

# Dissection of the critical binding determinants of cellular retinoic acid binding protein II by mutagenesis and fluorescence binding assay

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## ABSTRACT

The binding of retinoic acid to mutants of Cellular Retinoic Acid Binding Protein II (CRABPII) was evaluated to better understand the importance of the direct protein/ligand interactions. The important role of Arg111 for the correct structure and function of the protein was verified and other residues that directly affect retinoic acid binding have been identified. Furthermore, retinoic acid binding to CRABPII mutants that lack all previously identified interacting amino acids was rescued by providing a carboxylic acid dimer partner in the form of a Glu residue.

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**Key words:** electrostatic interactions; hydrophobic effect; ordered water network; carboxylic acid dimer; all-*trans*-retinoic acid.

## INTRODUCTION

Retinoic acid (RA), the carboxylic acid form of vitamin A, has been identified as one of the most active naturally occurring retinoids.<sup>1,2</sup> The presence of RA is crucial for the proliferation and differentiation of a series of cell lines including skin, epithelial, cartilage, hematopoietic and neuronal cell lines.<sup>3–10</sup> In addition, it has been observed that either a deficiency or a large excess of RA during fetal development can result in severe developmental defects.<sup>1,2,5,11–14</sup> Therefore, regulating the cellular RA concentration is critical for maintaining normal cell growth and morphogenesis.

Two separate classes of proteins have been identified to bind all-*trans*-retinoic acid and assist its function.<sup>15</sup> Nuclear retinoic acid receptors (RARs) mediate the regulatory effects of RA<sup>16–22</sup> and two cellular retinoic acid binding proteins, CRABPI and CRABPII are responsible for modulating its intracellular concentration.<sup>23–25</sup> Although the exact biological function of each of the latter two homologous proteins is not yet clear, it is known that CRABPII serves as a mediator for delivering retinoic acid to the transcription factors, activated by RAR.<sup>26–32</sup>

Both CRABPI and CRABPII are members of a family of intracellular lipid binding proteins that bind small hydrophobic ligands. The homology within this family, which includes retinoid and fatty acid binding proteins is rather low (~20% sequence identity).<sup>33</sup> However, according to the available crystal structures, all members of the lipid binding protein family share a similar flattened  $\beta$ -barrel fold formed by two nearly orthogonal five-stranded  $\beta$ -sheets.<sup>34,35</sup> A helix-turn-helix motif appears to act as the entrance of the deep, hydrophobic ligand binding pocket.

The binding of RA in both CRABP proteins is stabilized by a series of electrostatic interactions.<sup>36</sup> Recently, we have determined the structure of the CRABPII-RA complex to an improved resolution of 1.48 Å.<sup>37</sup> In the case of CRABPII, the carboxylic acid is bound through a salt bridge to Arg132 and a hydrogen bond with Tyr134 while it interacts with a second arginine (Arg111) and a threonine (Thr54) residue through an ordered water molecule. The crucial role of the two arginine residues (Arg132, Arg111) for retinoic acid binding has been discussed previously, and through site directed mutagenesis the effect of each residue has been evaluated. By applying a radioactivity based competitive binding assay Yan and coworkers estimated relative  $K_d$  values for single mutants R132M and R111M and double mutant R132M:R111M.<sup>38</sup> The results indicated that both residues are important for RA binding, with removal of Arg111 resulting in a greater loss of RA binding.

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Although these experiments provided some information on the importance of the arginine residues present in the CRABP II binding site, the method has had limited use due to the rather complex setting of the experiment, based on the use of [11, 12 <sup>3</sup>H]-RA, and the fact that the  $K_d$  values obtained always refer to relative affinities, as compared to WT-CRABP II binding. In addition, to date the effect of Tyr134 and Thr54 on RA binding has not been evaluated, although the crystal structure shows a direct interaction with the bound ligand. In fact, no comprehensive study of the direct effect of different CRABP II mutations on RA binding affinity has been performed and no apparent  $K_d$  values have been reported.

Recently, we determined the first crystal structure of apo-CRABP II and our results directly contradict previous speculations regarding the mechanism of ligand entry.<sup>37</sup> In the course of our attempts to reengineer CRABP II into a retinal binding protein<sup>39,40</sup> several mutants were constructed and their binding affinities for RA were evaluated. The data reported herein present a comprehensive and quantitative study of the effect of different amino acids within the CRABP II cavity on RA binding. (The choice of CRABP II for our studies was strongly influenced by reports suggesting that proteins of this family are highly tolerant to amino acid mutations.<sup>41</sup>) The presence of a cluster of amino acid residues and the ordered-water network within the binding cavity is critical for efficient RA binding. In addition, through different point mutations, we were able to separate the electrostatic versus hydrophobic binding of RA within the CRABP II cavity and quantify the effect of the two different binding mechanisms. Furthermore, we were able to regain RA binding affinity, even after all previously identified interacting amino acids were removed, by providing a dimer partner in the form of a Glu residue. The high resolution crystal structure of the latter mutant in complex with all-*trans*-retinoic acid, reveals a novel binding motif for this family of proteins.

## METHODS

Fluorescence spectra were recorded using a SPEX<sup>®</sup> Fluorolog-3 fluorometer (HORIBA JOBIN YVON). UV-vis spectra were recorded with a Cary300 BioWinUV spectrophotometer (Varian, Inc). All-*trans*-retinoic acid was purchased from Aldrich and was used as received.

