# Holo-Cellular Retinol-Binding Protein: Distinction of Ligand-Binding Affinity from Efficiency as Substrate in Retinal Biosynthesis†

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ABSTRACT: Microsomal enzymes that catalyze the first step in the biosynthesis of retinoic acid from retinal, retinol dehydrogenases (RDHs), access retinol bound to cellular retinol-binding protein (CRBP). This study tested the hypothesis that the RDHs interact with the region in CRBP designated as the "helical cap" by evaluating single site-directed mutations, namely, L29A, I32E, L35A, L35E, L35R, L36A, F57A, R58A, and R58E. UV analysis showed mutants had similar conformations of retinol in their binding pockets. Nevertheless, the mutants bound retinol with affinities 2−5-fold lower than wild type, except for L35 mutants, which had affinities similar to wild type. All mutants' holoforms had more relaxed conformations about their helical caps, judged by sensitivity to partial protease digestion. Mutants showed no significant differences in $K_m$ values, but two (L36A, R58A) had increased $V_m$ values and L35 mutants had decreased $V_m$ values. Overall, the data indicate that the residues tested contribute in varying degrees to CRBP rigidity, retinol binding, and RDH recognition/access to bound retinol. The extent of contributions can be distinguished for several residues. For example, L35 mutants had lower $k_{cat}$ values than wild-type CRBP; thus, L35 seems important for RDH access to retinol. F57, on the other hand, a suspected key residue in controlling retinol entrance/exit, does not make a singular contribution to retinol binding. These results suggest a role for the helical cap region as a locus for RDH interaction and as a portal for ligand access to CRBP, and show that the affinity ($K_d$) of CRBP for retinol alone does not determine the efficiency of holo-CRBP as substrate. These are the first experimental data of enzyme recognition by a specific exterior residue of CRBP (L35).

Various tissues and cell types convert retinol (vitamin A) into *all-trans*-retinoic acid (*1*−*3*), a hormone that regulates a wide array of biological processes in vertebrates (*4*, *5*). Physiologically, retinol occurs tightly bound with CRBP[1] ($K_d$ ∼0.1 nM) (*6*). Several enzymes that metabolize unbound retinol and/or retinal also access CRBP-bound retinoids, whereas others cannot catalyze retinoid metabolism in the presence of CRBP (*7*−*14*). This suggests that use of CRBP-bound retinoids occurs through specific interactions with select enzymes and thereby confers specificity on retinoid metabolism. In support of this concept, holo-CRBP, but not apo-CRBP, derivatized with a radioiodinated heterobifunctional cross-linking reagent, cross-links covalently with RDH, and cross-linking occurred only in the presence of cofactor (*15*). These results illustrate two points. First, RDH apparently distinguishes between the holo and apo forms of CRBP. The insensitivity of RDH-catalyzed retinal synthesis from holo-CRBP to equimolar concentrations of apo-CRBP provides further support for this first notion (*10*). Second, cross-linking (i.e., CRBP recognition by RDH) proceeded by the ordered bi-substrate mechanism anticipated for a substrate−dehydrogenase interaction. That is, coenzyme binding occurred first, followed by substrate binding (*16*). Both of these observations are consistent with a specific substrate−enzyme relationship between RDH and holo-CRBP. The Michaelis−Menten kinetics of retinal generation catalyzed by RDH from holo-CRBP also indicate a substrate−enzyme interaction between the binding protein and RDH. Recognition of retinoid-binding protein associated substrate by select enzymes appears to be a general feature of retinoid metabolism (*17*−*21*). To date, at least 12 retinoid-binding protein interactions have been reported with enzymes as substrates and/or affectors of enzyme activities (*1*−*3*, *22*).

CRBP, a member of the family of intracellular lipid binding-proteins (*23*, *24*), consists of a flattened β-barrel that encloses retinol in a seemingly inaccessible cavity covered by two α-helices joined by a loop (*25*). Only minor differences have been observed between the structures of apo and holo lipid binding-proteins, giving no clear indication about the mechanism of binding and release of their ligands. Crystallographic studies of these proteins, however, do suggest increased mobility of the backbone and several residues in the region comprising helix αII and residues from turns βC-βD and βE-βF, supporting the hypothesis that this region constitutes a portal for ligand entry (*23*, *24*, *26*, *27*).

