Quantitative Dissection of the Binding Contributions of Ligand Lysines of the Receptor-associated Protein (RAP) to the Low Density Lipoprotein Receptor-related Protein (LRP1)*

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Background: Only small effects on affinity are seen for seemingly critical lysines of LRP1 ligand RAP.

Results: Lysines act in pairs and fully account for overall affinity to LRP1. Mutagenesis reduces binding up to 100,000-fold.

Conclusion: Two pairs of paired lysine interactions can give high affinity binding of protein ligands to LRP1.

Significance: Rules for identifying high affinity receptor binding sites are established.

Although lysines are known to be critical for ligand binding to LDL receptor family receptors, relatively small reductions in affinity have been found when such lysines have been mutated. To resolve this paradox, we have examined the specific binding contributions of four lysines, Lys-253, Lys-256, Lys-270, and Lys-289, in the third domain (D3) of receptor-associated protein (RAP), by eliminating all other lysine residues. Using D3 variants containing lysine subsets, we examined binding to the high affinity fragment CR56 from LRP1. With this simplification, we found that elimination of the lysine pairs Lys-253/Lys-256 and Lys-270/Lys-289 resulted in increases in $K_d$ of 1240- and 100,000-fold, respectively. Each pair contributed additively to overall affinity, with 61% from Lys-270/Lys-289 and 39% from Lys-253/Lys-256. Furthermore, the Lys-270/Lys-289 pair alone could bind different single CR domains with similar affinity. Within the pairs, binding contributions of Lys-270 > Lys-256 > Lys-253 ≈ Lys-289 were deduced. Importantly, however, Lys-289 could significantly compensate for the loss of Lys-270, thus explaining how previous studies have underestimated the importance of Lys-270. Calorimetry showed that favorable enthalpy, from Lys-256 and Lys-270, overwhelmingly drives binding, offset by unfavorable entropy. Our findings support a mode of ligand binding in which a proximal pair of lysines engages the negatively charged pocket of a CR domain, with two such pairs of interactions (requiring two CR domains), appropriately separated, being alone sufficient to provide the low nanomolar affinity found for most protein ligands of LDL receptor family members.

The low density lipoprotein receptor-related protein (LRP1)2 is a constitutively active receptor of the LDL receptor family and is capable of binding a wide range of structurally distinct protein ligands (1). Binding occurs to regions within the receptor that are composed of multiple copies of small, disulfide-rich, calcium-dependent domains, termed complement-like repeats (CR). Given the importance of LRP1, as well as of relatives such as LDLR and very low density lipoprotein receptor, in both ligand clearance and signaling functions, it is critical to understand on a quantitative level the molecular basis for the ability of a given member to bind unrelated proteins with high affinity.

From a number of mutagenesis studies on different receptor ligands, it is clear that ligand lysines are important for binding (2–12). However, if lysines alone are necessary and sufficient for high affinity binding of ligands in the low nanomolar range, then mutation of critical lysines should have correspondingly large effects on overall ligand affinity. Where quantitation has been carried out, this does not appear to be the case. Thus, the definitive study on the third domain (D3) of the receptor-associated protein (RAP) that established Lys-256 and Lys-270 as critically important lysines for binding of RAP to LRP1 found reductions in affinity of only 26- and 28-fold, respectively, for K256A and K270E mutations, representing losses of only ~17% each in binding energy. Another group found a similar 17% reduction in binding energy for the K256A mutation but only a 12% reduction for a K270A mutation (10). Even more perplexing are mutagenesis studies on the interaction of the serpin plasminogen activator inhibitor-1 with LRP1 in which mutation of a number of plausible receptor-binding lysines or arginines, either singly or in combination, reduced affinity, but by no more than a factor of 3, corresponding to a cumulative loss of less than 7% of the binding energy (13).

An x-ray structure of D3 in complex with a two-CR domain ligand-binding fragment (LB34) from LDLR sheds some light on this question. The structure shows that lysines 256 and 270 on D3 engage clustered acidic residues in each CR domain and stack with the single aromatic side chain present in each of the two CR domains (14), with other potentially important interactions coming from two additional, immediately proximal lysines: Lys-253 adjacent to Lys-256 and Lys-289 adjacent to Lys-270. Although there might be some concern that RAP is not a true physiological ligand for LDLR (in contrast to other family members such as LRP1) and binds to LDLR with a $K_d$ of...
only ~0.5 μM (15) compared with ~3 nM to LRP1, the clear importance of Lys-256 and Lys-270 in the complex structure qualitatively parallels the conclusions from the mutagenesis studies on the RAP-LRP1 system (4). This suggests that the contacts observed in the D3-LB34 complex are indeed the critical ones and may also apply to the RAP-LRP1 system as well. If this is so, however, it fails to explain why Lys-256 and Lys-270 together account for no more than 29–34% of the binding energy. Do Lys-253 and Lys-289 make up the remaining 66–71% of the binding energy, or is there compensation by other lysines in D3? One possibility is that, given that CR domains are joined to one another by short flexible linkers (16), there may be sufficient scope for repositioning that permits an alternative, albeit somewhat lower affinity site to replace the mutated one. In RAP D3 alone there are six lysines in addition to Lys-253, Lys-256, Lys-270, and Lys-289.