### Site directed mutagenesis of CRABP II

Site-directed mutagenesis was performed using the CRABP II-pET17b plasmid following Stratagene's Quik-change<sup>®</sup> Kit protocol. The primers used for the mutations described in this manuscript were: R132K forward: 5'-GACGTTGTGTGCACCAAGGT-CTACGTCCGAGAG-3', reverse: 5'-CTCTCGGACGTAGA-CCTTGGTGCACAC AACGTC-3'; R132K:Y134F forward: 5'-GTTGTGTGCAC

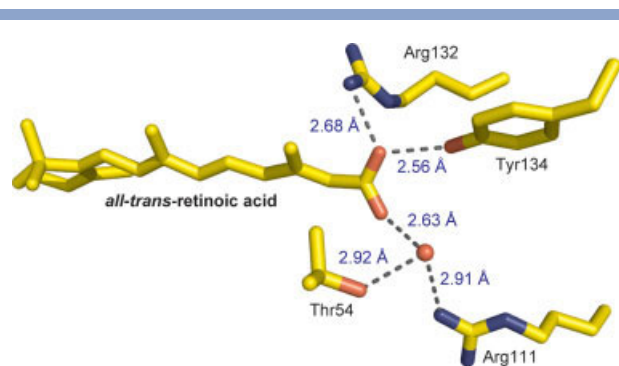
CAAGGTCTTCGTCCGAGAGCTCGAG-3', reverse: 5'-CTCGAGCTCTCGGACGAAGACCTTGGTGCA-CACAAC-3'; R111L forward: 5'-CCCAAGACCTCGTG-GACCCTA GAACTGACCAACGATGG-3', reverse: 5'-CC-ATCGTTG GTCAGTTCTAGGGTCCACGAGGTCTTGGG-3'; R132L forward: 5'-GTTGTGTGCACCCTGGTCTACGTCCG-3', reverse: 5'-CDDACGTAGACCAGGGTGCACACAAC-3'; T54V: forward: 5'-CTACATCAAAGTCTCCACCACCGT GC-G-3', reverse: 5'-CGCACGGTGGTGGAGACTTTGAT GTAG-3'; R59L: forward: 5'-CCTCCACCACCGTGTGA CCACAG-AG-3'; reverse: 5'-CTCTGTGGTCAACACGGT GGTGGAGG-3'; R111M: forward: 5'-GACCTCGTGGAC CATGGAAGTACC-ACC-3', reverse: 5'-GGTCAGTTCCATGG TCCACGAGGTC-3'; R111E: forward: 5'-CCTCGTGGAC CGAGGAACTGACC-AACG-3', reverse: 5'-CGTTGGTC AGTTCCTCGGTCCACG-AGG-3'; R111K: forward: 5'-CGTGGACCAAAGAACTGAC-C-3', reverse: 5'-GGTCAG TTCTTTGGTCCACG-3'; R111H: forward: 5'-GACCTC GTGGACCCACGAACTGACC-3', reverse: 5'-GGTCA-GT TCGTGGTCCACGAGGTC ; W109L: forward: 5'-GAC CTCGCTGACCAGAGAACTGAC-C-3', reverse: ; E73A: forward: 5'-GGAGTTTGTAGGCGCAG-ACTGTGG-3', reverse: 5'-CCACAGTCTGCGCCTCAAACCT-CC-3'; L121E: forward: 5'-GATGGGAACTGATCGAGAC-CATGACGG CGGATGA-G-3', reverse: 5'-CTCATCCGCCG-TCATGG TCTCGATCAG-TTCCCCATC-3'; L121Q: forward: 5'-GGGAACTGATCC-AGACCATGACGGCG-3', reverse: 5'-CGCCGTCATGGTCTGGATCAGTTCCCC-3'; L121D: forward: 5'-GGGAACTGATCGACACCATGACGGCG-3', reverse: 5'-CGCCGTCATG-GTGTGATCAGTTCCCC-3'.

### CRABP II protein expression and purification

The expression of the CRABP II proteins was carried out as previously described.<sup>40</sup> Briefly, expression of the CRABP II mutants in the pET17b vector was performed at 32°C for 5 h in *Escherichia coli* strain BL21(DE3)pLacI. The overexpressed proteins were purified by ion exchange chromatography using Q Sepharose<sup>™</sup>, Fast Flow resin followed by FPLC (Source 15Q resin).

### Determination of extinction coefficients of CRABP II mutants

The absorption extinction coefficients ( $\epsilon$ ) were determined according to the method first described by Gill and von Hippel.<sup>42</sup> The calculated values are (in M<sup>-1</sup> cm<sup>-1</sup>): WT: 20,215; R132L: 19,631; R132K: 19,202; R132K:Y134F: 18,317; R132K:R111L:20,846; R132L: R111L:21,239; R132K :Y134F:R111L:18,422; R132L: Y134F:R111L:29,970; R132K :Y134F:T54V:20,934; R132K: Y134F:R111L:T54V:17,798; R132K:R111L:R59L:15,726; R132K:R111M:23,108; R132K: R111E:19,748; R132K: R111K:20,389; R132K: R111H: 20,768; W109L:16,865; R132K:W109L:16,789; R132K:E73A:18,369; R132K: W109L:L121E:15,289; R132K:E73A:L121E:20,298; R132K:

**Figure 1**

Crystal structure of WT-CRABP II bound to all-*trans*-retinoic acid (PDB ID: 2fr3). [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

Y134F:R111L: L121E:21,500; R132K: Y134F:R111L:L121E: T54V:19,362; R132K:Y134F:R111L: L121Q:T54V:22,821; R132K:Y134F: R111L:L121D:T54V: 22,056; R132K:Y134F: L121E:18,422; R132K:L121E:17,981; R132K:R111L:L121E: 23,136; R132K:R111L:L121E:T54V:19,706.

