change in adjacent receptors that decreases their affinity (20). This type of negative cooperativity may cause the dissociation rate to increase with increases in receptor occupancy. In a receptor cross-linking model, a multivalent ligand may cross-link receptors, but as receptor occupancy increases, univalent binding becomes more predominant and is manifest by a more rapid dissociation rate. Therefore, with respect to dissociation kinetics, negative cooperativity may be indistinguishable from receptor cross-linking (21–23).

However, the dissociation rates of $^{125}\text{I}$-LDL were similar at four different levels of receptor occupancy (Fig. 6 and Table I). Furthermore, inclusion of an excess of unlabeled LDL (100 $\mu$g of protein/ml) in the dissociation medium did not increase the dissociation rate (not shown). Thus, neither negative cooperativity nor receptor cross-linking of the types described above are likely to have been present. Nonspecific binding was less than 10% of the total when measured as binding in the presence of a 50-fold excess of unlabeled LDL and less than 20% of the total binding when measured as the radioactivity that was resistant to heparin displacement (not shown). Nonspecific binding did not change much during dissociation.

The dissociation was biphasic. A model with one saturable site (Equation 1) could not describe the dissociation of $^{125}\text{I}$-LDL as well as a model with two saturable sites (Equation 2).

**FIG. 4.** Association of $^{125}\text{I}$-LDL fractions to normal human fibroblasts at 4°C. The assay was performed as described in the Fig. 1 legend. LDL fractions were isolated by ultracentrifugation of the $d = 1.006-1.05$ g/ml lipoproteins in an SW41 rotor (Beckman). Fraction 2 particles were 27 nm in diameter and bound via both apoE and apoB-100, whereas fraction 4 particles were 23 nm in diameter and bound predominantly via apoB-100 (5). Media containing $^{125}\text{I}$-labeled fraction 2 (dashed lines) or fraction 4 (solid lines) at the concentrations indicated was allowed to associated with cells. Non-specific binding was defined as $^{125}\text{I}$-fraction 2 or 4 bound in the presence of a 50-fold excess of unlabeled LDL.

**FIG. 5.** Relationship of the observed pseudo-first-order rate constants of association ($k_{obs}$) with $^{125}\text{I}$-LDL concentration. Association data for $^{125}\text{I}$-LDL binding at 4°C under pseudo-first-order conditions were fitted as described under “Experimental Procedures.” The observed rate constants ($k_s$, from Equation 4) did not increase linearly with ligand concentrations but instead, began to plateau as predicted by an isomerization model (Scheme 1). The solid line is the best fit to Equation 5 which describes such a model. Rate constants were obtained from $^{125}\text{I}$-LDL binding shown in Fig. 2. Kinetic parameters derived from the isomerization model are summarized in Table III.
FIG. 6. Dissociation of 125I-LDL from normal human fibroblasts at 4 °C at four levels of receptor occupancy. Media containing 125I-LDL at various concentrations, as indicated, were incubated with cells in duplicate wells for 60 min. After association, each well was washed rapidly five times with ice-cold buffer and incubated with fresh dissociation buffer for the times indicated. Specific binding is expressed as the percentage bound at t = 0. Nonspecific binding was defined as radioactivity that was resistant to heparin displacement. Total binding is not shown. A, after association of 125I-LDL at concentrations of 0.4, 3.9, 13, or 39 μg of protein/ml, the specifically bound 125I-LDL at t = 0 was 3.6, 15, 24, or 31 ng, respectively. B, data shown in A (□) for the dissociation of 125I-LDL after association at a concentration of 39 μg/ml were fitted to one (dashed line) or two (solid line) saturable-site models as described under "Experimental Procedures." The two-site model (Equation 2) described the data better as indicated by a decrease in the estimated standard error by a factor of four and an F statistic which exceeded the critical value for p < 0.01. Binding parameters derived from the two-site model are shown in Table I.