To unambiguously assess the quantitative roles of Lys-253, Lys-256, Lys-270, and Lys-289 of D3 in binding to LRP1, we have taken a different mutagenesis approach from others. In this approach, we have eliminated all lysines in D3 except those being directly examined. This involved making D3 variants containing either all four of Lys-253, Lys-256, Lys-270, and Lys-289, or else subsets in which one or two of these lysines were selectively removed. The results of binding studies of these species to the high affinity fragment CR56 from LRP1 show that these lysines alone are sufficient to account for all of the binding energy and that Lys-256 and Lys-270 together account for 83% of the energy of interaction. The finding that Lys-289, although alone relatively unimportant, can partially compensate for the loss of Lys-270 establishes the feasibility of lysine substitution and so provides an answer to earlier underestimation of the importance of critical lysines in D3 and perhaps other LRP ligands such as plasminogen activator inhibitor-1.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis of RAP D3**—The cDNA encoding the third domain of RAP (D3, residues 206–323) was cloned in pQE30, modified to contain a tobacco etch virus cleavage site (17). All mutagenesis was performed using the QuickChange protocol (Agilent Technologies). First, lysines at positions 238, 248, 262, 298, 305, and 306 were changed to alanines to generate the variant K4, in which the only lysines remaining were at positions 253, 256, 270, and 289. K4 served as the template for subsequent mutagenesis to yield the various K3 and K2 variants. All mutations were verified by DNA sequencing before protein expression.

**Expression and Purification of D3 Variants**—Wild type and lysine-containing variants of RAP D3 were expressed as previously described (17). D3 was expressed in SG13009 cells, grown in 2YT medium to A600 of 0.6–1.0 and induced with 1 mM isopropyl β-D-thiogalactopyranoside. The cells were harvested after 4–5 h. After sonication, the clarified lysate was loaded on GSH-Sepharose, eluted with 5 mM GSH, and cleaved with tobacco etch virus protease while dialyzing at 4 °C overnight against 4 × 4 liters of 20 mM Tris-HCl, 50 mM NaCl, 4 mM EDTA, pH 8.0. Untagged CR56 was found in the flow through from a second passage on the GSH column. Final purification was obtained by ion exchange chromatography. CR56 was dialyzed against 20 mM Tris-HCl, 1 mM CaCl2, pH 7.4, the mixture was loaded on a GSH-Sepharose column. Folded CR56 was eluted from the column-bound GST-RAP with 8 mM β-mercaptoethanol, untagged CR56 was eluted with a gradient of 0–1000 mM NaCl in 20 mM Tris-HCl, 6 mM urea, 0.1% β-mercaptoethanol, pH 8.0.

CR56 (0.1–0.2 mg/ml final concentration) was folded in the presence of an equimolar amount of GST-RAP by dialysis against 50 mM Tris-HCl, 10 mM CaCl2, pH 8.5, containing 0.1% β-mercaptoethanol and 0.1% 2-hydroxyethylsulfide for 24 h at room temperature, followed by 24 h at 4 °C. After dialysis against 20 mM Tris-HCl, 50 mM NaCl, 1 mM CaCl2, pH 7.4, the mixture was loaded on a GSH-Sepharose column. Folded CR56 was eluted from the column-bound GST-RAP with 8 mM β-mercaptoethanol, pH 8.0, followed by reverse phase chromatography on a Discovery BIO wide bore C18 column, eluted with a gradient of 9–72% acetonitrile in 0.1% TFA. A stoichiometric titration of CR56 with D3 was carried out to determine the fraction of CR56 that was correctly folded and had high affinity, assuming that the stoichiometry of complex between WT D3 and CR56 was 1:1 (this had been previously confirmed by analytical ultracentrifugation studies (17)). The sample used in the present study was 44% active. All concentrations of CR56 reported and used in the fitting of experimental data are of fully active material.

**Isothermal Titration Calorimetry**—ITC experiments were performed on a VP-ITC instrument (MicroCal). All proteins were dialyzed against the same 2 × 4 liters of 20 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl2, 0.02% NaN3, pH 7.4, and an aliquot of the latter dialysis buffer was used for dilutions and blank experiments, as necessary. CR56 (1–2 μM active concentration) was titrated with 5–10-μl injections of D3 (25–135 μM). Active concentration was measured by a stoichiometric titration of WT D3 with CR56 and corroborated the value of 44% found separately by fluorescence titration. Binding isotherms were fitted to a single binding site model using Origin, with the value of n, the number of binding sites per CR56, as a variable. An n value of 1 thus indicates that concentration of binding sites equals the CR56 concentration modified by a factor of 0.44 to take into account fully active high affinity material. It should be noted that in none of the titrations was there any evidence for a second weaker binding to the residual 56% of CR56 that was not active.
**CD of D3 Variants**—CD spectra of wild type D3 and of variants K4, K3-1, and K2-1 were recorded using a Jasco 710 CD spectrometer. Proteins were dialyzed against 4×4 liters of 10 mM NaP, pH 7.4, prior to analysis. Spectra were recorded at a protein concentration of 10 μM, scanning from 260 to 195 nm with 0.5-nm steps and averaging five scans.

**Fluorescence Spectroscopy**—All fluorescence measurements were made on a PTI Quantumaster spectrophotometer, equipped with double monochromators on both excitation and emission sides. Excitation was at 280 nm for emission spectra. For measurements of emission intensity at a single wavelength, used in ligand titrations, excitation was at 280 nm, except for weak binding ligands, where excitation was at 290 or 295 nm to avoid inner filter effects from the higher protein concentrations used. For single wavelength measurements, the emission wavelength was chosen as the wavelength of maximum change (335 nm). For emission spectra, slits of 1 nm for excitation and 2 nm for emission were used. For single wavelength measurements, slits of 1 nm for excitation and 8–10 nm for emission were used.