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[1] Abbreviations: CRBP, cellular retinol-binding protein; RDH, retinol dehydrogenase.

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In addition, partial endoproteolytic digestion revealed a more relaxed conformation of helix αII in apo-CRBP vs holo-CRBP (*28*). Newcomer and Ong have suggested that the same region may interact with enzymes (*23*, *28*).

A cluster of hydrophobic amino acids with bulky side chains (L29, I32, L35, L36, and F57) and a basic amino acid (R58) lay exposed on the surface of CRBP in the putative portal/enzyme recognition region. Based on crystallographic data, previous work (*28*), and computer modeling, it appears possible that these residues may be important to the postulated function of this region. Therefore, we used site-directed mutagenesis of CRBP to replace residues either in the "helical cap" (L29, I32, L35, L36) or which make contact with the helical cap (F57, R58) to test whether they contribute to a site of RDH recognition and/or a portal for ligand entry and exit. If amino acid residues in this region were involved in RDH recognition, changing them should affect the CRBP−RDH interaction, and may affect CRBP conformation and affinity for retinol. Moreover, positively charged residues from the homologous regions of some fatty acid-binding proteins are involved in collisional exchange of fatty acids with membranes (*29*). Similarly, a positive surface potential around the putative portal of CRBP could contribute to its interaction with RDH. To disrupt this positive surface potential, we replaced I32, L35, or R58 with glutamate.

Our results show that the residues tested contribute differently to retinol binding, conformation of the helical cap region, and recognition by RDH. Of these, L35 mutants had lower $k_{cat}$ values than wild-type CRBP. Therefore, L35 seems a key residue for RDH accessing retinol. Moreover F57 surprisingly does not serve as a key residue for retinol binding. This work provides further insight into ligand binding by CRBP, suggests a role for the "helix cap" region as a portal for ligand access and enzyme recognition, and provides evidence suggesting a function for a specific exterior CRBP residue (L35).

## EXPERIMENTAL PROCEDURES

*Construction of Mutants.* The entire plasmid pMONCRBP (*30*) served as template for circular polymerase chain reaction with the following primers: L29A-1, 5′-GATGTCAACGTG-GCCGCGCGAAAAAT; L29A-2, 5′-GAGCGCACGCAG-GTACTCCT; I32E-1, 5′-TTGCGAAAAGAGGCCAACAAC-TTGCT; I32E-2, 5′-GGCCACGTTGACATCGAGCGCA; L35A, 5′-TTGCGAAAAATCGCCAACGCGCTGAAGC-CG; L35E, 5′-TTGCGAAAAATCGCCAACGAGCTGAA-GCCGGACAA; L35R, 5′-TTGCGAAAAATCGCCAACAG-GCTGAAGCCGGACAAA; L36A, 5′-TTGCGAAAAAT-CGCCAACTTGGCGAAGCCGGA; F57A-1, 5′-ATCCG-CACGCTGAGCACTGCTCGAAACTAT; F57A-2, 5′-GAT-CATGTGGTCGCCATCCT; R58A-1, 5′-GCACTTTTGCAA-ACTATATCATGGA; R58A-2, 5′-TCAGCGTGCGGATGA-TCA; R58E, 5′-GCACTTTTGAAAACTATATCATGGA. Primers were used in the following combinations: I32E-1/I32E-2 for I32E, L35A/I32E-2 for L35A, L35E/I32E-2 for L35E, L35R/I32E-2 for L35R, L36A/I32E-2 for L36A, F57A-1/F57A-2 for F57A, R58A-1/R58A-2 for R58A, and R58E/R58A-2 for R58E. Reaction mixtures contained 0.1 μg of single-stranded DNA-binding protein. Amplification products were circularized and sequenced.

