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Elucidating the exact role of engineered CRABPII residues for the formation of a retinal protonated Schiff base

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ABSTRACT

Cellular Retinoic Acid Binding Protein II (CRABPII) has been reengineered to specifically bind and react with all-*trans*-retinal to form a protonated Schiff base. Each step of this process has been dissected and four residues (Lys132, Tyr134, Arg111, and Glu121) within the CRABPII binding site have been identified as crucial for imine formation and/or protonation. The precise role of each residue has been examined through site directed mutagenesis and crystallographic studies. The crystal structure of the R132K:L121E-CRABPII (PDB-3I17) double mutant suggests a direct interaction between engineered Glu121 and the native Arg111, which is critical for both Schiff base formation and protonation.

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Key words: Schiff base; Bürgi–Dunitz angle; cellular retinoic acid binding protein; ordered water network; rhodopsin protein mimic.

INTRODUCTION

Successful application of rational protein redesign for altered activities requires a detailed understanding of the stability and activity determinants of proteins. The specific orientation of amino acid side chains is one of the most important factors for correct protein folding and proper enzyme function.[1–10] In most cases, even residues that do not directly interact with a bound substrate are important for binding through a variety of indirect mechanisms. An example of the latter is the Cellular Retinoic Acid Binding Protein II (CRABPII), a small cytosolic protein that binds all-*trans*-retinoic acid,[1] modulating its intracellular concentration and delivering it to the appropriate targets.[11–15] The affinity of CRABPII for all-*trans*-retinoic acid is remarkably high (2.0 ± 1.2 n*M*) and, as Figure 1 depicts, the binding is achieved through a series of electrostatic interactions with neighboring residues.[16–18] In particular, retinoic acid is bound via a direct salt bridge and hydrogen bond with residues Arg132 and Tyr134, respectively, through one of its oxygen atoms, while the second carboxylate oxygen is hydrogen bonded to an ordered water molecule. This water molecule is held in place through interactions with two neighboring amino acids, Thr54 and Arg111. Although it has been shown that both residues are important for retinoic acid binding, the presence of Arg111 is more critical because it is also involved in additional hydrogen bonds with water molecules that define an ordered water network within the binding pocket of the protein.[17–20]

Recently, we reported the reengineering of CRABPII into a protein that can bind a non-native substrate, all-*trans*-retinal, as a protonated Schiff base (PSB) [Fig. 2(a)][21,22] mimicking the binding of 11-*cis*-retinal in rhodopsin, the protein responsible for vision.[23–33] This redesigned system is a convenient model for studying the stereoelectronic principles that govern wavelength regulation and enable color vision. The rational redesign of CRABPII into a protein that can bind retinal as a PSB requires a successful approach to the issue of proper amino acid side chain conformation.

Schiff base (SB) and PSB-bound intermediates are found in many enzymes with different functions. Understanding the exact mechanism of PSB formation by manipulating amino acid residues of the binding cavity is important for further protein engineering projects involving this unique binding motif. Successful PSB formation in a protein system results when two different events take place, sequentially. First and foremost, a nucleophilic Lys residue must be appropriately positioned to attack the electrophilic carbonyl. In addition to proximity, the exact orientation of the nucleophile relative to the carbonyl's plane is critical for SB formation. Following the basic principles of organic chemistry, optimal attack occurs when the nucleophile approaches at the Bürgi–Dunitz angle (107°). In CRABPII, replacement of Arg132 with a Lys (R132K)

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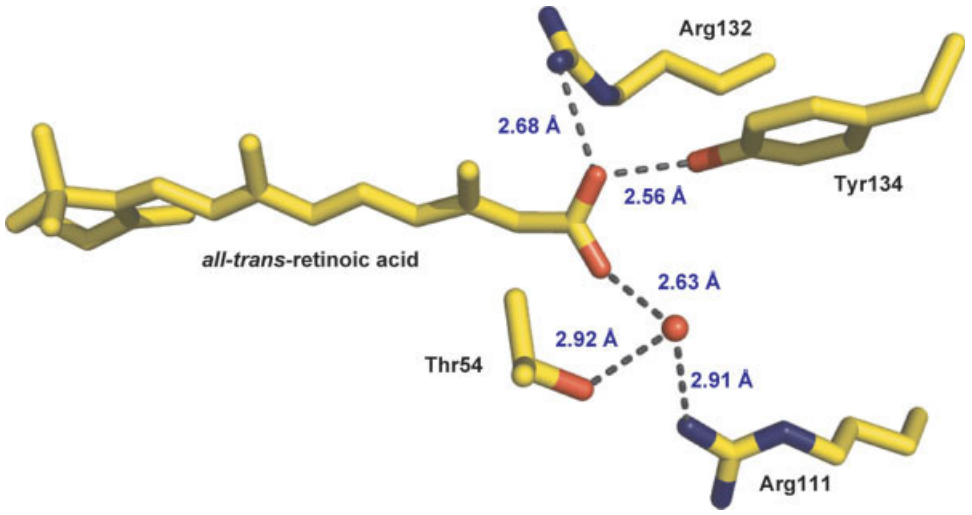