Samples were 1.2 ml in 1-cm cuvettes and contained 0.1–3 μM CR56, depending on the anticipated Kd. The pH 7.4 buffer contained 20 mM Tris-HCl, 1 mM CaCl2, and 0.1% PEG 8000. NaCl was added from a stock 5 M solution according to the desired final ionic strength.

Emission spectra were recorded from 310 to 450 nm in 2-nm steps with 2 s of dwell time and were the averages of three scans. Single wavelength measurements at a given ligand concentration were made over 120 s and averaged. The data in each case were corrected for contributions from a buffer blank. Correction to the fluorescence intensity was made for contributions from the added D3 and for any dilution of the protein resulting from the addition of D3 ligand. In most cases such dilution was less than 2%. Temperature was maintained by a circulating water bath. All titrations were carried out at 298 K.

Kd values were determined by nonlinear least squares fitting of the binding data to a simple single-site binding isotherm. Fitting was done in KaleidaGraph (Synergy Software). Titrations were carried out a minimum of three times, with means and ranges given in Table 1.

## RESULTS

### Effect of D3 Mutations on Structure—RAP D3 is a 110-residue three-helical bundle that contains 10 lysine residues and a single tryptophan (18). In the complex of D3 with the two CR domains, LB3 and LB4, from LDLR, contact occurs between lysines 253 and 256 and aspartates 147, 149, and 151 surrounding the Ca2+-binding pocket of LB4 and between lysines 270 and 289 and equivalent aspartates (108, 110, and 112) on LB3 (Fig. 1) (14). An additional contact is with an aromatic side chain that is tryptophan in LB4 and phenylalanine in LB3 (19).

For the present study, D3 variants were created in which only these four lysines (K4) or the various permutations of three (K3-x) or two (K2-x) of these lysines remained (Table 1), with the other lysines being mutated to alanine.

### Table 1

**Binding of D3 variants to CR56 monitored by fluorescence**

| Abbreviation | 253 | 256 | 270 | 289 | ΔFmax | Kd | ΔG° |
|--------------|-----|-----|-----|-----|-------|----|------|
| WT           | K   | K   | K   | K   | 83 ± 10 | 1 ± 0.4 | -12.3 ± 0.5 |
| K4           | K   | K   | K   | K   | 84 ± 8  | 5 ± 2 | -11.4 ± 0.3 |
| K3-1         | K   | K   | K   | K   | 94 ± 2  | 39 ± 8 | -10.2 ± 0.2 |
| K3-2         | K   | K   | K   | K   | 67 ± 4  | 220 ± 62 | -8.8 ± 0.2 |
| K3-3         | K   | K   | K   | K   | 62 ± 10 | 1900 ± 600 | -7.8 ± 0.2 |
| K3-4         | K   | K   | K   | K   | 77 ± 10 | 35 ± 10 | -10.2 ± 0.2 |
| K2-1         | K   | K   | K   | K   | 101 ± 15 | 90 ± 23 | -9.7 ± 0.2 |
| K2-2         | K   | K   | K   | K   | 153 ± 50 μM | 153 ± 50 μM | -5.2 ± 0.3 (−4.5) |
| K2-3         | K   | K   | K   | K   | 52 ± 2  | 6200 ± 800 | -7.1 ± 0.1 |
| K2-4         | K   | K   | K   | K   | 69 ± 8  | 430 ± 60 | -8.7 ± 0.1 |
| K2-5         | K   | K   | K   | K   | 94 ± 50 μM | 94 ± 50 μM | -5.5 ± 0.5 |
| K2-6         | K   | K   | K   | K   | ND | ND | ND |

* Mutations of lysines were to alanine in all cases.

* Fitted fluorescence change at saturation expressed as a percentage of starting CR56 fluorescence, corrected for contribution from D3. The values given are the means and half of range of separate titrations. Individual fits gave smaller variation.

* Kd values in μM at I = 0.17 unless noted otherwise.

* By extrapolation from higher ionic strength.

* Measured at I = 0.02.

* Not detectable, because of either small fluorescence change upon binding or no binding under the conditions used.

* The value in parentheses is from Kd of 500 μM extrapolated from I = 0.02 to I = 0.17 and assuming two charge-charge interactions.
To determine whether the multiple lysine to alanine mutations altered the structure of the D3 variants, CD spectra were recorded for wild type D3, the K4 variant, and one each of the K3 and K2 variants. The spectrum of wild type D3 showed minima at 208 and 222 nm, as expected from an all-α-helical protein (Fig. 2). Each of the three variants examined gave CD spectra with the same shape and nearly identical intensity, confirming that the mutations had not caused misfolding or loss of helical content (Fig. 2).

As a further measure of correct folding, we recorded tryptophan fluorescence emission spectra for all variants. The single tryptophan is mostly buried in WT D3 at a position where the three helices come together and has a fluorescence emission spectrum with a wavelength maximum of 330 nm, reflecting the tryptophan’s hydrophobic environment (data not shown). The very similar emission spectra for the D3 variants, with maxima between 330 and 332 nm, support minimal perturbation to the D3 structure. Taken together with the CD spectra, these findings suggest that the various D3 species differ minimally from wild type in both secondary and tertiary structure.