TABLE I

125I-LDL dissociation parameters after 60 min of binding to fibroblasts

<table>
<thead>
<tr>
<th>125I-LDL concentration (μg/ml)</th>
<th>Specific binding (ng)</th>
<th>B1</th>
<th>B2</th>
<th>k1 (10⁻² min⁻¹)</th>
<th>k2 (10⁻² min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>3.6</td>
<td>23</td>
<td>77</td>
<td>3.3</td>
<td>1.8</td>
</tr>
<tr>
<td>3.9</td>
<td>15</td>
<td>(2)</td>
<td>(1)</td>
<td>(2.7)</td>
<td>(0.2)</td>
</tr>
<tr>
<td>13</td>
<td>24</td>
<td>18</td>
<td>82</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>39</td>
<td>31</td>
<td>19</td>
<td>80</td>
<td>0.8</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* 125I-LDL at the concentrations indicated were allowed to associate to cells for 60 min prior to beginning the dissociation.

† Specific binding per well at the beginning of dissociation.

‡ B1, B2, k1, and k2 are the amount of 125I-LDL bound and the rate constants derived for the rapidly or slowly dissociating sites, respectively. Standard deviations are in parentheses.

For example, data from Fig. 6A (open squares) were fitted to one- or two-site models to generate the lines shown in Fig. 6B. Predicted rate constants (Table I) for the more rapidly dissociating site (k2) were on average 110-fold higher (1.6 × 10⁻² min⁻¹ vs. 1.4 × 10⁻¹ min⁻¹) than those for the second site (k1). The percentage of 125I-LDL bound to the more slowly dissociating site (B2) was 4-fold greater than B1. Neither the rate constants nor the percentage bound to each site were much affected by changes in the degree of receptor occupancy (Table I). However, contrary to the prediction of models including negative cooperativity or receptor cross-linking, both k1 and k2 tended to decrease with increasing receptor occupancy.

Dissociation rates of 125I-LDL fractions 1 and 7 did not vary much at three different levels of receptor occupancy (Fig. 7). Although fraction 1 particles bound via both apoB and apoB-100, their dissociation rate was not accelerated by a 3-fold increase in receptor occupancy (Fig. 7 and Table II) as might be expected if they were to cross-link receptors. On average, 91% of large LDL bound to the more slowly dissociating site (B2) which had a rate constant (k2) that was about one-fourth that of small LDL (0.38 vs. 1.4 × 10⁻³ min⁻¹, Table II). Rate constants and percentages bound to the more rapidly and slowly dissociating sites for small or unfractionated LDL were in general agreement (Figs. 6 and 7, Tables I and II).

Isomerization Model—The failure of kobs to increase linearly with 125I-LDL concentrations (Fig. 5) does not suggest simple bimolecular binding to one or two independent saturable sites. However, it is a pattern predicted by an isomerization mech-
anism in which the initial binding of $^{125}$I-LDL is followed by slower isomerization to a complex with higher affinity, as described in Scheme 1 (17). Data fitted to Equation 5, which describes such a model, generated the rate and equilibrium dissociation constants shown in Table III. The equilibrium dissociation constant ($K = 24 \mu g$ of protein/ml) predicted for the first step in Scheme 1 was 16-fold larger than the overall $K_D$ for both steps ($K_D = 1.5 \mu g$ of protein/ml). The latter agrees more closely with the $K_D$ for the high-affinity sites of Scatchard plots (0.5 $\mu g$ of protein/ml) (5). The isomerization model (Scheme 1) predicts that the initial receptor-ligand complex should dissociate relatively rap-ldy, whereas the complex formed by isomerization should dissociate more slowly (17). To test this, we varied the association time by incubating $^{125}$I-LDL (5 $\mu g$ of protein/ml) with fibroblasts at 4 °C for 2, 15, or 120 min and then followed the dissociation rates (Fig. 8). The percentage of $^{125}$I-LDL that dissociated rapidly was greatest after 2 min of binding (36%) and smallest after 120 min (11%) (Table IV). Two independent estimates of nonspecific binding were used (Fig. 8, A and B). The percentage of $^{125}$I-LDL that was resistant to displacement by a 50-fold excess of unlabeled LDL was less than that resistant to heparin displacement. However, nonspecific binding was highest after association for 2 min when measured using the former technique (Fig. 8A), and lowest when measured by the latter technique (Fig. 8B).