*Expression and Purification of Wild-Type and Mutant Proteins.* Proteins were expressed and purified by the methods of Levin et al. (*30*) and Posch et al. (*10*). Fractions with a ratio of $A_{348}/A_{280} > 1.4$ were pooled (*31*). For competition binding and endoproteinase digestion studies, protein samples were further purified by Mono Q fast-protein liquid chromatography (*30*). To generate His-CRBP, the CRBP cDNA was inserted into pET14b, expressed in BL21(DE3) pLysS *E. coli* cells (Novagen, Madison, WI), and purified according to manufacturer's instructions. Purified proteins were dialyzed against 10 mM Tris-HCl, pH 7.9. To prepare wild-type and mutant holo-proteins, an aliquot of each apo-protein in a volume of 0.5 mL was equilibrated with excess retinol for 1 h at 25 °C. To separate holo-protein and unbound retinol, the mixture was loaded at 4 °C onto a column (1 × 4 cm) packed with 2 mL of Sephadex G-25 and eluted in 2 mL of RDH (20 mM Hepes, 150 mM KCl, pH 8.0). Holo-His-CRBP had a UV absorbance spectrum and kinetic characteristics with RDH similar to native CRBP (data not shown).

*Competition Binding Assays.* One micromolar His-CRBP was incubated with 0.25 μM [11,12-$^3$H]retinol (12.6 Ci/mmol) in 0.5 mL of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) for 1 h at 25 °C. Unbound ligand was removed with a Sephadex G-25 column as described above, and wild-type or mutant CRBP (1 μM) then was allowed to incubate with His-CRBP for 1 h at 25 °C. The solution was loaded onto a column (1 × 5 cm) of His.Bind resin (150 μL) which was washed with 3 mL of binding buffer to collect unretained protein. Bound protein was eluted with 3 mL of elution buffer. The radioactivity in the fractions was measured by liquid scintillation counting.

*Protease Digestion.* Partial proteolysis with Arg-C (Clostripain, Sigma) was done in 0.1 mL of 20 mM Tris-HCl, pH 7.8, 1 mM $CaCl_2$, 2 mM dithiothreitol at 37 °C, as described by Jamison et al. (28). Each reaction mixture contained 0.5 mg/mL protein and Arg-C, in a ratio of 5:1 (w/w), and excess retinol to ensure saturation of CRBP. At various times 1 μL of 1 M EDTA of was mixed with 20 μL aliquots, which were analyzed by 15% SDS−PAGE. Gels were stained with Coomassie blue. Bands were quantified with a GS700 Imaging Densitometer and Molecular Imager software (BioRad).

*Enzyme Assays*. Assays of RDH activity were done in duplicate at 37 °C for 30 min with microsomes prepared from the livers of male Sprague−Dawley rats (200 g) by differential centrifugation as described (10). The reaction mixtures contained a 3:5 molar ratio of apo-CRBP/holo-CRBP to ensure that only CRBP-bound retinol was available. Retinal was quantified by high-performance liquid chromatography (*32*, *33*).

*Protein Assays.* Molar concentrations were calculated for apo-proteins with a molar extinction coefficient at 280 nm of 28 080 $M^{-1}$ $cm^{-1}$ (*30*) and for holo-proteins from the absorbance at 348 nm with a molar extinction coefficient of 50 200 $M^{-1}$ $cm^{-1}$ (*34*).

## RESULTS

*Selection of Residues To Mutate.* A hydrophobic patch in the helical cap of CRBP near its putative portal was selected for mutagenesis as a possible enzyme recognition site (Figure 1). These helical-cap hydrophobic residues (L29, I32, L35, L36) contact two residues outside of the helical loop: one bulky and hydrophobic (F57) and the other large and basic

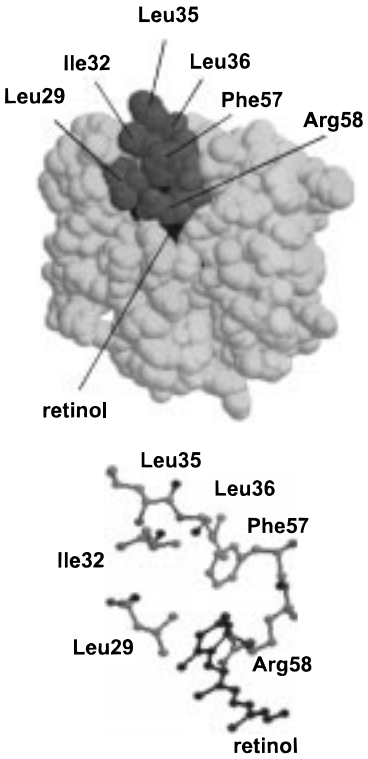


FIGURE 1: Mutated amino acids of CRBP. Top, space-filling model of holo-CRBP showing the mutated residues in dark shading. Bottom, representation of the relationship between the mutated amino acids and retinol.