Figure 1

Crystal structure of WT-CRABPII bound to all-*trans*-retinoic acid. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

was the best choice for optimal positioning of the nucleophile. Equally important, however, is the positioning of the electrophilic carbonyl. Binding of retinal with CRABPII places the carbonyl in an unproductive orientation with respect to the putative nucleophilic lysine residue. This orientation is stabilized by interaction with an ordered water molecule that in turn is oriented by Arg111. Therefore, the additional replacement of Arg111 by a Leu residue was required to remove this water and promote efficient PSB formation and stabilization. Subsequent to nucleophilic attack and loss of water, PSB formation requires the protonation of the SB nitrogen, which is promoted by stabilization of a positive charge at this position. In an attempt to mimic the arrangement in the native rhodopsin system, a counter anion (Glu121) was placed close to the imine nitrogen to achieve this stabilization. The above mutations led to the R132K:R111L:L121E triple mutant that binds all-*trans*-retinal as a PSB with high affinity (K_d = 1.4 n*M*). This binding was further verified by a high-resolution crystal structure [Fig. 2(a)], which reveals a significant rotation of the chromophore (retinal), ~50° about its long axis when compared with the retinoic acid bound in the wild-type protein. This rotation was necessary for adopting the needed orientation for effective bond formation.[21] The tightly hydrogen bonded W1 with Arg111 in the WT structure is moved out of play in the R132K:R111L:L121E-CRABPII triple mutant [Fig. 2(b)], thus freeing the carbonyl of retinal to assume the necessary orientation for nucleophilic attack. The removal of this ordered water molecule (W1), by replacing the Arg residue closely associated with it was considered a crucial element for SB formation and stabilization. In this article, the goal is to explicitly and systematically test the assumptions behind the engineering of a retinal-SB forming protein as we predict that it is the specific amino acid interactions that dictate the critical roles that each residue plays for the formation and stabilization of a PSB. In particular, we will further examine the effect of Arg111 for the efficient formation of a CRABPII-retinal PSB. In addition, the exact role of Glu121, the residue engineered as a PSB counter anion, is further evaluated. The crystal structure of R132K:L121E-CRABPII double mutant suggests that the presence and relative orientation of Glu121, as dictated by its interaction with Arg111, or lack of it, is critical for both the formation and stabilization of the retinal-PSB. Furthermore, the exact role of Tyr134, a residue that has been proven critical for retinal-imine formation, will be addressed.

MATERIALS AND METHODS

Fluorescence spectra were recorded using a SPEX® Fluorolog-3 fluorometer (HORIBA JOBIN YVON). UV–vis spectra were recorded with a Cary300 BioWinUV spectrophotometer (Varian). All-*trans*-retinal was purchased from Fluka and was used as received.

Site-directed mutagenesis of CRABPII

Site-directed mutagenesis was performed using the CRABPII-pET17b plasmid following Stratagene's Quikchange® Kit protocol. The primers used for the mutations described in this manuscript were: **R132K** forward: 5′-GACGTTGTGTGCACCAAGGTCTACGTCCGAGAG-3′, reverse: 5′-CTCTCGGACGTAGACCTTGGTGCACACAA CGTC-3′; **R132K:Y134F** forward: 5′-GTTGTGTGCACCA AGGTCTTCGTCCGAGAGCTCGAG-3′, reverse: 5′-CTCG AGCTCTCGGACGAAGACCTTGGTGCACACAAC-3′; **R111L**