### Tryptophan fluorescence quenching

Measurements of tryptophan fluorescence quenching and  $K_d$  calculation were carried out as previously described.<sup>39,40</sup> Briefly, protein fluorescence spectra were recorded at 25°C in a 0.01% gelatin containing PBS buffer (4 mM NaH<sub>2</sub>PO<sub>4</sub>, 16 mM Na<sub>2</sub>HPO<sub>4</sub>, 150 mM NaCl, pH = 7.3), containing 0.5 μM of protein. The sample was excited at 283 nm with an excitation slit width of 1.5 nm. The fluorescence was measured at the peak maximum (345 nm). Retinoic acid was added at varying equivalents from a 1.5 mM stock solution in ethanol, maintained in the dark. Care was taken to ensure that the final EtOH volume remained below 2%. A measurement was taken for each chromophore addition at the same wavelength. This was plotted as concentration of chromophore versus the relative fluorescence intensity. The titration was complete when there was no observable quenching of fluorescence upon addition of the chromophore. The  $K_d$  for each CRABP II mutant was determined according to the method previously described by Wang *et al.*,<sup>38</sup> using Sigmaplot software and is reported as the mean value ± SD based on one specific titration. (Most of the proteins were expressed several times with similar results. Every time the binding affinity of RA, according to the fluorescence quenching assay, is reproducible, within error.)

## RESULTS AND DISCUSSION

The exact mode of binding of retinoic acid to WT-CRABP II is shown in Figure 1. One oxygen of the carboxylic acid is bound through direct hydrogen bonding

with Arg132 (2.68 Å) and Tyr134 (2.56 Å) while the other interacts with an ordered water molecule set in place by two neighboring residues, Arg111 (2.91 Å) and Thr54 (2.92 Å) (see Fig. 1).

To evaluate the effect of Arg132 on RA binding, single mutant R132K was prepared. This electronically conservative mutation places a Lys residue in the center of the binding pocket, maintaining an amine functionality in the vicinity of the bound carboxylic acid. However, because of the geometrical differences between an Arg and a Lys residue, the trajectory for interacting with RA could be different. In addition, since the pK<sub>a</sub> of a Lys residue is lower than that of Arg in solution (10.5 vs 12, respectively) Lys132 may remain deprotonated, in the relatively hydrophobic cavity (our previous studies using the R132K mutant further verify this suggestion<sup>39</sup>).

WT-CRABP II binds RA with high affinity. The literature reported  $K_d$  values vary from 0.1–2 nM, depending on the method utilized.<sup>38,43</sup> The apparent  $K_d$  value as calculated by fluorescence quenching assay is 2.0 ± 1.2 nM (Table I, entry 1). Replacement of Arg132 with a Lys residue results in reduced RA affinity (30-fold reduced,  $K_d$  = 65 ± 14 nM, Table I, entry 4). The model

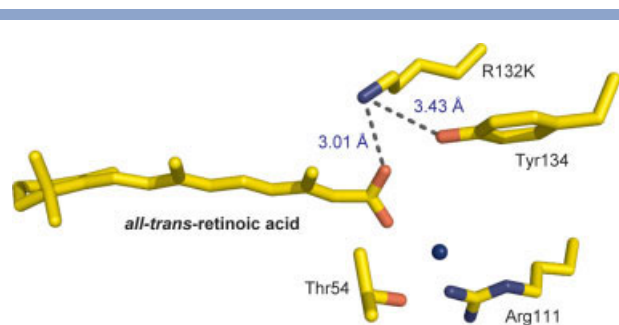
**Table I**

Binding Affinities of Various CRABP II Mutants with all-*trans*-Retinoic Acid as Determined by Fluorescence Quenching<sup>a</sup>

	CRABP II mutant	$K_d$ (nM)
1	WT-CRABP II	2.0 ± 1.2
2	R132M	12–16 <sup>b</sup>
3	R132L	189 ± 11
4	R132K	65 ± 14
5	R111M	80–90 <sup>b</sup>
6	R132K:Y134F	100 ± 7.1
7	R132K:R111L	736 ± 114
8	R132L:R111L	553 ± 38
9	R132K:Y134F:R111L	1000 ± 28
10	R132L:Y134F:R111L	940 ± 39
11	R132K:Y134F:T54V	565 ± 79
12	R132K:Y134F:R111L:T54V	900 ± 64
13	R132K:R111L:R59L	2044 ± 248
14	R132K:R111M	699 ± 71
15	R132K:R111E	1088 ± 95
16	R132K:R111K	5016 ± 633
17	R132K:R111H	1362 ± 95
18	W109L	2692 ± 361
19	R132K:W109L	3196 ± 252
20	R132K:E73A	564 ± 56
21	R132K:W109L:L121E	93 ± 9
22	R132K:E73A:L121E	353 ± 35
23	R132K:Y134F:R111L:L121E	770 ± 61
24	R132K:Y134F:R111L:L121E:T54V	250 ± 19
25	R132K:Y134F:R111L:L121Q:T54V	1739 ± 94
26	R132K:Y134F:R111L:L121D:T54V	760 ± 43
27	R132K:Y134F:L121E	1400 ± 63
28	R132K:L121E	1260 ± 73
29	R132K:R111L:L121E	426 ± 47
30	R132K:R111L:L121E:T54V	400 ± 36

<sup>a</sup>The ± errors signify SD and are produced after the data fitting by the software.