(R58), which also were selected for mutagenesis. Alanine was substituted for each residue in turn to reduce the contribution of the hydrophobic side chains. Of these residues, L35 projects prominently from the surface of CRBP, which along with its size and hydrophobicity makes it a probable candidate for interacting with proteins that would approach CRBP. Accordingly, with L35 glutamate and arginine, substitutions were made to evaluate the effects of sizable side chains with negative and positive charges, respectively. Also with R58, a glutamate substitution was made to evaluate the possible contribution of charge to its role.

*Properties of the Purified Mutant Proteins.* The UV absorption spectrum of holo-CRBP reflects the microenvironment of the retinol-binding pocket (*35*). Therefore, to evaluate the possible effects of point mutations on the conformation of retinol in the binding pocket and the structural integrity of the mutant proteins, UV spectra of mutant holoproteins were compared to the spectrum of wild-type CRBP. The spectra of most mutants were virtually superimposable with that of wild type, indicating that the point mutations did not affect the conformations of binding pockets (data not shown). Some mutant proteins had absorption maxima slightly different than from that of the wild type at 348 nm: L35E, 349 nm; L36A, 346 nm; R58A and R58E, 350 nm (Figure 2). The fine structure of the UV spectrum of L36A also was somewhat different from the wild type, and its $A_{348}/A_{280}$ ratio was no higher than 1.2, possibly reflecting a different conformation of retinol in its binding pocket.

*Determination of Relative Affinities for Retinol.* To determine the extent to which the mutations affected CRBP's affinity for retinol, wild-type CRBP and each mutant were allowed to compete with His-tagged CRBP for [$^3$H]retinol (Table 1). The differences in retinol binding between CRBP and mutants under these standard conditions reflect differences in their $K_d$ values. The three mutations at L35 did not change the affinity for retinol. The R58A mutation had 2-fold lower affinity compared to wild-type CRBP. I32E and F57A showed 3-fold lower affinities. L29A, L36A, and R58E had 5-fold lower affinities for retinol.

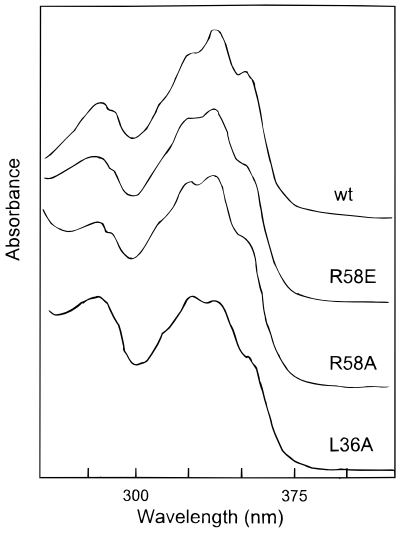


FIGURE 2: Absorption spectra of liganded CRBP and mutant binding proteins. Spectra were obtained as described under Experimental Procedures. Spectra were normalized to the same scale. Three mutants that produced noticeably different spectra are compared to that of wild-type CRBP.

Table 1: Competition between His-CRBP and Wild-Type CRBP or Mutant Proteins[a]

| protein | % total [$^3$H]retinol peak 1[b] | % total [$^3$H]retinol peak 2 | relative $K_d$[c] |
|---|---|---|---|
| wild type | 67 ± 2 | 33 ± 2 | 1 |
| L29A | 29 ± 6[d] | 71 ± 6 | 5 |
| I32E | 45 ± 6[d] | 55 ± 6 | 3 |
| L35A | 73 ± 8 | 27 ± 8 | 1 |
| L35E | 73 ± 7 | 27 ± 7 | 1 |
| L35R | 76 ± 10 | 18 ± 15 | 1 |
| L36A | 28 ± 6[d] | 71 ± 6 | 5 |
| F57A | 42 ± 5[d] | 58 ± 5 | 3 |
| R58A | 47 ± 2[d] | 52 ± 2 | 2 |
| R58E | 30 ± 2[d] | 69 ± 3 | 5 |