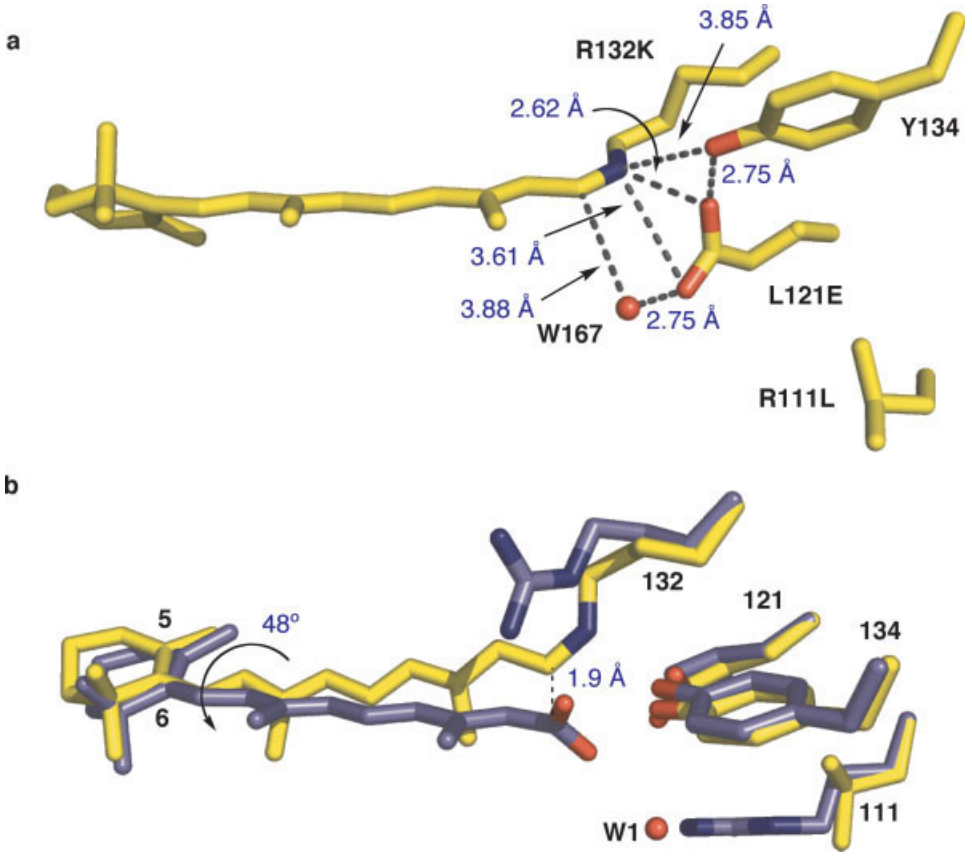

Figure 2

(**a**) Retinal bound to R132K:R111L:L121E-CRABPII as a Schiff base through Lys132. (**b**) Overlay of retinal bound to R132K:R111L:L121E -CRABPII (yellow carbon atoms) and RA bound to WT-CRABPII (blue carbon atoms). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

forward: 5′-CCCAAGACCTCGTGGACCCTAGAACTGACCAA CGATGG-3′, reverse: 5′-CCATCGTTGGTCAGTTCTAGGGTC CACGAGGTCTTGGG-3′; **T54V**: forward: 5′-CTACATCAAAG TCTCCACCACCGTGCG-3′, reverse: 5′-CGCACGGTGGTG GAGACTTTGATGTAG-3′; **R111M**: forward: 5′-GACCTCGTG GACCATGGAACTGACC-3′, reverse: 5′-GGTCAGTTCCAT GGTCCACGAGGTC-3′; **R111V**: forward: 5′- GACCTCGTG GACCGTAGAACTGACC-3′, reverse: 5′- GGTCAGTTCTACG GTCCACGAGGTC-3′; **R111E**: forward: 5′-CCTCGTGGACC GAGGAACTGACCAACG-3′, reverse: 5′-CGTTGGTCAGTTCC TCGGTCCACGAGG-3′; **R111K**: forward: 5′-CGTGGAC CAAAGAACTGACC-3′, reverse: 5′-GGTCAGTTCTTTGGTC CACG-3′; **R111H**: forward: 5′-GACCTCGTGGACCCAC GAACTGACC-3′, reverse: 5′-GGTCAGTTCGTGGGTCCAC GAGGTC; **L121E**: forward: 5′-GATGGGGAACTGATCGA GACCATGACGGCGGATGAG-3′, reverse: 5′-CTCATCCGCC GTCATGGTCTCGATCAGTTCCCCATC-3′.

CRABPII protein expression and purification

The expression of the CRABPII proteins was carried out as previously described.[21] Briefly, expression of the CRABPII mutants in the pET17b vector was performed at 32°C for 5 h in *Escherichia coli* strain BL21(DE3)-pLysS. The overexpressed proteins were purified by ion exchange chromatography using Q Sepharose™, Fast Flow resin followed by FPLC (Source 15Q resin).

Determination of extinction coefficients of CRABPII mutants

The absorption extinction coefficients (ε) were determined according to the method first described by Gill and von Hippel.[34] The calculated values are (in M^{-1} cm^{-1}): **R132K**:19,202; **R132K:Y134F**:18,317; **R132K:Y134F:R111L**:16,654; **R132K:R111L**:20,846; **R132K:Y134F:L121E**:18,422; **R132K:L121E**:17,981; **R132K:Y134F:R111L:L121E**:21,500; **R132K:R111L:L121E**:23,136; **R132K:Y134F:R111L:L121E:T54V**:19,362; **R132K: R111L:L121E:T54V**:19,706; **R132K:L121E:T54V**:20,925; **R132K:R111L:T54V**:21,190; **R132K:Y134F:R111L:T54V**:17,798; **R132K:R111M:L121E**:18,678; **R132K:R111V:L121E**:19.027; **R132K:R111H:L121E**:19,550; **R132K:R111K:L121E**:15,068; **R132K:R111E:L121E**:17,611.

Tryptophan fluorescence quenching

Measurements of tryptophan fluorescence quenching and K_d calculations were carried out as previously described.[21,22] Briefly, protein fluorescence spectra were recorded at 25°C in a 0.01% gelatin containing PBS buffer (4 m*M* NaH_2PO_4, 16 m*M* Na_2HPO_4, 150 m*M* NaCl, pH = 7.3), containing 0.5 μ*M* 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, emission slit 12 nm). All-*trans*-retinal was added at varying equivalents from a 1.5 m*M* 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 after the addition of each aliquot of chromophore. The obtained values were 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 CRABPII mutant was determined according to the method previously described by Wang *et al.*,[18] using Sigmaplot software.

Reductive amination of the protein/retinal complex and MALDI-TOF analysis

CRABPII proteins (0.5 mg) in Tris buffer (1 mL, 10 m*M* TRIS-HCl, pH = 8) were mixed with 1.0 equiv of retinal in ethanol (final ethanol volume less than 2%). The mixture was incubated at 23°C, shielded from light for 1 h after which 10 μL of 5*M* $NaCNBH_3$ in 1*N* NaOH was added. The reaction was incubated for an additional hour before it was applied to a Q Sepharose™ Fast Flow column (quaternary ammonium resin, strong anion exchanger). The column was washed with 10 m*M* TRIS HCl, pH = 8.0 and the protein was eluted using 10 m*M* TRIS-HCl, 100 m*M* NaCl, pH = 8.0 buffer. The fractions containing protein were identified using UV–vis (A_{280}) and were concentrated to a 50 μL final volume using micro-centrifuge filters (Millipore Ultrafree® 0.5 Centrifugal Filters, NMWL: 5000). MALDI-TOF spectra were obtained using sinapic acid as the matrix.