<sup>b</sup>Estimated values obtained through a competitive binding assay ( $K_d$  (R132M)/ $K_d$  (WT) = 6–8;  $K_d$  (R111M)/ $K_d$  (WT) = 40–45).<sup>38</sup>



**Figure 2**

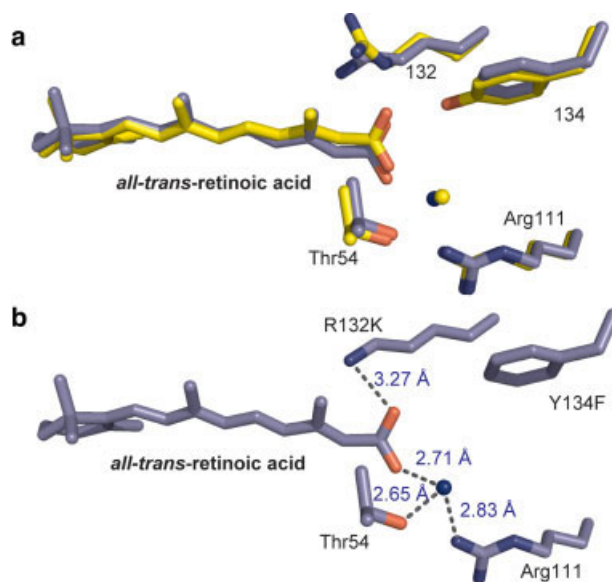
Model structure of R132K-CRABP II with all-*trans*-retinoic acid. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

places the newly introduced Lys132 3.0 Å away from the RA (see Fig. 2), still within a hydrogen bond forming distance. However, the interaction with the incoming RA is not as efficient as that with Arg132. As compared to the reported R132M mutant,<sup>38</sup> it appears that placement of a Lys residue at position 132 reduces the RA affinity more than if a Met residue is present ( $K_d$  values are 65 nM vs 12–16 nM for R132K and R132M mutants, respectively). However, as the two  $K_d$  values were obtained using different methods, a direct comparison of the two might not be in order. A better comparison would be that with single mutant R132L (Table I, entry 3,  $K_d = 189 \pm 11$  nM) in which the hydrophobic Leu residue leads to similar binding affinity as Lys, thus supporting our previous suggestion that a lysine residue at position 132 does not significantly assist in RA binding.

To date, the effect of Tyr134 on RA binding to CRABP II has not been evaluated. To investigate this, double mutant R132K:Y134F was constructed [Note: Y134F single mutant yielded a protein that was miss-folded and was not usable. On the other hand, retaining the R132K mutation led to the isolation of a well-behaved double mutant protein (R132K:Y134F)]. Empirical results in our lab indicate that mutant proteins derived from R132K-CRABP II are stable and well-folded in all cases. On the other hand, some mutants containing Arg132 were not well-behaved (either denatured easily under routine experimental conditions or were sensitive to small amounts of added organic solvents used to introduce RA) and were not well-folded according to CD analysis (data not shown). Thus, the R132K mutation was retained in most of the proteins discussed in this manuscript since its effect on RA binding is well-understood.] Maintaining the R132K mutation, Tyr134 was replaced by a Phe residue. This structurally conservative mutation only removes the phenolic —OH from the binding cavity eliminating one of the hydrogen bonds that holds the retinoic acid in place. As expected, upon mutation the RA binding affinity further declines ( $K_d = 100 \pm 7.1$ , Table I, entry 6). However, although the RA affinity has

decreased  $\sim 50$ -fold as compared to WT-CRABP II, we were able to crystallize the R132K:Y134F mutant bound to retinoic acid (PDB ID: 2G78).<sup>40</sup> As depicted in Figure 3(a), the RA is still bound in a position similar to that of WT-CRABP II, the two molecules virtually overlapping, whereas no significant movement is observed due to the loss of two major hydrogen bonds responsible for RA binding in the WT structure. As expected, the interaction of the engineered Lys132 with the carboxylic acid moiety is considerably weaker than that of the original Arg132 (3.27 Å vs 2.68 Å, respectively). As shown in Figure 3, the carboxylic acid end of the ligand maintains a tight hydrogen bond (2.71 Å) with the water molecule associated with Arg111 (2.83 Å) and Thr54 (2.65 Å). It appears that, in agreement with what was previously reported, the effect of Arg111 on the RA binding is more significant than that of Arg132, although the interaction of Arg111 with the ligand is indirect, through an ordered water molecule.

To further evaluate the role of Arg111, double mutant R132K:R111L was formed. Replacement of Arg111 by hydrophobic Leu results in a further decrease of the RA binding affinity ( $K_d = 736 \pm 114$  nM, Table I, entry 7), similar to what has been reported for R111M.<sup>38</sup> Leu was chosen because of its relatively similar size as compared to the original Arg residue, while its hydrophobic nature can prevent any interaction with neighboring water molecules. Recall that in the CRABP II-WT/RA structure an ordered water molecule, stabilized by hydrogen bonds



**Figure 3**

(a) Overlay of the crystal structures of WT-CRABP II (yellow carbon atoms) and R132K:Y134F mutant (blue carbon atoms) bound to all-*trans*-retinoic acid. (b) Crystal structure of R132K:Y134F-CRABP II mutant bound to all-*trans*-retinoic acid. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

with Arg111 and Thr54, directly contributes to the RA binding (see Fig. 1). Upon formation of the R111L mutation, this water would no longer be well-ordered and/or would further move towards Thr54. Similar results were obtained when double mutant R132L:R111L was formed ( $K_d = 553 \pm 38$  nM, Table I, entry 8), supporting the suggestion that Lys132 does not assist RA binding. Upon additional replacement of Tyr134 with Phe (R132K:Y134F:R111L) the binding affinity declines further ( $K_d = 1000 \pm 28$  nM, Table I, entry 9). In the absence of both Arg132 and Arg111, it was Tyr134 that was responsible for the remaining RA affinity. This result further verifies that the hydrogen bond between Lys132 and retinoic acid is either not forming or is not strong enough to maintain significant retinoic acid binding affinity. To further prove the latter point, triple mutant R132L:Y134F:R111L, which lacks the Lys132, yields a similar binding constant ( $K_d = 940 \pm 39$  nM, Table I, entry 10).