[a] Values are means ± SD of four experiments, except for I32E ($n = 3$). [b] Peak 1 corresponds to the retinol fraction eluted from the Ni-column, i.e., retinol bound to the non-His-tagged binding proteins, whereas peak 2 represents retinol bound to His/CRBP. [c] The relative $K_d$ was calculated from the formula: (% wild-type/% His/CRBP)/(% mutant/% His/CRBP). [d] Significantly different from the wild-type value, $p < 0.001$.

*Partial Protease Digestion.* Based on differential sensitivities to endoproteinase digestion, the helical cap in apo-CRBP seems to be in a comparatively open or relaxed conformation, whereas the cap in the holo-protein seems to be secured by van der Waals interactions between retinol and CRBP residues L29, L36, and F57 (28). We used limited digestion by endoproteinase Arg-C, which proteolyzes CRBP at R30, to test whether mutants affected the conformation of holo-CRBP. Digestion produced fragments of ~12 kDa and ~3 kDa (Figure 3). All mutants were much more rapidly cleaved than wild-type CRBP with $t_{1/2}$ values ~3−6-fold shorter, with the exception of L35A which had a half-life <2-fold shorter (Table 2).

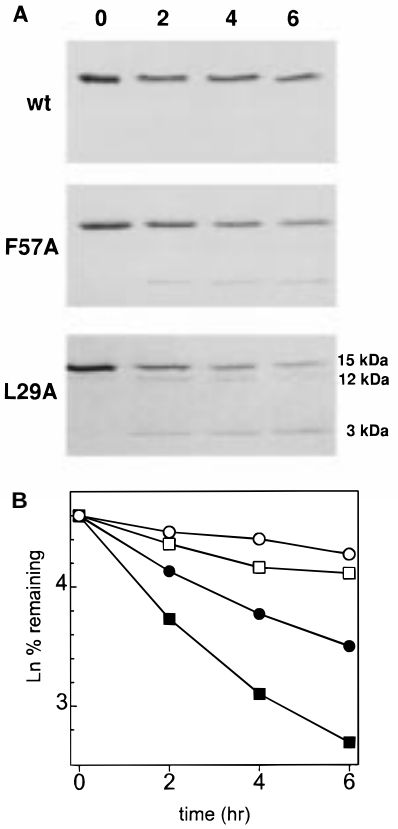


FIGURE 3: Arg-C-catalyzed proteolysis of holo-CRBP and mutant binding proteins. Wild-type and mutant holo-binding proteins (0.5 mg/mL) were digested with Arg-C and analyzed by SDS−PAGE as described under Experimental Procedures. (A) Examples of SDS−PAGE analyses of digestion by Arg-C. The upper band is CRBP, the intermediate band represents the 12 kDa fragment, and the lower band is 3 kDa. (B) Representative time courses of endoproteolytic digestions. Each point represents the average of two experiments (○, wild-type; □, L35A; ●, F57A; ■, L29A).

Table 2: Rates of Proteolysis of CRBP Relative to Single-Residue Mutants

| CRBP | % remaining[a] | $t_{1/2}$ (h) |
|---|---|---|
| wt | 87 | 10 |
| L29A | 42 | 1.6 |
| I32E | 45 | 1.8 |
| L35A | 78 | 5.8 |
| L35E | 55 | 2.3 |
| L35R | 64 | 3.2 |
| L36A | 53 | 2.2 |
| F57A | 62 | 2.9 |
| R58A | 44 | 1.7 |
| R58E | 39 | 1.5 |

[a] Percent remaining after 2 h of incubation with Arg-C. The half-lives ($t_{1/2}$) were calculated from the formula: $t_{1/2} = 0.693/([4.6 - \ln \%$ remaining at 2 h$]/2)$, because the rates of proteolysis decreased after 2 h, possibly because of enzyme instability or resistance of holo-protein to further proteolysis. Therefore, the first 2 h of incubation was selected as representative of the initial rates of proteolysis.