Crystallization conditions

Crystals of the apo-R132K:L121E-CRABPII (KE) were grown in 0.1*M* bis-tris-propane (BTP), pH 9.5, and 30% PEG 4000 using the hanging drop vapor diffusion method at room temperature. Crystals appeared after 7 days. The crystals were briefly immersed in a cryo solution containing 20% glycerol before they were flash frozen in liquid nitrogen. A dataset was collected at the Advanced Photon Source LS-CAT 21-ID-D at Argonne National Laboratory. Processing and scaling was done using HKL2000.[35] For the dataset of apo-R132K:L121E-CRABPII (1.68 Å, P1), the crystal to detector distance was 150 mm and 400° of data were collected with an oscillation of 1°. Structure of the apo-R132K:L121E-CRABPII was solved using Molrep[36] in CCP4[37] with the R132K:R111L:L121E-CRABPII structure as the search model (PDB ID:2G7B). Model building was accomplished in both TURBO-FRODO and COOT.[38] REFMAC5[39–42] within the CCP4 suite of programs was used to refine the structures against 95% of the data, while 5% of data was chosen randomly for cross validation, R_{free}.

Characterization of retinal binding to CRABPII mutants

Binding of retinal to CRABPII and formation of a PSB is monitored via the following experimental techniques. The formation of retinal-CRABPII SB can be followed by mass spectrometry. In particular, after the protein–retinal mixture is submitted to reductive amination conditions (addition of $NaCNBH_3$) the SB forming proteins will show a $[M + 268]^+$ peak in the MALDI spectrum (where *M* is the mass of the CRABPII mutant and 268 is the mass of the covalently bound retinal molecule, after SB formation). After the imine is formed, the protonation of the SB nitrogen is thought to be stabilized by placing a counter anion Glu residue in close proximity. The formation of a retinal-PSB is followed by UV–vis spectroscopy. In solution, typical *N*-retinylidene-n-alkylamines absorb at ~365 nm. In their protonated state there is a bathochromic shift to ~440 nm. PSB formation of retinal by various CRABPII mutants is evaluated via UV–vis spectroscopy. In addition, based on a Trp-fluorescence quenching assay, the K_d values for the overall binding of all-*trans*-retinal to CRABPII can be calculated.

RESULTS AND DISCUSSION

As previously reported three mutations were necessary for the engineering of CRABPII into a protein that can bind retinal as a PSB with high affinity. Namely, in addition to engineering Lys132 (R132K mutant), Arg111 was removed (R111L), presumably allowing retinal to adopt a conformation that can undergo SB formation. It is believed that by substituting Arg111 with a hydrophobic residue an ordered water molecule was removed that was otherwise hindering the rotation of the carbonyl to adopt the necessary orientation for nucleophilic attack. Furthermore, introduction of a negative charge close to the formed imine should stabilize its protonated form, acting as a counter anion. Engineered Glu121 (L121E) can assume this role, leading to the best retinal-PSB forming CRABPII mutant produced thus far (triple mutant R132K:R111L:L121E, $K_d = 1.4 \pm 4.9$ n*M*).

On the other hand, removal of Tyr134 is detrimental for both SB and PSB formation. As shown in Table I

Table I
Retinal Bound to CRABPII Mutants

	CRABPII protein	K_d (n*M*)	λ_{max}[a] (nm)	Red. Am.[b]	pK_a[c]
1	R132K	280 ± 17	379	Yes	<6.5
2	R132K:Y134F	120 ± 5	404	No	N/A
3	R132K:Y134F:R111L	160 ± 7	400	Yes	<6.5
4	R132K:R111L	567 ± 36	408	Yes	<6.5
5	R132K:Y134F:L121E	160 ± 10	381	Yes	<6.5
6	R132K:L121E	104 ± 11	457	Yes	7.8
7	R132K:Y134F:R111L:L121E	200 ± 8	446	Yes	6.5
8	R132K:R111L:L121E	1.4 ± 4.9	449	Yes	8.7
9	R132K:Y134F:R111L:L121E:T54V	2.7 ± 1.7	446	Yes	6.5
10	R132K:R111L:L121E:T54V	2.0 ± 4.1	449	Yes	8.9
11	R132K:L121E:T54V	276 ± 23	456	Yes	n.d.
12	R132K:R111L:T54V	72 ± 12	418	Yes	n.d.
13	R132K:Y134F:R111L:T54V	84 ± 12	412	Yes	n.d.
14	R132K:R111M:L121E	164 ± 32	449	Yes	8.8
15	R132K:R111V:L121E	31 ± 63	449	Yes	8.9
16	R132K:R111H:L121E	4.1 ± 4.7	447	Yes	7.8
17	R132K:R111K:L121E	236 ± 18	460	Yes	8.1
18	R132K:R111E:L121E	894 ± 120	390	No	N/A

[a]Deconvolution of overlapping UV–vis spectra is detailed previously.[22]
[b]Yes/No refers to the results obtained from MALDI-TOF analysis (presence of $[M + 268]^+$) of protein-retinal complex that has been subjected to reductive amination conditions.
[c]pK_a values determined by UV–vis acid-base titration.