Binding parameters derived from Fig. 8 illustrate the time-dependence of $^{125}$I-LDL binding to the more rapidly or slowly dissociating sites (Table IV). Total receptor occupancy increased by approximately 3-fold as association time increased from 2 to 120 min. This increase was due primarily to binding to the slowly dissociating site ($B_2$). Binding to the rapidly dissociating site ($B_1$) was relatively unchanged. When expressed as the percentage of total binding, $B_1$ decreased, whereas $B_2$ increased with association time. These findings were confirmed in other experiments and contrasted with the relatively small changes in $B_1$ and $B_2$ when receptor occupancy was varied while keeping the association time constant at 60 min (compare Figs. 6 and 8). The dissociation rate constants for the two sites decreased slightly with longer association times (Table IV). The average value for $k_1$ was $0.78 \times 10^{-1}$ min$^{-1}$ which was 64-fold larger than the average value of $k_2$(1.2 $\times 10^{-3}$ min$^{-1}$).

In contrast to small LDL (fraction 7), large LDL (fraction 1) did not undergo a time-dependent isomerization to higher affinity during binding (Fig. 9). After associating for 2 min, 90% of large LDL dissociated slowly, whereas after 120 min of binding 92% dissociated slowly. Receptor occupancy after 2 versus 120 min of binding increased 2-fold from 13 to 28 ng, and rate constants were close to those shown for large LDL in Table II (average $k_1 = 1.1 \times 10^{-1}$ min$^{-1}$, average $k_2 = 0.31 \times 10^{-3}$ min$^{-1}$). Isomerization during binding of small LDL (Fig. 9) was similar to that of unfractionated LDL (Fig. 8 and Table IV) with 31, 21, and 13% dissociating rapidly after 2, 15, or 120 min of association, respectively. Receptor occupancy after 2 versus 120 min of binding increased 4.5-fold from 7.3 to 33 ng, and rate constants were close to those for unfractionated LDL in Table IV (average $k_1 = 0.51 \times 10^{-1}$ min$^{-1}$, average $k_2 = 1.5 \times 10^{-3}$ min$^{-1}$).

**DISCUSSION**

Our studies of LDL binding kinetics have revealed a binding mechanism that could not have been suspected from studies at equilibrium, namely that isomerization to a higher affinity occurs during apoB-100 binding to LDL receptors. In addition, the kinetics of LDL binding is consistent with a lattice model, but not with other explanations for concave-upward Scatchard plots including negative cooperativity, two independent saturable sites, or receptor cross-linking. Dissociation rates did not increase with increases in receptor occupancy (Fig. 6A and Table I). Therefore, negative cooperativity in which LDL binding induces a conformational change in adjacent receptors is unlikely (20). Lovastatin treatment up-regulated LDL binding but had no other effect on equilibrium (5) or kinetic binding parameters. Since LDL receptors are the only known sterol-responsive binding sites for LDL (1, 19), regulation of binding by lovastatin indicates that other receptors were probably not involved. Also, binding to null.