*CRBP Mutants as Substrates for Microsomal RDH.* If access of RDH to CRBP-bound retinol represents the rate-limiting event in retinal synthesis, then changes in the interaction surface (helical cap?) should alter the kinetics of the reaction. None of the mutant CRBPs had statistically significantly different $K_m$ values from wild-type CRBP, but three mutants (L35A, L36A, R58A) had statistically significantly different $V_m$ values (Figure 4, Table 3).

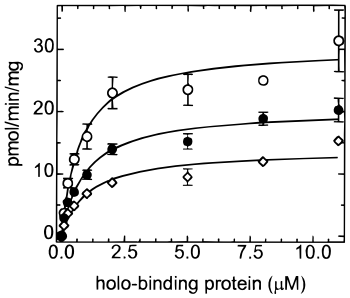


FIGURE 4: Retinal synthesis vs concentrations of representative holo-binding proteins. Reactions were run in the presence of excess apo-CRBP or mutant apo-binding proteins as described under Experimental Procedures: wild type (●), L36A (○), and L35A (◇). Data represent mean ± SEM.

Table 3: Comparison of Kinetic Constants of CRBP and Mutants[a,b]

| mutant | $K_M$ | $V_m$ | $n$ |
|---|---|---|---|
| WT | 0.9 ± 0.1 | 20 ± 0.8 | 5 |
| L29A | 0.5 ± 0.1 | 23 ± 1.3 | 2 |
| I32E | 0.6 ± 0.1 | 23 ± 1.0 | 2 |
| L35A | 1.0 ± 0.2[c] | 14 ± 0.7[c] | 4 |
| L35E | 0.7 ± 0.3 | 17 ± 1.6 | 3 |
| L35R | 0.7 ± 0.2[c] | 15 ± 1.0[c] | 3 |
| L36A | 0.8 ± 0.2[c] | 30 ± 2.0[c] | 3 |
| F57A | 0.5 ± 0.2 | 22 ± 1.6 | 3 |
| R58A | 0.9 ± 0.2[c] | 28 ± 0.2[c] | 2 |
| R58E | 0.9 ± 0.1 | 23 ± 1.0 | 2 |

[a] Reaction mixtures contained a 3:5 molar ratio of apo-CRBP relative to holo-CRBP, to ensure that only CRBP-bound retinol was available. [b] Values represent means ± SEM from nonlinear regression analyses using the program *GraphPad Prism*. [c] Values are statistically different from the wild-type value ($p < 0.02$) calculated by ANOVA.

## DISCUSSION

This study examined the relationship among CRBP flexibility, its affinity for retinol, and its efficiency as a substrate for retinal formation as a function of specific surface amino acid residues. For the most part, the results did not reveal simple relationships among these factors and suggest that the surface residues tested contribute cumulatively to the factors measured. Nevertheless, several specific insights were provided. For example, the mutants tested had a more relaxed helical cap and/or weaker interaction between the helical cap and the main chains than wild-type CRBP. Apparently CRBP has evolved to provide a holoform with an optimally rigid and "closed" conformation. Both side-chain size and charge were important for maximum effect. In one case, reducing the side chain size had as much effect (R58A) as changing the charge (R58E). Given the locus of R58, it is not surprising that changing its size or charge would affect the flexibility of holo-CRBP. With the protruding L35 side chain (L35A), however, reducing the size resulted in the most modest change in flexibility observed. Inserting charged L35 side chains (L35E, L35R) resulted in greater disruption of the CRBP structure than simply reducing size, likely reflecting electrostatic repulsion between L35 mutants and neighboring residues.