(entry 1), the single mutant R132K is capable of SB formation, as evident from the positive reductive amination result. However, the additional mutation of Tyr134 into the structurally conservative Phe residue resulted in a CRABPII mutant (R132K:Y134F, Table I, entry 2) that can bind retinal with relatively high affinity (120 ± 5 n*M*) but is incapable of forming an imine (SB) (according to results from both reductive amination and crystallography). With this double mutant in hand, we had an excellent starting point for investigating the mechanism of SB formation. A series of CRABPII mutants involving the three aforementioned positions (Tyr134, Arg111, Glu121) were generated based on the above observations. The experimental results obtained for each family of CRABPII mutants, as presented in Table I, are summarized below:

Tyr134

While the single mutant R132K was able to form an SB, removal of the Y134 hydroxyl by construction of the double mutant R132K:Y134F resulted in a protein that was unable to form an imine in the presence of all-*trans*-retinal, as proven by spectroscopic and crystallographic results.[21] This indicates that the hydroxyl of Tyr134 is essential for SB formation. Figure 1 shows that in WT-CRABPII, Tyr134 makes a hydrogen bond to the carbonyl oxygen of retinoic acid, indicating that it is this carbonyl position that is required for SB formation. The structure of the inactive mutant R132K:Y134F-CRABPII bound to all-*trans*-retinal shows the chromophore bound to the protein via hydrogen bonds with Lys132 and an ordered water molecule, held in place by Arg111 and Thr54. Although the distance between Lys132 and the carbonyl carbon is close enough to react, it appears that this particular binding motif locks the carbonyl in an unfavorable conformation that would not allow the nucleophilic attack by Lys132. We postulated that by removing Arg111 and/or Thr54 the ordered water molecule that forms a hydrogen bond with retinal's carbonyl oxygen will be also removed, allowing for a conformation that can lead to imine formation. Therefore, Arg111 was replaced with a Leu residue (R111L). The resulting triple mutant R132K:Y134F:R111L regains the ability for imine formation (Table I, entry 3, K_d = 160 ± 7 nM, λ_{max} = 400 nm), confirming the postulate. When the same mutation is applied in the presence of Tyr134 the resulting double mutant (R132K:R111L mutant, Table I, entry 4) exhibits similar behavior. These results directly suggest that the presence of Tyr134 can assist SB formation, presumably by interacting with the carbonyl of the aldehyde to hold it in a reactive conformation and activate it for the nucleophilic attack. In other words, Tyr134 can direct retinal's carbonyl toward Lys132, opposing the effect of the ordered water molecule associated with Arg111 (single mutant R132K). However, when the associated water molecule is moved out of play as a result of substituting Arg111 with Leu, the presence or absence of Tyr134 is not as critical, resulting in similar spectroscopic behavior of both R132K:Y134F:R111L and R132K:R111L mutants.

Interestingly, introduction of a counter anion at position 121 (L121E) can also restore imine formation within the protein's binding pocket. Triple mutant R132K:Y134F:L121E binds retinal as a SB (Table I, entry 5, K_d = 160 ± 10 n*M*, λ_{max} = 381 nm) but does not form a protonated SB at physiological pH, indicating that Glu121 can compensate for loss of Tyr134, probably by directly interacting with the retinal carbonyl oxygen. On the other hand, when Tyr134 is restored and the counter anion Glu121 is maintained, the resulting double mutant R132K:L121E can successfully form a retinal PSB (Table I, entry 6, λ_{max} = 457 nm). The presence of Tyr134 appears to have a dramatic effect on this set of mutants, which translates into a 76 nm red shift. This result suggests a direct interaction between the two residues, Tyr134 and Glu121, in a way that can enhance the ability of Glu121 to act as a counter anion (compare the PSB pK_a values of the two mutants: R132K:Y134F:L121E < 6.5; R132K:L121E = 7.8).

Since both mutations R111L and L121E are important for SB and PSB formation, the next logical step was to apply them simultaneously. Therefore mutants R132K:Y134F:R111L:L121E and R132K:R111L:L121E were produced and their retinal binding affinities were evaluated (Table I, entries 7, 8). When Tyr134 is removed (tetra mutant R132K:Y134F:R111L:L121E), the protein can bind retinal as a PSB (λ_{max} = 446 nm) albeit with a

rather low pK_a (PSB pK_a = 6.5). This is an interesting result considering that none of the previously studied triple mutants that lack Tyr134 (R132K:Y134F:R111L and R132K:Y134F:L121E) could form a PSB with retinal, although both do form a stable SB. It appears that when Tyr134 is removed the presence of Arg111 prevents Glu121 from acting as an efficient PSB counter anion (mutant R132K:Y134F:L121E), presumably as a result of ionic interactions. However, when Arg111 is also removed, tetra mutant R132K:Y134F:R111L:L121E can bind retinal as a PSB. This result suggests that there is an interaction between Arg111 and the engineered Glu121, which can be detrimental for Glu121's ability to act as an efficient PSB counter anion. This fact is even more pronounced in the series of mutants where Tyr134 is restored. As previously discussed, double mutant R132K:R111L can form a SB but cannot stabilize a PSB (Table I, entry 4). On the other hand, double mutant R132K:L121E can form a PSB with retinal with a pK_a value of 7.8 (Table I, entry 6). When both mutations are combined, the resultant triple mutant R132K:R111L:L121E (Table I, entry 8) is a low nM retinal binder with a high apparent PSB pK_a of 8.7. Removal of Arg111 in the presence of Glu121 results in $\sim$1 pK_a unit increase, further indicating an interaction between the two amino acids that is detrimental for yielding PSB forming mutants with high pK_a.