Mode of Binding of D3 Variants to CR56—In the D3-LB34 x-ray structure, the hydrophobic portion of the side chain of Lys-256 stacks against the indole ring of the single tryptophan of the LB4 domain. An homologous tryptophan is present in most other CR domains of principal ligand-binding regions of LDL receptor family members, including the LRP1 domains CR5 and CR6 present in the CR56 used in this study. We have previously interpreted the strong blue shift of the tryptophan fluorescence of CR domains upon forming complexes with RAP domains D1, D2, and D3, and with other protein ligands, plasminogen activator inhibitor-1 and α2-macroglobulin, as resulting from an analogous interaction between ligand lysine and receptor tryptophan (8, 17, 20). Such perturbation provides both a useful signal for monitoring ligand binding and evidence of whether different ligands bind equivalently.

Because D3 itself contains a single tryptophan, the fluorescence perturbation of the CR domains is most clearly seen in a difference spectrum, which for wild type D3 binding to CR56 consists of a maximum at 335 nm and a smaller negative peak, with a minimum at 400 nm (Fig. 3). Representative D3 variants containing four, three, or two lysines gave very similar difference spectra (Fig. 3), consistent with analogous engagement of the two CR domains by lysine residues. This perturbation also provided a ready means of following complex formation.

Affinities of D3 Lysine Variants for CR56—The affinities for CR56 of the D3 variants containing four, three, or two lysines were obtained by titration of the D3 species into a fixed concentration of CR56 and following binding to saturation from change in fluorescence emission at the wavelength of maximum change (335 nm) (Table 1). Data were fitted by nonlinear least squares analysis to a single-site binding model, as described under “Experimental Procedures.” Representative titrations, together with fitted curves, are given in Fig. 4. A control of WT D3, containing all 10 lysines was also carried out. For WT and K4 D3, the affinities were very high at physiological ionic strength and gave 1:1 binding stoichiometry. However, the high affinity made it difficult to directly obtain accurate $K_d$ values. Accordingly, titrations were carried out at several higher ionic strengths, and the values were extrapolated to physiological ionic strength. The converse problem arose for the extremely weak binding species K2-2, K2-5, and K2-6, where it was difficult to saturate the CR56 binding sites at feasible D3 concentrations. For these species, titrations were carried out at low ionic strength ($I = 0.02$). Even so, the affinities were still so low that only partial saturation was obtained at the highest D3 concentrations used (30 μM). By assuming that the magnitude of the fluorescence perturbation at saturation would
be similar to those for tighter binding complexes (typically ~80% enhancement at 335 nm), an estimate of $K_d$ was made by fitting the data, with the maximum fluorescence change fixed at this value. Although the errors introduced might be much larger than from titrations conducted to saturation, they are not likely to be off by more than a factor of 2 and consequently to result in errors in $\Delta G^\circ$ of no more than a few tenths of a kcal mol$^{-1}$. For K2-6 no reliable fluorescence perturbation was observed, suggesting that the affinity was too weak to measure, or else that with both Lys-256 and Lys-270 absent, binding did not perturb the tryptophans of CR56.

$K_d$ values for binding of WT D3 and the K4 variant to CR56 were obtained at different ionic strengths to enable values to be obtained at physiological ionic strength. For WT the $K_d$ extrapolated to $I = 0.17$ was $\sim 1$ nM, whereas for K4 the extrapolated $K_d$ was $\sim 5$ nM, both of which are close to literature values of $\sim 3$ nM for WT D3 binding to intact LRP1, determined by others using other approaches (4), and suggests that the interaction of K4 D3 with CR56 is a good representation both of WT D3 from the viewpoint of the protein ligand and of intact LRP1 from the viewpoint of the receptor. An additional benefit of making these measurements was in obtaining the number of ionic interactions involved in the interaction, from the slope of the plot of log $K_d$ against $I^{1/2}$ (Fig. 5). This gave a value of 4.1 for WT and of 3.9 for K4, suggesting that four ionic interactions are involved for each species and hence that all four lysines are involved in binding. For each of the four variants containing three lysines, the affinity was weakened, providing further evidence that each lysine is involved in binding to CR56. The effects of removing each lysine were, however, very different,
RAP Lysine Binding

being greatest for Lys-256 (380-fold), less for Lys-270 (44-fold), and much less for Lys-253 (7-fold) and Lys-289 (8-fold).

As a result of varied separations between the four lysines (Fig. 6), the six variants containing only two lysines should be considered in two distinct groups. One group contains variants in which the lysines are so far apart that each lysine is expected to interact with a different CR domain (K2-1, K2-4, K2-5, and K2-6). The other group contains variants in which both lysines are so close to one another that they are expected to interact with only a single CR domain (K2-2 and K2-3). Affinities of variants in the first group varied over more than 3 orders of magnitude. K2-1, which contains both Lys-256 and Lys-270, was 1.6 μM for K2-3 (6.2 μM measured at I = 0.17) and ~150 μM for K2-2. This 90-fold difference shows that the Lys-270/Lys-289 pair binds much more tightly than the Lys-253/Lys-256 pair. Assuming the involvement of two charge interactions for binding of the weaker K2-3, the Kd at I = 0.17 is predicted to be ~500 μM.