**TABLE II**

$^{125}$I-LDL fractions dissociation parameters after 60 min of binding to fibroblasts at 4 °C

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific binding</th>
<th>Bound</th>
<th>Rate constants</th>
</tr>
</thead>
<tbody>
<tr>
<td>dg/ml</td>
<td>ng</td>
<td>%</td>
<td>$10^{-1}$ min$^{-1}$</td>
</tr>
<tr>
<td>0.25</td>
<td>9.9</td>
<td>8.6</td>
<td>91</td>
</tr>
<tr>
<td>0.91</td>
<td>16</td>
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<td>92</td>
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<td>8.8</td>
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</tr>
<tr>
<td>1.0</td>
<td>18</td>
<td>20</td>
<td>79</td>
</tr>
<tr>
<td>9.9</td>
<td>44</td>
<td>17</td>
<td>82</td>
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</tbody>
</table>

$^{125}$I-LDL fraction 1 or 7 at the concentrations indicated were allowed to associate to cells for 60 min prior to beginning the dissociation.

$^*$Specific binding per well at the beginning of dissociation.

$^*$Parameters derived from an isomerization model for LDL binding

Pseudo-first-order rate constants of association derived from a model including one saturable and one nonsaturable site (Equation 4) were fitted to the isomerization model described by Scheme 1 and Equation 5. Experiment 1 is shown in Fig. 1; experiment 4 is shown in Fig. 2. Standard deviations are shown in parentheses. Correlation coefficients for each fit were 0.99 or greater.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$K^*$</th>
<th>$k_1$</th>
<th>$k_2$</th>
<th>$K_D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>mu protein/ml</td>
<td>min$^{-1}$</td>
<td>$10^{-1}$ min$^{-1}$</td>
<td>$10^{-3}$ min$^{-1}$</td>
<td>mu protein/ml</td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>1.8</td>
<td>(0.3)</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>29</td>
<td>1.3</td>
<td>(0.3)</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>22</td>
<td>1.1</td>
<td>(0.6)</td>
<td>0.72</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>0.5</td>
<td>(0.1)</td>
<td>4.4</td>
</tr>
<tr>
<td>Mean</td>
<td>24</td>
<td>1.2</td>
<td>(0.1)</td>
<td>0.77</td>
</tr>
</tbody>
</table>

$^*$K is the equilibrium dissociation constant ($k_0/k_1$) for the first step in the isomerization model. $k_1$ and $k_0$ are the forward and reverse rate constants for the second step in the isomerization model, respectively. $K_D$ is the equilibrium dissociation constant for both steps in the isomerization model and is calculated using Equation 6.
bound to two independent sites. A more plausible explanation is that initial LDL receptor-ligand complexes slowly rearrange over the cell surface (1, 24). Theoretically, these two populations of LDL receptors could interact with LDL differently, but as described below, binding kinetics do not support the existence of two independent, saturable sites.

Pseudo-first-order rate constants of association \( k_{\text{on}} \) did not increase linearly with increases in LDL concentrations (Fig. 5). This suggests an isomerization mechanism but not simple bimolecular binding to one or two independent, saturable sites (17). The percentage of LDL that dissociated rapidly was dependent on the duration of association (Figs. 8 and 9) but not on the degree of receptor occupancy (Figs. 6 and 7). These observations are difficult to explain if LDL bound to two independent sites. A more plausible explanation is that initial LDL receptor-ligand complexes slowly rearrange to form complexes with higher binding affinity which is evident by slower dissociation.

Cross-linking of LDL receptors by apoE-HDL, a multivalent lipoprotein, has been proposed by other investigators (2, 3). Receptor cross-linking is a potential factor in our studies because both apoE and apoB-100 contributed to binding by large LDL (fractions 1–3) (5). Kinetically, receptor cross-linking by a multivalent ligand may occur by relatively slow, univalent binding followed by more rapid cross-linking to other sites. The cross-linking step is usually faster because the effective concentration of receptors on the cell surface is greater than in free solution (21–23). Because univalent binding is usually rate limiting, the observed rate constant of association \( k_{\text{on}} \) increases linearly with increasing ligand concentrations (22). However, \( k_{\text{on}} \) for LDL association did not follow this pattern (Fig. 5).