Finally, the role of Thr54, the second residue associated with the critical ordered water molecule (W1, Fig. 1) in the CRABPII cavity, was probed. Based on the spectroscopic results, when Tyr134 is replaced (Y134F), additional mutation of Thr54 into the structurally conserved Val (T54V) producing penta mutant R132K:Y134F:R111L:L121E:T54V exhibits improved retinal binding as compared to the corresponding tetra mutant R132K:Y134F:R111L:L121E (lower K_d, Table I, entries 9 and 7, respectively). On the other hand, in the presence of Tyr134, the T54V mutation resulting in tetra mutant R132K:R111L:L121E:T54V (Table I, entry 10) exhibits a spectroscopic profile virtually identical to that of the corresponding triple mutant R132K:R111L:L121E (Table I, entry 8), rendering the extra mutation T54V unnecessary for successful PSB formation. As was previously discussed, both Arg111 and Thr54 form hydrogen bonds with the same ordered water molecule that is identified to prevent SB formation with retinal (Fig. 1). However, the polar hydrogen bond between the water molecule and positively charged Arg111 is expected to contribute considerably more, up to 3000-fold, to the overall binding than the neutral hydrogen bond between the water molecule and Thr54.[43–45] In agreement with this observation, in the presence of Arg111, the T54V mutation does not assist in either retinal binding or PSB formation as evident from comparing double mutant R132K:L121E (Table I, entry 6, λ_{max} = 457 nm, K_d = 104 $\pm$ 11 n*M*) with triple mutant R132K:L121E:T54V (Table I, entry 11, λ_{max} = 456 nm, K_d = 276 $\pm$ 23 n*M*). On the other hand, in the absence of Glu121, which is suspected to act both as a carbonyl activator and a PSB counter anion, replacement of both Arg111 and T54 with hydrophobic residues significantly improves retinal binding (R132K:R111L:T54V, Table I, entry 12, λ_{max} = 418 nm, K_d = 72 $\pm$ 12 n*M*) as compared to the R132K:R111L double mutant (Table I, entry 4, λ_{max} = 408 nm, K_d = 567 $\pm$ 36 n*M*). A similar trend is observed in the absence of Tyr134, since the binding of retinal to tetra mutant R132K:Y134F:R111L:T54V (Table I, entry 13, K_d = 84 $\pm$ 12 n*M*) is worse than that observed for triple mutant R132K:Y134F:R111L (Table I, entry 3, K_d = 120 $\pm$ 5 n*M*). Overall, we can see that Thr54 is important for retinal binding only when both potential carbonyl activators, Tyr134 and Glu121 are absent.

To summarize, we can conclude that Tyr134 is important for SB formation. When Tyr134 is removed, other additional mutations are required to restore the protein's ability to form a SB (R111L, L121E). At the same time, Tyr134 also assists in the protonation of the imine. When Tyr134 is present, the above mutations can result in improved retinal binding, both as a SB and PSB, resulting in the triple mutant R132K:R111L:L121E identified thus far as the best retinal-PSB forming CRABPII mutant.

Arg111

As previously reported, replacing Arg111 with a hydrophobic residue (R111L) was critical for the successful reengineering of CRABPII into a retinal-PSB forming protein.[21] This mutation was responsible for the removal of a highly ordered water molecule (W1, Fig. 1) within the CRABPII binding cavity that was set in place by hydrogen bonds with Arg111 and Thr54. We had postulated that the presence of this water molecule prevented retinal's carbonyl from adopting a favorable trajectory for nucleophilic attack by the active site Lys residue (R132K) and consequently hindered SB formation. This hypothesis evolved from studying the crystal structures of CRABPII mutants that did not bind retinal as a SB, such as the double mutant R132K:Y134F.[21] Removal of W1, accomplished by removing its hydrogen-bond partner Arg111 enabled the carbonyl to rotate such that it could adopt an orientation necessary for SB formation.

To test this hypothesis we evaluated whether other residues besides Leu at position 111 would effectively yield a functional PSB forming protein. The working hypothesis is that any amino acid that leads to the removal of W1 (the ordered water molecule) should effectively yield a CRABPII mutant capable of binding retinal as a PSB. For this purpose, a series of CRABPII mutants were produced (Table I, entries 14–18).