Calorimetric Measurements—To determine the contributions of enthalpy and entropy to binding of RAP D3 to CR56, ITC was used to follow binding and to obtain values of ΔH°. Titrations were carried out for those D3 species with Kd values tighter than 10 μM for which satisfactory titrations to saturation could be performed. Titrations of D3 variants into CR56 together with control titrations of D3 into identical buffer, as well as the experimental fits, are given in Fig. 7. For both WT D3 and K4 D3, large negative ΔH° values were obtained that were greatly in excess of ΔG°, calculated from Kd values obtained by fluorescence titration (Table 2). The use of fluorescence-derived Kd values rather than those from the ITC fits represented what we considered the best compromise for obtaining a consistent set of accurate ΔG° values over a wide range of affinities, because the ITC measurements were carried out at 1–2 μM and thus would not give as accurate values for Kd (and hence ΔG°) for the very high affinity binding species where Kd << [CR56]. Nevertheless, there was general agreement between ΔG° values obtained by the two approaches, with the same finding of ΔH° values being greatly in excess of ΔG°. Differences of +4.2 to +8.7 kcal mol⁻¹ between ΔG° and ΔH° were found (Table 2), indicating that enthalpy drives binding and is opposed by a large unfavorable entropic contribution.
TABLE 2

| D3 species       | $K_d$  | $\Delta G^\circ$ | $\Delta H^\circ$ | $\Delta S^\circ$ |
|------------------|--------|------------------|-------------------|------------------|
| WT               | $3.2 \times 10^{-5}$ | -12.3 ± 0.5 | -16.5 ± 0.8 | -4.2 |
| K4 (253,256,270,289) | 5 ± 2  | -11.4 ± 0.3 | -20.1 ± 0.8 | -8.7 |
| K3-1 (253,256,270) | 39 ± 8 | -10.2 ± 0.2 | -14.5 ± 0.7 | -4.3 |
| K3-2 (253,256,289) | 220 ± 62 | -9.1 ± 0.2 | -6.6 ± 0.1 | -2.5 |
| K3-3 (253,256,270,289) | 1900 ± 600 | -7.8 ± 0.2 | -9.6 ± 0.9 | -1.8 |
| K3-4 (256,270,289) | 35 ± 10 | -10.2 ± 0.2 | -16.6 ± 0.2 | -6.4 |
| K2-1 (256,270) | 90 ± 23 | -9.7 ± 0.2 | -16.8 ± 0.1 | -7.1 |
| K2-3 (270,289) | 6200 ± 800 | -7.1 ± 0.1 | -11.1 ± 1.4 | -4.0 |
| K2-4 (256,289) | 430 ± 60 | -8.7 ± 0.1 | -5.9 ± 0.1 | -2.8 |

$^aK_d$ and $\Delta G^\circ$ from fluorescence measurements.
$^b\Delta H^\circ$ from ITC measurements (mean and half of range of replicate titrations).

To obtain further insight into which lysines were responsible for the favorable enthalpic contributions, we also determined $\Delta H^\circ$ for the four K3 species and for three K2 species. Elmination of either Lys-253 (K3-4) or Lys-289 (K3-1) had little effect on the total enthalpy of binding (Table 2), suggesting that the more important Lys-256 and Lys-270 were alone responsible for all or most of the favorable $\Delta H^\circ$. This was supported by the $\Delta H^\circ$ for K2-1, which contains only Lys-256 and Lys-270 and yet has a $\Delta H^\circ$ value similar to those of K4 and of the two K3 species. When Lys-256 was mutated to alanine (K3-3), the $\Delta H^\circ$ dropped by 10.5 kcal mol$^{-1}$ compared with K4, suggesting that the interaction of Lys-256 with CR56 contributes a favorable enthalpy of 6–10 kcal mol$^{-1}$ (comparison with WT D3 and K4 D3, respectively). Similarly, K3-2, in which Lys-270 has been removed, has a much reduced $\Delta H^\circ$ of only $-6.6$ kcal mol$^{-1}$, suggesting that the enthalpy contribution from Lys-270 is somewhat greater than from Lys-256.

Only two variants had favorable entropic contributions to binding. These variants were K3-2 and K2-4, which are the only two of the variants examined that do not have Lys-270 and show a large reduction in $\Delta H^\circ$. The favorable entropic contribution, however, results in overall quite high affinities (220 and 430 nM, respectively).

**Binding of K2-3 to Different CR Domains**—Given the finding above that the lysine pair Lys-253/Lys-256 binds to a CR domain with $K_d$ 80–90-fold weaker than does the pair Lys-270/Lys-289, we were interested to examine whether this difference was the result of intrinsic differences in the properties of the lysine pairs or in the CR domain to which each bound. We therefore examined the binding of K2-3 (Lys-270/Lys-289) to the single domains CR3, CR5, and CR8 (K2-2, containing the Lys-253/Lys-256 pair bound too weakly to obtain accurate comparative data). Measurements were made at low ionic strength in anticipation of possible weakened binding for some of the CR domains. However, we found that there was only small variation in affinity, despite large sequence differences outside of the conserved residues. $K_d$ values of 2 μM were obtained for CR3 and CR8 and 1.1 μM to CR5.