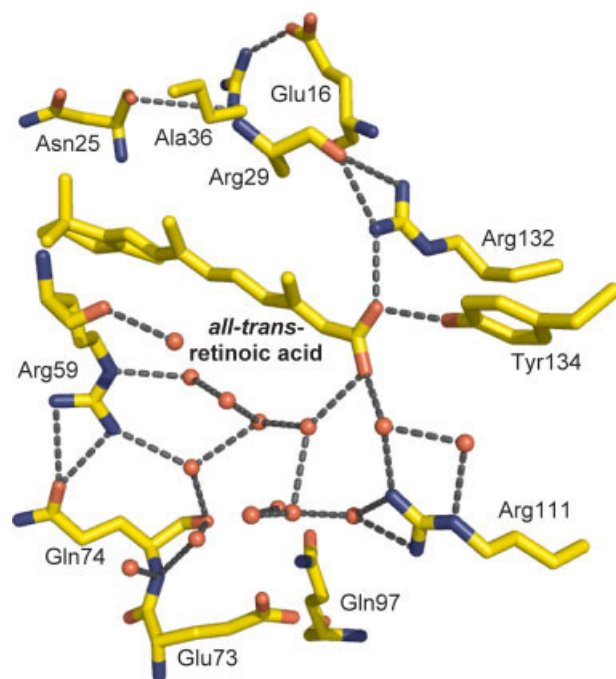
To evaluate the relative contribution of Thr54 to the RA binding, triple mutant R132K:Y134F:T54V was formed. The binding efficiency of the latter mutant decreased ( $K_d = 565 \pm 79$  nM, Table I, entry 11) as compared to the corresponding double mutant R132K:Y134F ( $K_d = 100 \pm 7.1$  nM, Table I, entry 6). However, the effect is not as dramatic as when Arg111 is removed (the corresponding  $K_d$  value for the R132K:Y134F:R111L mutant is  $1000 \pm 28$  nM, Table I, entry 8). Finally, by removing all the residues that have been identified to directly interact with RA, mutant R132K:Y134F:R111L:T54V was formed. The binding affinity of the latter mutant for RA ( $K_d = 900 \pm 64$  nM, Table I, entry 12) is very similar to that of the R132K:Y134F:R111L triple mutant ( $K_d = 1000 \pm 28$  nM, Table I, entry 9) verifying the suggestion that Arg111 is more crucial for RA binding than Thr54. This result agrees with what has been previously observed, according to which neutral-neutral hydrogen bonds (such as that between Thr and water) contribute considerably less (up to 1000-fold less) to the overall binding than the polar-neutral hydrogen bond (Arg-water).<sup>44–46</sup>

At this point, it is important to point out that even when all the residues that, according to the crystal structure, directly interact with RA (Arg132, Arg111, Tyr134, Thr54) are removed the binding constant remains rather high. This result suggests that other interactions with the protein, rather than just hydrogen bonding and electrostatic stabilization, are important for RA binding to CRABP II. On the basis of our data, a rough contribution of hydrophobicity and polar effects to the CRABP II-RA binding affinity can be surmised. One can presume that the hydrophobic nature of the CRABP II binding pocket, and its appropriately large volume ( $\sim 600$  Å<sup>3</sup>) yields a cavity that exhibits  $\sim 1$  μM binding affinity. Further increase in binding affinity to achieve single nM values necessitates the introduction of the four critical hydrogen

bonding interactions shown in Figure 1. In addition, our results are consistent with the previously published suggestion that strategically placed CRABP II Arg residues can create a positive potential, extending from the mouth of the cavity to the binding site that directs the carboxylic acid into the CRABP II.<sup>47</sup> This is supported by the fact that the RA binding affinity is significantly reduced when Arg59, located at the entry portal of the protein, is mutated along with Arg132 and Arg111, versus when only the latter two are mutated (mutant R132K:R111L,  $K_d = 736 \pm 114$  nM versus R132K:R111L:R59L,  $K_d = 2044 \pm 248$  nM, Table I, entry 7 and 13, respectively).

Recently, we have further evaluated the direct effect of removing Arg111 by determining the crystal structure of apo-WT CRABP II, at high resolution.<sup>37</sup> When the structure was compared with the previously published structure of the R111M mutant clear structural differences were noticed in the  $\alpha 2$  helix and the  $\beta C$ - $\beta D$  hairpin loop as compared to the apo-WT structure. Since removal of Arg111 appears to induce the greatest decline in RA binding, different residues, in addition to the previously discussed Leu, were introduced in position 111 in order to evaluate their effect. In the case of R132K:R111M, the binding constant ( $K_d = 699 \pm 71$  nM, Table I, entry 14) was similar to that of R132K:R111L ( $K_d = 736 \pm 114$  nM, Table I, entry 7). Residue 111 is embedded in the middle of a hydrophobic cavity. When a negatively charged residue replaces the native Arg111 (mutant R132K:R111E), the binding of RA declines significantly ( $K_d = 1088 \pm 95$ , Table I, entry 15). A Glu residue at position 111 would be too far away to be involved in any direct interaction with the ligand. It is possible, though, that it can be surrounded by water molecules, which, in turn, can coordinate to the incoming RA. Because of the differences in size and polarity of the functional groups present, such a coordination could disrupt the ordered water network previously observed in the CRABP II-WT/RA crystal structure, that begins from Arg111 and extends all the way to Arg59 and the helical entrance of the CRABP II cavity (see Fig. 4). In particular, all hydrogen bonding interactions that were previously observed in the presence of Arg111 may now be either eliminated or reversed in polarity, based on the protonation state of Glu. Glu111 may prefer to be protonated when inside the hydrophobic CRABP II binding pocket. Either way, one can argue that the ordered water molecules held in place by their interaction with Arg111 may be significantly altered in the presence of Glu111.