These proteins retain the nucleophilic Lys132 and the PSB counter anion Glu121, while the residue at position

111 is varied. The replacement of Arg111 by Leu (R111L) minimized any potential problems associated with conformational changes of the overall protein structure, as evident from the reported crystal structure.[21] The question remains, however, whether or not Leu is critical for the successful PSB formation or any other hydrophobic mutation, which can also result in the removal of the coordinated water molecule, would be equally efficient. If the removal of the ordered water molecule is key, as we have previously speculated, similar results (successful PSB formation) should be achieved if hydrophobic amino acids, other than Leu, are introduced in position 111. On the other hand, polar amino acids that can retain the highly ordered water molecule, thus imitating Arg111, should not allow for efficient PSB formation. The spectroscopic results obtained from a number of mutants, as presented in Table I, can be summarized into the following:

(i) Replacement of Arg111 with hydrophobic residues other than Leu (Met and Val entries 14–15, Table I) yields mutants with identical UV-vis absorption maxima (449 nm) as the R132K:R111L:L121E-CRABPII triple mutant, which has Leu at position 111. Similar results were also obtained when neutral His replaced the charged Arg residue at position 111 (entry 16, Table I). These results are consistent with the hypothesis that removal of the polar residue at position 111 leads to loss of the coordinated water molecule and allows for efficient PSB formation.

(ii) Replacement of Arg111 with the electronically conserved Lys (Table I, entry 7) results in the formation of a red-shifted PSB, with an observed λ_{max} at 460 nm. The spectroscopic behavior of this mutant is similar to that of the R132K:L121E double mutant, where the original Arg111 is maintained (Table I, entry 6). In both cases the presence at position 111 of a residue that can hydrogen bond to water in a similar way can result in maintaining the highly ordered W1 at its original position. The ~10 nm red shifting associated with the presence of Lys111 could be attributed to the interaction of Glu121 with the ϵ-amino group of Lys 111. As such, one would expect a red shift as a result of greater iminium ion conjugation since the cation is not as stabilized locally on the iminium nitrogen atom due to the attenuated counter anion. A weaker counter anion (due to the above interaction) can also lead to reduced PSB pK_a as evident from the data in Table I (entries 6, 8). Overall, the red shift characteristics and the pK_a of the PSB formed with positively charged amino acids at position 111 indicate a lessened interaction of Glu121 with the iminium ion.

(iii) When Glu replaces Arg111 the resulting protein (Table I, entry 18) loses its ability to bind retinal as a SB, as apparent by the negative reductive amination result. [The presence of a retinal-SB can be verified by mass spectrometry (MALDI-TOF). The presence of a mass of $[M + 268]^+$ (where M is the mass of the protein) is indicative of a single retinal-SB adduct (positive result). A negative reductive amination result refers to lack of the $[M + 268]^+$ peak in the MALDI-TOF spectrum]. Since the protein retains μM affinity for retinal (K_d = 894 ± 120 nM) one can argue that the polar Glu111 can reverse the polarity of the hydrogen-bond network with the participation of Glu121, thus in effect preventing Glu121 from activating retinal's carbonyl oxygen for nucleophilic attack by Lys 132. Alternatively, in the midst of the otherwise hydrophobic environment of the CRABPII cavity, the two Glu residues (Glu121 and Glu111) can be both protonated leading to the formation of a carboxylic acid dimer (we have previously observed a similar arrangement between Glu121 and the carboxylic acid of all-*trans*-retinoic acid, the native substrate of CRABPII).[20] Such an arrangement would inhibit Glu121 to act either as a carbonyl activator or a possible PSB counter anion, consistent with the observed results.

Glu121

As evident from the results summarized earlier the exact role of the engineered Glu121 in this system is closely connected to the nature of the residue at position 111. As we have previously seen, in the absence of the L121E mutation the protein's ability for PSB formation is compromised (R132K:R111L mutant, Table I, entry 4). When there is no counter anion to stabilize the PSB, the protein retains its ability for SB formation, albeit with a depressed iminium pK_a (pK_a < 6.5 for R132K:R111L, Table I, entry 4 vs. pK_a = 8.7 for the R132K:R111L:L121E mutant, Table I, entry 8). On the other hand, R132K:L121E double mutant binds retinal as a PSB; however, with a 10-fold lower affinity as compared to R132K:R111L:L121E triple mutant that lacks Arg111 (Table I, entries 6, 8). In addition, the λ_{max} of the resulting PSB peak is significantly red-shifted for this double mutant (457 nm for R132K:L121E vs. 449 nm for R132K:R111L:L121E) indicating that in the presence of Arg111, the Glu121 counter anion might be involved in additional interactions, either through the ordered water molecule W1 or directly with Arg111. This putative interaction can affect both Glu's efficiency as a counter anion and its ability to activate retinal's carbonyl oxygen for nucleophilic addition, according to the mechanism previously proposed.[21]

The UV–vis data in Table I indicates that on PSB formation double mutant R132K:L121E red shifts more than triple mutant R132K:R111L:L121E by ~8 nm. At the same time, there is a significant change in the pK_a value of the formed PSB (Table I). In particular, when

there is no counter anion present (Leu121) double mutant R132K:R111L does not successfully form a PSB at physiological pH (PSB pK_a < 6.5, Table I, entry 4). In the presence of Glu121, when Arg111 is not removed (R132K:L121E, Table I, entry 6) the pK_a for the formed PSB is 7.8. When both R111L and L121E mutations are applied the triple mutant R132K:R111L:L121E successfully binds retinal as a PSB with a pK_a value of 8.7 (Table I, entry 8). It appears that, in the presence of Glu121, replacement of Arg111 with the hydrophobic Leu results in increased PSB pK_a values by ~1 unit. Similar results are obtained when Arg111 is replaced with other hydrophobic residues besides Leu (mutants R132K:R111M:L121E and R132K:R111V:L121E, Table I, entries 14 and 15, pK_a values 8.8 and 8.9, respectively). However, when Arg111 is replaced by either a Lys or a His residue, the pK_a of the corresponding PSB is lower. As shown in Table I, entries 17 and 18, the pK_a values for R132K:R111K:L121E and R132K:R111H:L121E are 7.8 and 8.1, respectively. Those values are similar to that of the R132K:L121E mutant (pK_a = 7.8, Table I, entry 6).