**Effect of Lysine Separation**—In D3 the two most important lysines are Lys-256 and Lys-270, each of which is expected to bind to a separate CR domain of the CR56 pair, as they do in the complex with LB3-LB4. In that x-ray structure, the separation between the ε-amino groups of Lys-256 and Lys-270 is 21 Å (Fig. 6). Although CR domains are connected by flexible linkers (16, 21, 22), there must be constraints on how close or how far apart binding sites on adjacent CR domains can be that are determined by the physical size of each CR domain, the length of the linker, and the relative orientation of the CR domains with respect to one another. To further examine the effect of interlysine separation on binding to CR56, we examined two new K2 variants of D3 in which the strongly interacting Lys-270 was retained and a second lysine was introduced by mutagenesis on the same α-helix either one turn closer than Lys-256 or one turn further away than Lys-253 (two turns further away than Lys-256). This involved the mutations Y260K and H249K, respectively (Fig. 6). In this way we obtained interlysine separations (between ε-amino groups) expected to be 16 Å (Lys-260–Lys-270) and 31 Å (Lys-249–Lys-270), based on the x-ray structure of D3. Whereas K2-1 (Lys-256 and Lys-270) bound to CR56 with a $K_d$ of 90 nM (Table 1), there was no detectable binding of either the Lys-249/Lys-270 or the Lys-260/Lys-270 variant, even when 30 μM D3 was added. This indicated that affinity for CR56 had been reduced well over 3 orders of magnitude compared with K2-1. In comparison, K2-5, which contains Lys-253 and Lys-270 (separation 25.3 Å), bound to CR56 with a $K_d$ of 94 μM (Table 1).

**DISCUSSION**

The rationale for conducting the present study was to try and resolve the paradox between the identification of lysines as being important for binding of D3 to LRP1 and the rather small effects that mutating them to alanine, or even glutamic acid, had on affinity. For this, we used a novel mutagenesis approach involving elimination of all lysines from the ligand D3 other than those being evaluated for their role in binding to CR56. Gratifyingly, removal of the other six lysines gave a D3 species, K4, that closely recapitulated the properties of wild type D3, by binding to CR56 with an affinity of 5 nM at physiological ionic strength, which compares very favorably with our value for WT D3 of ~1 nM and values of ~3 nM routinely reported for binding of D3 to intact LRP1. Also the effects of removing these six lysines, as well as additional of the target lysines, had minimal effect on the structure of D3, judged by CD and fluorescence emission spectrosopies. With this approach we have been able to quantitatively probe the roles of each of the lysines Lys-253, Lys-256, Lys-270, and Lys-289, which are the likely candidates for mediating binding to LRP1 and to obtain major new insights into the specificity of such binding that may also be applicable to other LDL receptor family members.

**Binding Involves Four Lysines**—The first important finding was that all four of the lysines Lys-253, Lys-256, Lys-270, and Lys-289 are involved in binding to CR56, because mutation of any one of them to alanine reduced the affinity for CR56 by 7–380-fold. The largest effects were for Lys-256 (380-fold) and Lys-270 (44-fold), with much smaller effects for Lys-253 (7-fold) and Lys-289 (8-fold). Although others have found reductions in affinity for Lys-256 and Lys-270, when mutated on a WT background, the magnitudes were much smaller (20-fold or 8-fold) (4, 10). The only report of a K253A mutation found no effect (10), while no one has examined the role of Lys-289. Consistent with the involvement of four lysine-aspartate charge-charge interactions in binding, the Debye-Hückel plot of affinity as a function of ionic strength gave a slope of 4 for...
K4 and WT D3, whereas the same plot gave a slope of close to 2 for the K2-1 variant that contains only Lys-256 and Lys-270. Such binding appears to be driven by large favorable enthalpies arising from Lys-256 and Lys-270, because the $\Delta H^o$ value for K2-1 containing only Lys-256 and Lys-270 is similar to that of both K4 and WT D3, whereas K2-3, which lacks both Lys-253 and Lys-256, has a much smaller $\Delta H^o$ ( 10.5 kcal mol$^{-1}$). This is also supported by the large reductions in $\Delta H^o$ when either Lys-256 or Lys-270 is removed (K3-3 and K3-2). Entropy appears to oppose binding in all cases, except those in which Lys-270 is missing (see below).

Lysines Fully Account for Binding Energy—The second important finding is that, acting in pairs, the lysines seem to account for all of the binding energy between D3 and CR56 and to do so in an additive rather than synergistic manner, which had been suggested by others for the binding of a two-domain fragment of LDLR to an apoE fragment (22). Thus, considering each pair in turn, we found that the variant K2-2, which contains only the Lys-253/Lys-256 pair, and so is expected to be able to interact with only one CR domain of CR56, had a $K_d$ of $\sim 500 \mu M$ extrapolated to $I = 0.17$ (4.5 kcal mol$^{-1}$). For K2-3, which contains only the Lys-270/Lys-289 pair, the affinity was reduced to a $K_d$ of 6.2 $\mu M$ (7.1 kcal mol$^{-1}$). If each lysine pair interacts with one CR domain of CR56 and contributes to overall affinity in an additive manner, the predicted affinity for the D3-CR56 interaction would be the sum of these affinities. The predicted $\Delta G^o$ obtained by adding the contribution of each pair is $-11.6$ kcal mol$^{-1}$, corresponding to a $K_d$ of 3.1 nM. These are very close to the values of $-11.4$ kcal mol$^{-1}$ and 5 nM, respectively, observed for K4, which contains both pairs of lysines. This is shown schematically in Fig. 8C.

Using these affinities, the interaction of Lys-253/Lys-256 with one CR domain is calculated to contribute 39% of binding energy, whereas the Lys-270/Lys-289 interaction with one CR domain is calculated to contribute the remaining 61%. This establishes experimentally for the first time that the lysines alone are the sole direct contributors to binding of D3, which is consistent with the x-ray structure of the LB3-LD3 complex, where only these lysines from D3 contact the LDLR fragment (14).