The presence of an ordered water network in the hydrophobic interior of proteins has been implicated in a number of structural and functional tasks. In the case of the structurally related Fatty Acid Binding Proteins (FABP), a similar water network has been suggested to be the source of ligand selectivity between different proteins.<sup>48,49</sup> In the case of the adipocyte lipid binding proteins it has been suggested that the ordered water net-



**Figure 4**

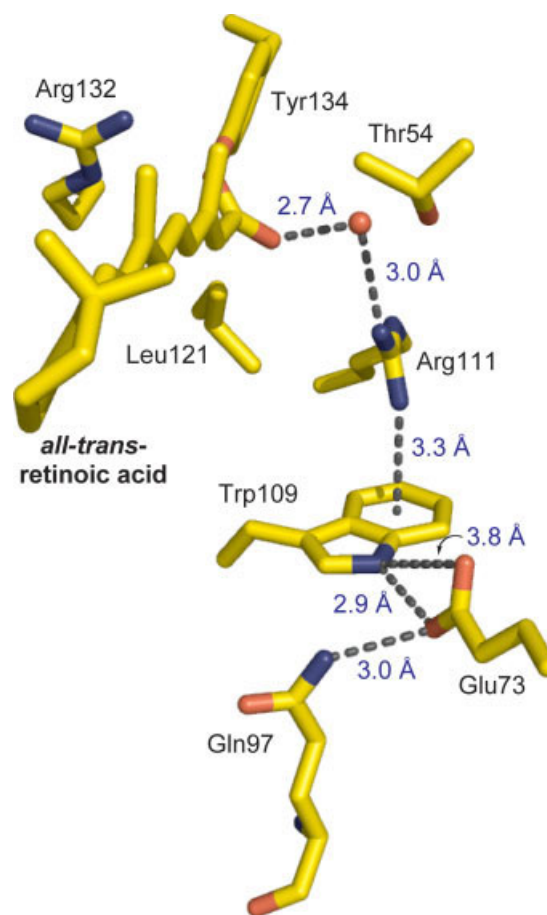
Crystal structure of the WT-CRABP II-RA complex depicting specific interactions within the binding pocket. The water molecules that participate in the Arg111 centered water network are shown in red spheres. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

work can form the equivalent of a fixed hydrophobic surface.<sup>49</sup> As pointed out for this family of proteins, one cannot study the formation and function of the binding site without considering the ordered water network present. This water network involves, through a hydrogen bond cascade, most of the internal polar side chains and appears mostly intact in both the apo- and the holo-form of the proteins.<sup>47</sup> This observation suggests that the presence of this water network must be related to the overall stabilization of the protein itself which provides an interface between the apolar molecule and the polar residues within the binding site (similar to the behavior of an apolar molecule in an aqueous medium). The fact that the water found in the FABP-cavity is intrinsically involved in the binding process was also verified by a series of simulations.<sup>50–52</sup>

The recent crystal structure of the apo-CRABP II verifies that the water network is maintained, and is similar in both the apo- and the holo-forms of the protein.<sup>37</sup> However, the ordered water molecule is significantly altered in the R111M mutant, as first reported by Chen *et al.*<sup>47</sup> Although this change in the protein interior, which was also accompanied by structural changes, especially in the  $\alpha$ -helical part of the protein, was initially attributed to a three-step mechanism for ligand entry and binding to CRABP II, our results indicate that the

observed changes are exclusively due to the R111M mutation. Therefore, our data support the hypothesis that any mutation that causes disruption of the organized water network can lead to significant structural changes in the protein and can therefore result in lower RA affinity.

In a similar manner, the disruption of the ordered water network within the protein interior can also explain the decrease in RA binding observed when a Lys or a His residue is placed at position 111 (mutants R132K:R111K,  $K_d = 5016 \pm 633$  and R132K:R111H,  $K_d = 1362 \pm 95$ , Table I, entries 16 and 17, respectively). Both of these residues are capable of forming hydrogen bonds with water molecules similar to the native Arg. However, they did not facilitate RA binding in the same manner as the WT protein. This result further supports the suggestion that the exact positioning of the water molecules in the CRABP II cavity, as determined by their interactions with surrounding amino acid residues, is critical for ligand binding. The unusual sensitivity of the



**Figure 5**

Interactions within the binding site of the WT-CRABP II-RA complex. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

structure to alterations in this internal hydrogen bonding network is likely a result of the relatively large internal cavity ( $600 \text{ \AA}^3$ ) formed within the structure of such a small (136 residues) protein. This is a common characteristic of all the proteins in this family.

A closer look at the CRABPII-WT/RA crystal structure reveals that Arg111 is locked in a specific orientation, presumably due to its interaction with Trp109 via a  $\pi$ -cation interaction (see Fig. 5). In turn, Trp109 is held in place for the  $\pi$ -cation interaction through a hydrogen bond with Glu73, which is further stabilized through a hydrogen bond with Gln97. In addition, Glu73 is part of a group of residues that has been suggested to support the ordered water network described earlier.<sup>47</sup> To probe the effect of the locked Arg111 orientation, Trp109 was removed and replaced by a Leu residue (mutant R132K:W109L, Table I, entry 19). Upon mutation, the observed affinity for RA binding dramatically decreases ( $K_d = 3196 \pm 252 \text{ nM}$ ) as compared to single mutant R132K ( $K_d = 65 \pm 14 \text{ nM}$ , Table I, entry 4). Very similar results are obtained for single mutant W109L ( $K_d = 2696 \pm 361 \text{ nM}$ , Table I, entry 18) verifying that the R132K mutation does not interfere significantly with RA binding. It appears that when Trp109 is removed, the  $\pi$ -cation interaction with Arg111 is eliminated and Arg111 is free to adopt additional conformations. Thus, the freely rotating Arg111 cannot interact with the ordered water molecule network presumably without stiff entropic penalty, resulting in great loss of RA binding. However, verification of this hypothesis must await quantitative calorimetric measurements (ITC experiments) that compare WT and mutant thermodynamic quantities, especially entropy changes. Furthermore, Glu73 can turn further towards Gln97 (see Fig. 5), possibly disrupting the existing water network. Although the CD spectrum of the R132K:W109L mutant suggests that the overall protein tertiary structure does not significantly change, an extensive change of the ordered water network within the cavity can result in local conformational changes that have a detrimental effect on the RA binding constant but do not disturb the overall tertiary structure of the protein. Interestingly, Trp109 is not a conserved residue within this family of proteins<sup>35</sup> suggesting that it might in fact be a source of ligand selectivity.