Another difference between LDL binding and a cross-linking model is the dissociation kinetics. In a receptor cross-
ligand binding to asialoglycoprotein receptors, which may also potentially mimic isomerization kinetics in Scheme 1. However, neither the dissociation rate of unfractinated LDL nor LDL fractions varied with the degree of receptor occupancy (Figs. 6 and 7). On the contrary, the percentage of rapidly dissociating LDL was dependent on the duration of ligand association (Fig. 8 and Table IV). Theoretically, the cross-linking step could be slower than the rate of univalent binding (21–23). This mechanism of receptor cross-linking may partially mimic isomerization kinetics in Scheme 1. However, only large LDL, which account for a small percentage of the total LDL, appear to be multivalent by virtue of being able to bind via either apoE or apoB-100 (5), and large LDL did not bind via an isomerization mechanism. The vast majority of LDL in the d = 1.02–1.05 g/ml fraction are small LDL which bind exclusively via apoB-100 (5). ApoB-100 exists at a ratio of one molecule/particle and appears to be univalent based on inhibition of binding by mutation of a single amino acid or by a monoclonal antibody (5, 25–28).

One feature of LDL binding kinetics that is characteristic of binding to lattices is the slow approach to equilibrium. LDL binding did not reach true equilibrium by 5–6 h (Figs. 1, 2, and 4). Receptor lattices resist saturation because repositioning of bound ligands is necessary to maximize the accessibility of receptors for further binding (6, 29–31). For example, in Fig. 10A, two LDL particles are positioned on a lattice of four receptors such that a third particle cannot easily bind to one site (designated R*). With time, R* may become more accessible (Fig. 10B). Such repositioning is a general feature of binding to lattices and probably accounts for the gradual increase in LDL binding after 60 min of association which was empirically described by the nonsaturable site in Equation 4. Although LDL binding is said to reach equilibrium in 1–2 h (13), previously published association curves do not support this conclusion (2, 32). Of interest, ligand binding to asialoglycoprotein receptors, which may also conform to a lattice model, does not reach equilibrium under similar conditions to those used here (33). Experimentally achievable “equilibrium” conditions for LDL binding at 4 °C probably only approximate the true equilibrium. Based on Scatchard plots, the apparent affinity of the high affinity sites, as well as their number, continue to increase slowly with increasing association time (Figs. 1B and 2B).

Isomerization during apoB-100 binding (Scheme 1) was first suggested by the nonlinear increase in the apparent pseudo-first-order rate constants of association (Fig. 5) (17) and confirmed by the dependence of LDL dissociation on the duration of association (Figs. 8 and 9). These data indicate the existence of a time-dependent rearrangement of apoB-100/receptor complexes to more slowly dissociating complexes. Scheme 1, as used here, describes the rapid formation of the initial complex with slower isomerization to a higher affinity binding state. This mechanism of binding is common and includes binding to heme proteins, flavoproteins, and enzymes (34–36), muscarinic receptors (37–39), adrenergic receptors (40), insulin receptors (41), and bacterial periplasmic proteins (42). Of interest, there is precedence for ligand binding to a lattice to behave kinetically as predicted by Scheme 1. Certain drugs, including some acridines and tilorone, which intercalate into DNA are an example (43–45). Binding to DNA can be described by a one-dimensional lattice model in which intercalated drugs exclude binding to a variable number of adjacent base pairs. For some acridines and tilorone, but not for all drugs that intercalate into DNA, the kinetics of binding is consistent with Scheme 1 in which the isomerization step appears to involve transfer of the drug between the major and minor grooves of DNA (43–45).