It should be noted, however, that mutagenesis of these lysine pairs, Lys-253/Lys-256, or Lys-270/Lys-289, resulted in massive reductions in affinity of 1240-fold ($K_d$ of 6.2 $\mu M$ for K2-3) and 100,000-fold ($K_d$ of 50 $\mu M$ for K2-2), respectively, compared with K4. This establishes the importance of pairs of lysines in ensuring high affinity interactions. When only the single lysines Lys-256 or Lys-270 were mutated, the effects were much smaller (380- and 44-fold, respectively), although they are much larger than the values of 20- or 8-fold obtained by others (4, 10). The explanation for this latter discrepancy is still not clear.

Different Affinities of Lys-253/Lys-256 and Lys-270/Lys-289 Pairs—An unexpected finding from our set of binding measurements was that the lysine pair Lys-253/Lys-256 bound to a CR domain 80–90-fold more weakly than did the Lys-270/Lys-289 pair. However, the variation of no more than 2-fold in $K_d$ for binding of K2-3 to the three single CR domains CR3, CR5, and CR8 suggests that this results from intrinsically different properties of the two lysine pairs, rather than different interactions of a given pair with different CR domains.

Structures of CR3, CR7, and CR8, as well as of several other analogous domains from LDLR and other receptors, show a conserved fold, conserved disulphides, and a conserved calcium-binding site but great variability of surface residues elsewhere (14, 23–26). This raises the question of what the reason is for the large difference between the affinities of the two lysine pairs. Model compound data shed some light on this. The simple ligand lysine methyl ester (two free amino groups, depending on pH) binds to the single CR domains CR3, CR5, and CR8 with $K_d$ values of 350–400 $\mu M$ at $I = 0.17$ (12). First, this parallels the present findings that different CR domains that contain the critical DXDXXD motif in the calcium/ligand-binding site (true for all of CR3, CR5, CR6, and CR8) all bind lysine ligands with similar affinity, and second, this shows that the affinity of the Lys-253/Lys-256 pair closely resembles that of the simple model compound. An additional datum is available for a protein ligand of known structure: the receptor-binding domain of human $\alpha_1$-macroglobulin. The receptor-binding domain binds to CR3 through two lysines located adjacent to one another on the outer face of the single $\alpha$-helix. This is structurally analogous to the Lys-253/Lys-256 pair in D3 (5). The $K_d$ of 140 $\mu M$ for receptor-binding domain binding to CR3 at slightly lower ionic strength (25) is equivalent to $\sim 400 \mu M$ at $I = \frac{1}{2}$.

![Figure 8. Schematic of free energy contributions of individual lysines of D3 in binding to CR56.](image-url)

_Schematic of free energy contributions of individual lysines of D3 in binding to CR56. A and B, top panels, apparent $\Delta G^o$ values (in kcal mol$^{-1}$) of each of the lysines 253, 256, 270, and 289 deduced from comparison of observed affinities of each K$x$ species with that of K4 for binding to CR56. A, bottom panel, predicted affinity of K2-2 for sumamation of apparent individual lysine binding contributions, compared with observed affinity of K2-2 for CR56. Close agreement is obtained. B, bottom panel, predicted affinity of K2-3 from summation of apparent individual lysine binding contributions, compared with observed affinity of K2-3 for CR56. Note the large discrepancy between observed and predicted affinities, indicating that one or both individual contributions is greatly underestimated by the approach of comparing affinities of K3 species with K4 for these two lysines. C, good agreement between the affinity of K4 predicted from summation of the observed affinities of the lysine pairs Lys-253/Lys-256 (K2-2) and Lys-270/Lys-289 (K2-3) for CR56 and the affinity observed for K4 binding to CR56. This indicates independence of the two CR domain binding sites._
0.17, which is very close to the affinity of Lys-253/Lys-256 for CR56.

Together these results suggest that the Lys-253/Lys-256 pair is more typical for engagement of two lysines and that it is the Lys-270/Lys-289 pair that is somehow different and better. It seems to be unlikely that this arises from the closer proximity of Lys-253 to Lys-256 than of Lys-289 to Lys-270 (Fig. 5), because removal of both Lys-253 and Lys-289 (K2-1) reduced affinity only modestly (18-fold) and by an amount no more than expected from the summed contributions of Lys-253 and Lys-289. This implies that the large difference in affinity between the Lys-253/Lys-256 and Lys-270/Lys-289 pairs is retained even when Lys-253 and Lys-289 are absent. One possible explanation is that an arginine (Arg-285), which is in contact with Asp-110, one of the critical aspartates that interacts with Lys-270, might directly or indirectly augment the affinity of the Lys-270/Lys-289 pair.