When Trp109 is maintained but, instead Glu73 is replaced with a neutral residue (mutant R132K:E73A, Table I, entry 20) the binding affinity is reduced ( $K_d = 564 \pm 56 \text{ nM}$ ) as compared to that of the single mutant R132K ( $K_d = 65 \pm 14 \text{ nM}$ ). However, this decrease is not as dramatic as when Trp109 is removed, further suggesting that the locked orientation of Arg111 by the  $\pi$ -cation interaction with Trp109 is critical for retinoic acid binding. In an attempt to set the orientation of Arg111 by other means (in the absence of either Trp109 or Glu73) two sets of triple mutants were formed, R132K:W109L:L121E and R132K:E73A:L121E, Table I,

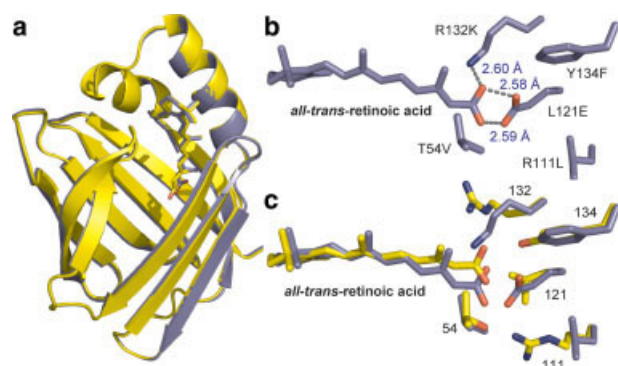
entries 21 and 22). In this case, a Glu residue replaced a neutral Leu at position 121, which is located, as shown in Figure 5, on the other side of Arg111. By promoting hydrogen bond formation between Arg111 and the newly introduced Glu121 we expected that RA binding affinity would be regained since the floppy Arg111 can be "locked" into position through Glu121 binding. The result was rather dramatic since, in the absence of Trp109, introduction of Glu121 increased the RA binding affinity by >34-fold (compare  $K_d$  values for R132K:W109L ( $3196 \pm 252 \text{ nM}$ , Table I, entry 19) vs R132K:W109L:L121E ( $K_d = 93 \pm 9 \text{ nM}$ , Table I, entry 21). However, when Trp109 is maintained and Glu73 is removed instead, introduction of Glu121 still increases RA binding but not as efficiently (the  $K_d$  values for R132K:E73A and R132K:E73A:L121E mutants are  $564 \pm 56 \text{ nM}$  and  $353 \pm 35 \text{ nM}$ , respectively). Overall, although the Arg111-Trp109  $\pi$ -cation interaction is more important than the possible Arg111-Glu121 salt bridge, freezing the conformation of Arg111 results in better RA binding.

The fact that introduction of a Glu residue at position 121 resulted in improved RA binding prompted us to introduce the same mutation (L121E) along with mutations of the residues that directly interact with RA (R132K:Y134F:R111L:T54V). Along these lines tetra mutant R132K:Y134F:R111L:L121E was formed. The introduction of Glu121 resulted in improved RA binding ( $K_d = 770 \pm 61 \text{ nM}$ , Table I, entry 23) as compared to the hydrophobic triple mutant R132K:Y134F:R111L ( $K_d = 1000 \pm 28 \text{ nM}$ , Table I, entry 9). The binding affinity is even further improved when the T54V mutation is included (R132K:Y134F:R111L:L121E:T54V ( $K_d = 250 \pm 19 \text{ nM}$ , Table I, entry 24) vs R132K:Y134F:R111L:T54V [ $K_d = 900 \pm 64 \text{ nM}$ , Table I, entry 12]). When all the polar residues that, according to the crystal structure, directly interact with the RA are removed (R132K:Y134F:R111L:T54V), introduction of a Glu at position 121 restores most of the RA binding affinity. These results were initially confusing since it was not clear how the presence of a single Glu in the binding pocket can result in significant RA binding. However, the crystal structure reveals the presence of a carboxylic acid dimer between the retinoic acid and Glu121 (see Fig. 6).<sup>53</sup> For such a binding motif to occur the carboxylic acids from both the RA and Glu121 have to remain protonated at physiological pH ( $\sim 7.3$ ). In solution, the  $pK_a$  of a typical carboxylic acid is  $\sim 4$ – $5$  and therefore, the carboxylate form is maintained at physiological pH. However, in the hydrophobic cavities of proteins the carboxylate-containing residues remain protonated since a negative charge cannot be stabilized by the neighboring nonpolar residues.<sup>54</sup> Literature reports point out that in the case of the structurally conserved fatty acid binding proteins that belong to the same family, different fatty acids in different proteins have been identified as being in either the carboxylate or the protonated acid form.<sup>51</sup> The

introduction of Glu121 into the binding pocket provides a hydrogen bond donor/acceptor system essential for RA binding when all other polar groups in the vicinity of the RA carboxylate (Arg132, Tyr134, Arg111, Thr54) have been removed. This mode of binding further explains why removal of all the polar group-bearing residues was essential to achieve good RA binding in the presence of the L121E mutation; the presence of any polar group (like the Thr54 —OH) in the vicinity of the RA-carboxylic acid moiety could alter the  $pK_a$  of either RA or Glu121 preventing the formation of a carboxylic acid dimer.