With the exception of L35, the "external" mutations reported here reduced the affinity of CRBP for retinol, indicating that the residues mutated make significant contributions to retinol binding. It is likely that the loss of van der Waals interactions among these residues and other amino acids and/or retinol led to lower binding affinities. Interest-

ingly, mutation of F57, a possible key residue in gating (*23*, *24*, *26*−*28*), did not decrease the affinity for retinol as much as mutating L29 or R58 and had no worse an effect than mutating I32. This suggests that F57 does not contribute as singularly to retinol retention as its nature and location suggest. It seems more likely that the contributions of several residues achieve a cumulative effect in the function of the portal. This is concordant with additivity of single mutations at the free energy level (*36*), as illustrated by mutagenesis of adipocyte lipid-binding protein (*37*). Individual mutations of the residues of adipocyte lipid-binding protein corresponding to I32 and F57 of CRBP resulted in 2−4-fold lower affinities for ligand, similar to our results with CRBP, whereas a double mutant (V32D/F57H) had a $K_d$ 9-fold higher. Removal of the entire helical region from another related fatty-acid binding protein produced a 20−100-fold reduction of affinity for ligand (*38*). All these data suggest that the helical loop serves as a binding cap.

The L35 mutations showed no change in $K_d$ values despite increased flexibility, suggesting that L35 makes little or no contribution to retinol binding. Two facts taken together, i.e., evolution of CRBP to optimum rigidity and no requirement for such rigidity to ensure efficient retinol binding, exemplified by the L35 mutants, provide additional support for the notion that the well-conserved exterior residues of CRBP serve a function other than in ligand binding (*22*, *23*). Notably, similar $K_d$ values for the wild type and two mutants (L35A, L35R) vs decreased $V_m$ values of the mutants relative to wild type also distinguish two "independent" domains of CRBP: ligand binding and substrate recognition. These data show that the efficacy of holo-CRBP as substrate for an enzymatic reaction does not reflect merely the affinity of CRBP for a given ligand.

Several mutants showed either increased (L36A, R58A) or decreased (L35) efficiency as substrates for retinal synthesis. Consideration of the kinetic differences between unbound retinol and CRBP-bound retinol helps place the magnitude of these changes into perspective. Microsomal RDH has a ~6-fold greater $V_m$ with unbound vs CRBP-bound retinol (*39*). The decrease in $V_m$ with CRBP−retinol most likely reflects the rate of transfer of retinol from CRBP to RDH, such that the rate-limiting event becomes RDH access to retinol, rather than dissociation of reduced cofactor, the usual rate-limiting event for a dehydrogenase. The changes in $V_m$ observed here are considerable if the $k_{cat}$ of RDH with unbound retinol can be viewed as an upper limit for its reaction with wild-type CRBP.[2] The increased $V_m$ (i.e., $k_{cat}$) of L36A and R58A indicates that these two residues not only maintain the helical cap in a fixed conformation but also contribute to a barrier that restricts access to retinol. The decrease in catalytic efficiency of the L35 mutants contrasts with the increases observed with L36A and R58A and supports a singular role, among the amino acid residues studied, for L35 in the events important to retinal formation. The L35A mutant is especially interesting because its decrease in $k_{cat}$ occurred with no change in the affinity for retinol and with the most modest change in the flexibility of CRBP. This suggests that both the bulky and hydrophobic nature of the leucine side chain contribute to an RDH−CRBP interaction important to RDH accessing retinol. These data also provide the first experimental evidence of a specific function for an external residue of CRBP in recognizing an enzyme associated with retinoid metabolism.

Our results indicate that the residues tested contribute in varying degrees to CRBP rigidity, retinol binding, and RDH recognition and access to bound retinol, and that the extent of contributions can be distinguished for several residues. For example, L35 appears to represent a key residue for RDH access to retinol whereas F57 does not make a singular contribution to retinol binding. These results also suggest a role for the helical cap region as a locus for RDH interaction and a portal for ligand access to CRBP and provide further insight into the mechanism of ligand binding by this class of lipid-binding proteins.

[2] Catalysis of CRBP-presented retinol dehydrogenation may occur either in two distinct steps (accessing retinol and catalyzing dehydrogenation) represented by two constants ($k^1_{cat}$ and $k^2_{cat}$) or by a concerted process represented by a single $k_{cat}$ value. If the former occurs, $V_m$ mutants should reflect changes only in $k^1_{cat}$. For simplicity, the two possibilities will not be distinguished here, i.e. $k^1_{cat}$ and $k^2_{cat}$ will be considered as $k^1_{cat} + k^2_{cat} \equiv k_{cat}$.

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