Compensation for Loss of Lys-270—Although analysis of the binding of pairs of lysines seems to be simply additive, analysis of some of the single lysine replacements is complicated by what seems to be evidence for the ability of Lys-289 to compensate for the loss of Lys-270. A straightforward way to examine whether each individual lysine contributes additively is to compare the summed effect of the apparent binding contributions of each lysine of the pair (from comparison of each K3 species with K4) with the observed affinity when either of the pairs 253/256 or 270/289 is present in the absence of the other. Such comparison of K3 species affinities with that of K4 implies affinity reductions of 7-fold for Lys-253, 38-fold for Lys-256, 44-fold for Lys-270, and 8-fold for Lys-289, corresponding to apparent \( \Delta G^0 \) binding contributions of \(-1.16, -3.54, -2.25, \) and \(-1.24 \) kcal mol\(^{-1}\), respectively, for each of these lysines (Fig. 8, A and B). Based on these values, it is predicted that K2-2, containing only Lys-253 and Lys-256, should have an affinity of \(-4.7 \) kcal mol\(^{-1}\), which compares to an observed \( \Delta G^0 \) (extrapolated to \( I = 0.17 \)) of \(-4.5 \) kcal mol\(^{-1}\) (Fig. 8A). This close agreement implies that the affinity contributions of Lys-253 and Lys-256 deduced from the K3 species are good representations for binding of each lysine and are approximately additive. In turn, this implies that Lys-253 and Lys-256 bind independently and that Lys-253 is not capable of partially substituting for loss of the more important Lys-256 in K3-3.

However, for K2-3, which contains only Lys-270 and Lys-289, the predicted affinity from summing apparent binding contributions of Lys-270 and Lys-289 is \(-3.49 \) kcal mol\(^{-1}\) (Fig. 8B), compared with an observed affinity of \(-7.1 \) kcal mol\(^{-1}\) (\( K_d \) of 6.2 \( \mu M \)). This massive discrepancy of 3.61 kcal mol\(^{-1}\) implies a gross underestimate of the contribution of Lys-270 and/or Lys-289 deduced from comparison of the affinities of K3-1 and K3-2 with K4. If one assumes that the contribution of Lys-289 is correctly reflected in the difference in affinity between K3-1 and K4 (39 versus 5 \( \mu M \) and implied binding contribution of \(-1.24 \) kcal mol\(^{-1}\)), this implies that the true binding contribution of Lys-270 (in the absence of any compensation) is the difference between the observed affinity of K2-3 (\(-7.1 \) kcal mol\(^{-1}\)) and the contribution of \(-1.24 \) kcal mol\(^{-1}\) from Lys-289. This gives a binding contribution of \(-5.86 \) kcal mol\(^{-1}\) for Lys-270, rather than the apparent value of \(-2.25 \) kcal mol\(^{-1}\) deduced from the 44-fold change in \( K_d \) of K3-2 compared with K4. In support of this analysis is the predicted affinity for K2-1, containing only Lys-256 and Lys-270. Using the revised binding contributions of \(-3.54 \) and \(-5.86 \) kcal mol\(^{-1}\), respectively, an affinity of \(-9.4 \) kcal mol\(^{-1}\) is predicted compared with the observed \(-9.7 \) kcal mol\(^{-1}\) (Table 1).

This suggests that, when Lys-270 is removed to create K3-2, Lys-289 or some other residue can partially compensate for the loss of Lys-270 to the extent of \(-3.61 \) kcal mol\(^{-1}\). The molecular nature of this compensation is unknown, although one possibility is that the nearby Arg-285, which was suggested above as a candidate for augmenting the affinity of the Lys-270/Lys-289 pair over that of the Lys-253/Lys-256 pair, might be able to play a different, more direct role when Lys-270 is absent. Importantly, however, these findings resolve the paradox of observations of others on the seeming low importance of Lys-270 when it is mutated to alanine or glutamate, but with Lys-289 still present (4, 10). Furthermore it establishes a different rank of importance for the binding contributions of the four lysines, with Lys-270 \( > \) Lys-256 \( > \) Lys-289 \( > \) Lys-253 (\(-5.86, -3.54, -1.16, \) and \(-1.24 \) kcal mol\(^{-1}\), respectively). Together, Lys-256 and Lys-270 account for \(~83\%\) of the binding energy.

General Consequences for Ligand Binding—Beyond establishing the quantitative roles of individual lysines in binding of RAP D3 to CR56, our findings have some important general consequences for the binding of any protein ligand to receptors that contain multiple CR domains. (i) Taking the Lys-253/Lys-256 interaction as the basic interaction with a CR domain (for reasons given above), an affinity of \(-500 \mu M \) is expected at physiological ionic strength for any CR domain that contains the DXDXD motif (true for most of LRP1 and very low density lipoprotein receptor, but only two domains of LDLR). (ii) Higher affinity may be achieved for interaction of such a lysine pair if additional adjacent arginine or lysine residues are present (cf. the Lys-270/Lys-289 pair). (iii) A ligand containing two such basic pairs capable of interacting with adjacent CR domains would have a minimum affinity of 500 \( \times \) 500 \( \mu M \) = 250 nm. (iv) Because one lysine of each pair interacts much more strongly, it alone could generate an affinity of 500 \( \times \) 8 \( \mu M \) = 4 nm (calculated from the effect of removing Lys-253 from the Lys-253/ Lys-256 pair). This is supported by data on the model compound N-acetyl lysine methyl ester, which binds to CR3, CR5, and CR8 with \( K_d \) values of 2.4, 2.5, and 3.1 nm, respectively, under physiological conditions (12). (v) A ligand containing two such single lysines appropriately spaced could generate a minimum affinity of 4 \( \times \) 4 nm = 16 nm, with higher affinity possible (e.g., K2-1 affinity of 90 nm) if there are other enhancing residues nearby. (vi) Separation of lysine pairs is critical to match the separation between the binding sites on adjacent CR domains. This will be influenced by the length of inter-CR domain linker and its flexibility. (vii) For the minimum strength interaction of \(~250 \) nm, there appears to be no special requirement for such lysine pairs other than accessibility, if the separation is appropriate.

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