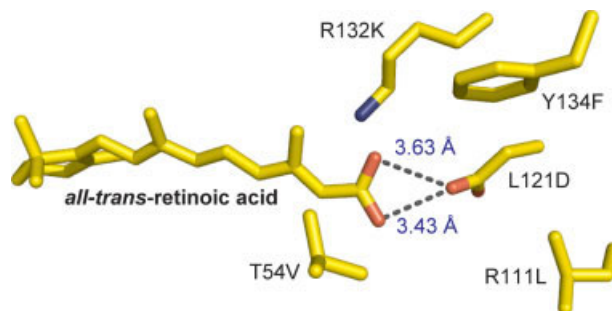
When an Asp instead of a Glu is introduced at position 121 (mutant R132K:Y134F:R111L:L1221D:T54V, Table I, entry 26) the binding affinity is reduced by  $\sim 3$ -fold ( $K_d = 760 \pm 43$  nM) as compared to the R132K:Y134F:R111L:L1221E:T54V mutant ( $K_d = 250 \pm 19$  nM). In this case, it appears that the shorter Asp121 cannot be placed close enough, in a face to face trajectory with the bound RA (see Fig. 7) resulting in significant loss of binding affinity. Exchange of the carboxylic acid group at position 121 with the structurally conservative amide Gln121 (penta mutant R132K:Y134F:R111L:L121Q:T54V, Table I, entry 25), results in a dramatic increase of the RA dissociation constant ( $K_d = 1739 \pm 94$  nM). This result might indicate that only in the presence of Glu121 the RA is protonated and can form a dimer. When Gln121 is present the RA is not protonated (carboxylate form), which might be the factor that leads to the higher  $K_d$ .

The effect of different mutations on RA binding was further evaluated by comparing a number of different mutants. In particular, by comparing triple mutant



**Figure 6**

(a) Overlay of the crystal structures of WT-CRABP II (yellow carbon atoms) and R132K:Y134F:R111L:L121E:T54V mutant (blue carbon atoms) bound to all-*trans*-retinoic acid. (b) Crystal structure of R132K:Y134F:R111L:L121E:T54V-CRABP II mutant bound to all-*trans*-retinoic acid through introduced Glu121 (PDB ID:3CWK). (c) Overlay of the binding cavity of WT-CRABP II (yellow carbon atoms) and R132K:Y134F:R111L:L121E:T54V mutant (blue carbon atoms) bound to all-*trans*-retinoic acid.



**Figure 7**

Model structure of R132K:Y134F:R111L:L121D:T54V-CRABP II mutant bound to all-*trans*-retinoic acid. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

R132K:Y134F:L121E ( $K_d = 1400 \pm 63$  nM, Table I, entry 27) with double mutant R132K:L121E ( $K_d = 1260 \pm 73$  nM, Table I, entry 28) we can see that mutation Y134F is not critical for retinoic acid binding. On the other hand, mutation R111L appears again to be more significant, resulting in a 3-fold increase in RA binding (compare triple mutant R132K:R111L:L121E ( $K_d = 426 \pm 47$  nM, Table I, entry 29) with double mutant R132K:L121E ( $K_d = 1260 \pm 73$  nM, Table I, entry 28). This is opposite to what was observed in the absence of L121E, since removal of Arg111 results in greatly decreased RA binding. As previously discussed, removal of R111 is important since it leads to increased hydrophobicity of the binding pocket which, in turn can promote the protonation of the carboxylate groups, resulting in carboxylic dimer formation, either through direct interactions or by disrupting the ordered water molecule network. On the other hand, the additional hydrophobic mutation T54V is proven to have no effect on RA binding (R132K:R111L:T54V:L121E, Table I, entry 30,  $K_d = 400 \pm 36$  nM). [As previously discussed, T54V does not appear to affect the water network (and binding) as dramatically as R111L].

## CONCLUSIONS

Overall, through a systematic replacement of amino acids within the CRABP II binding cavity we have identified and quantified the effect of additional residues on RA binding. We have shown that a specific water network of ordered water molecules is important for proper protein function and we have identified an orientation manifold for Arg111, a polar amino acid in the midst of a hydrophobic cavity that further verifies its importance for RA binding. Furthermore, it appears that RA responds to cavity shape in large measure since its binding trajectory remains the same even after all direct electrostatic interactions with residues within the binding cavity are significantly altered or removed. The  $\mu M$  bind-

ing results even when all direct electrostatic interactions with RA are removed, and thus providing evidence for an important contribution to binding from a ligand-driven hydrophobic effect. In addition, we have demonstrated that one can rescue the nM RA binding by removing all previously identified interacting amino acids and providing a dimer partner in the form of a Glu residue. On the basis of the data provided here, we have shown that appropriately placed hydrogen bond donors enhanced binding of RA by 3 orders of magnitude. In addition, the robustness of the overall structure of CRABPII has allowed us freedom for extensive manipulation of its binding cavity, making this scaffold a good candidate for further protein engineering projects.\* Since CRABPII does not possess any enzymatic activity it can be an ideal system for further studies on separating the effects of electrostatic versus hydrophobic binding of hydrophobic ligands to proteinic scaffolds.

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\*CD spectra and ~20 crystal structures of apo-CRABPII mutants that we have obtained verify that the CRABPII proteins are stable and correctly folded even when critical residues for binding have been replaced when multiple mutations are applied on the same protein.

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