In Equation 5, it was assumed that only the high affinity, slowly dissociating complex [LDL-R]* in Scheme 1 was measured experimentally (17). However, the data in Figs. 8 and 9 suggest that the rate of isomerization was slow enough that over 30% of the LDL was bound to the more rapidly dissociating intermediate [LDL-R] after 2 min of association and
decreased to 11% at 120 min of association (Table IV). Thus, although isomerization is suggested by the curvilinear relationship between the observed association rate constants ($k_{on}$) and LDL concentrations (Fig. 5), the magnitude of the binding parameters calculated using Equation 5 (Table III) should be interpreted with caution. Regardless of the limitations of the model used to fit the data in Fig. 5, the isomerization mechanism was demonstrated by the dependence of small and unfractionated LDL dissociation on the duration of association (Figs. 8 and 9). These experiments do not rely on the assumptions used in Equation 5 and independently confirm that isomerization was occurring.

In contrast to small LDL, isomerization to a higher affinity was not apparent during binding of large LDL. Varying the duration of association had no effect on dissociation of large LDL in fraction 1 (Fig. 9). Although it is possible that isomerization occurred too rapidly to be detected, it seems more likely that large and small LDL bind to receptors via different mechanisms. On average, the binding of fraction 1 particles was inhibited 37% by 1D7, a monoclonal antibody that specifically blocks apoE-mediated binding, whereas 4G3, a monoclonal antibody that specifically blocks apoB-100-mediated binding, caused 20% inhibition (5). Although both apoB-100 and apoE were present on large LDL, apoE binding predominated. The slower dissociation rate ($k_d$) of large LDL was one-fourth that of small LDL (Table II), in agreement with our previous estimate of the difference between apoE and apoB-100 dissociation rates (2). Taken together, these data suggest that apoE and apoB-100 bind to LDL receptors by different mechanisms.

The molecular basis for different mechanisms of large versus small LDL binding is unknown, but there are several interesting possibilities that could be tested in future experiments. For example, apoE and apoB-100 could interact with different sites on the LDL receptor molecule. There are seven cysteine-rich repeats that mediate binding to LDL receptors (46, 47). Because deletion or mutation of repeats 3–7 markedly decreased apoB-100 binding, but with the exception of repeat 5, apoE binding was relatively unaffected, Russell et al. (47) concluded that different combinations of repeats mediated binding to different ligands. It is also possible that apoB-100, which is a very large protein of $M_r$, 550,000, has a low affinity binding domain or conformation that forms the initial complex with LDL receptors followed by rearrangement of the ligand, receptor, or both to form the final high affinity binding state. ApoE, which is much smaller ($M_r$, 35,000), might not manifest such behavior.

Apart from the mechanism of isomerization, the physiologic significance of isomerization during binding of LDL is unknown but potentially important. The affinity of LDL for cell-surface receptors at 37 °C is said to be 10–15 μg of protein/ml (1). However, due to continuous internalization of LDL by cells at 37 °C, it is unlikely that equilibrium conditions existed as required for accurate measurements of binding affinities by Scatchard plots. In addition, van Driel et al. (48) showed that LDL receptors in a solid-phase assay displayed the same affinity for LDL at 4 and 37 °C. The isomerization model predicts that the initial complex of apoB-100 with receptors is formed rapidly. Therefore, it is intriguing that the estimated affinity at 37 °C is closer to the lower affinity complex (24 μg of protein/ml) than the overall $K_D$ (1.5 μg of protein/ml) (Table III). It is possible that the affinity of the initial complex of LDL with receptors is the functional affinity in vivo. Studies are planned at physiologic temperatures to test this hypothesis.

In summary, a lattice model in which isomerization occurs is the best model to describe both equilibrium and kinetic studies of LDL binding. If receptor cross-linking occurs, it is not manifest kinetically by slower univalent binding followed by faster cross-linking and does not account for the dependence on LDL size of the apparent number of high affinity sites at equilibrium. We found no evidence for cooperativity other than that due to steric hindrance. In contrast to small LDL, isomerization did not occur during binding of large LDL which bind via apoE to a greater extent than apoB-100. This suggests that apoE and apoB-100 bind by different mechanisms. More studies are needed to determine the molecular basis for the apparent isomerization during apoB-100 binding and to determine binding kinetics by metabolically active cells at 37 °C.

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REFERENCES

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