The latter data suggests that the presence of hydrophobic residues at position 111 results in elevated pK_a values for the PSB. On the other hand, any residue that is positively or potentially positively charged (Arg, Lys, His) lowers the pK_a. This is consistent with the idea that positively charged residues, such as Arg, will destabilize the positively charged PSB if in close proximity while removal of positive charges and dipoles from the PSB region can lead to increased pK_a. On the other hand, it has been shown that negatively charged residues close to the PSB can either directly or through polar hydrogen bonds with water molecules, stabilize the positive charge on the imine.[46–50] Therefore, the pK_a of a PSB can be electrostatically tuned via its interaction with charged amino acids within a protein binding site.

Besides altering the dipoles within the CRABPII cavity, removal of Arg111 can also affect the acidity of the nearby Glu121. The crystal structure of triple mutant R132K:R111L:L121E indicates that in the absence of Arg111, Glu121 directly interacts with both the PSB and Tyr134 [Fig. 3(a)]. However, as previously discussed, in the presence of Arg111 an interaction, either direct [Fig. 3(b)] or through a water molecule [Fig. 3(c)], between the positively charged Arg111 and Glu121 can weaken the counter-anion character of the latter, resulting in less stable (lower pK_a) and red shifted PSB. Replacement of Arg111 with hydrophobic residues can restore the counter anion ability of Glu121 as evidenced by the increased pK_a values concomitant with blue shifting of the PSB. This explanation is consistent with the data presented in Table I and initiated further investigation to probe the mode of interaction between Arg111 and Glu121 using crystallography.

Our efforts were focused on double mutant R132K:L121E that retains the original Arg111 and also provides Lys132 and the counter anion Glu121. Attempts to crystallize the holo structure of R132K:L121E bound to all-*trans*-retinal did not succeed. However, the apo form of R132K:L121E was crystallized (resolution 1.2 Å) revealing two orientations of equal occupancy for Glu121. As shown in Figure 4(a), both configurations of Glu121 interact with Tyr134 through a tight hydrogen bond (2.7 and 2.8 Å, respectively), while only one of them is engaged in a hydrogen bond (2.8 Å) with the imine-nitrogen of the Arg111 guanidinium. Superimposed structures of apo-R132K:L121E and apo-R132K:R111L:L121E reveal that the orientation of Glu121, which is pointing away from Arg111 is similar in both structures [Fig. 4(b)]. On the other hand, in the presence of Arg111 there is an alternate configuration of Glu121 that forms a hydrogen bond with the secondary

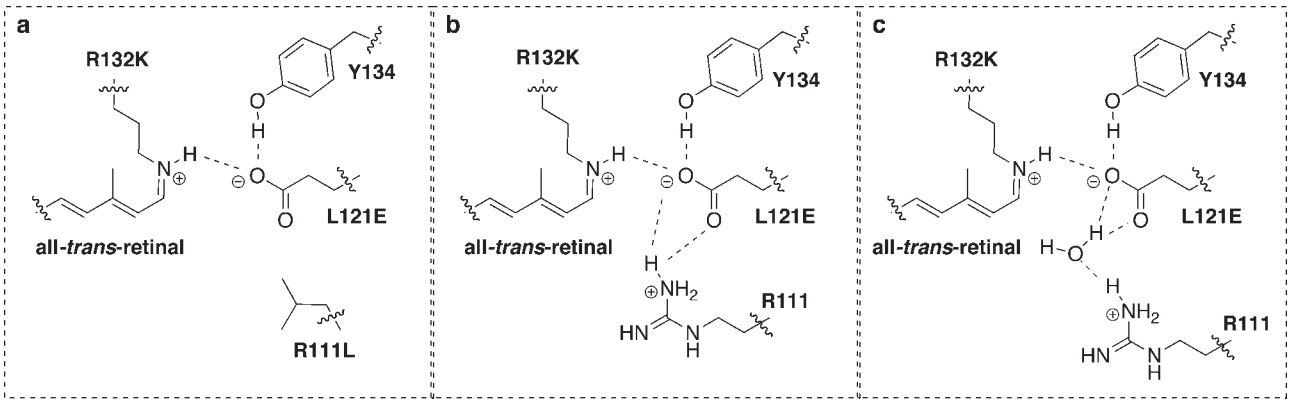

Figure 3

Possible interactions between Glu121 and Arg111. In the absence of Arg111, (**a**) Glu121 can act as a PSB counter anion while also hydrogen bonding with Tyr134. In the presence of Arg111, the positively charged guanidinium can form a hydrogen bond with Glu121, either directly (**b**) or through an ordered water molecule (**c**).

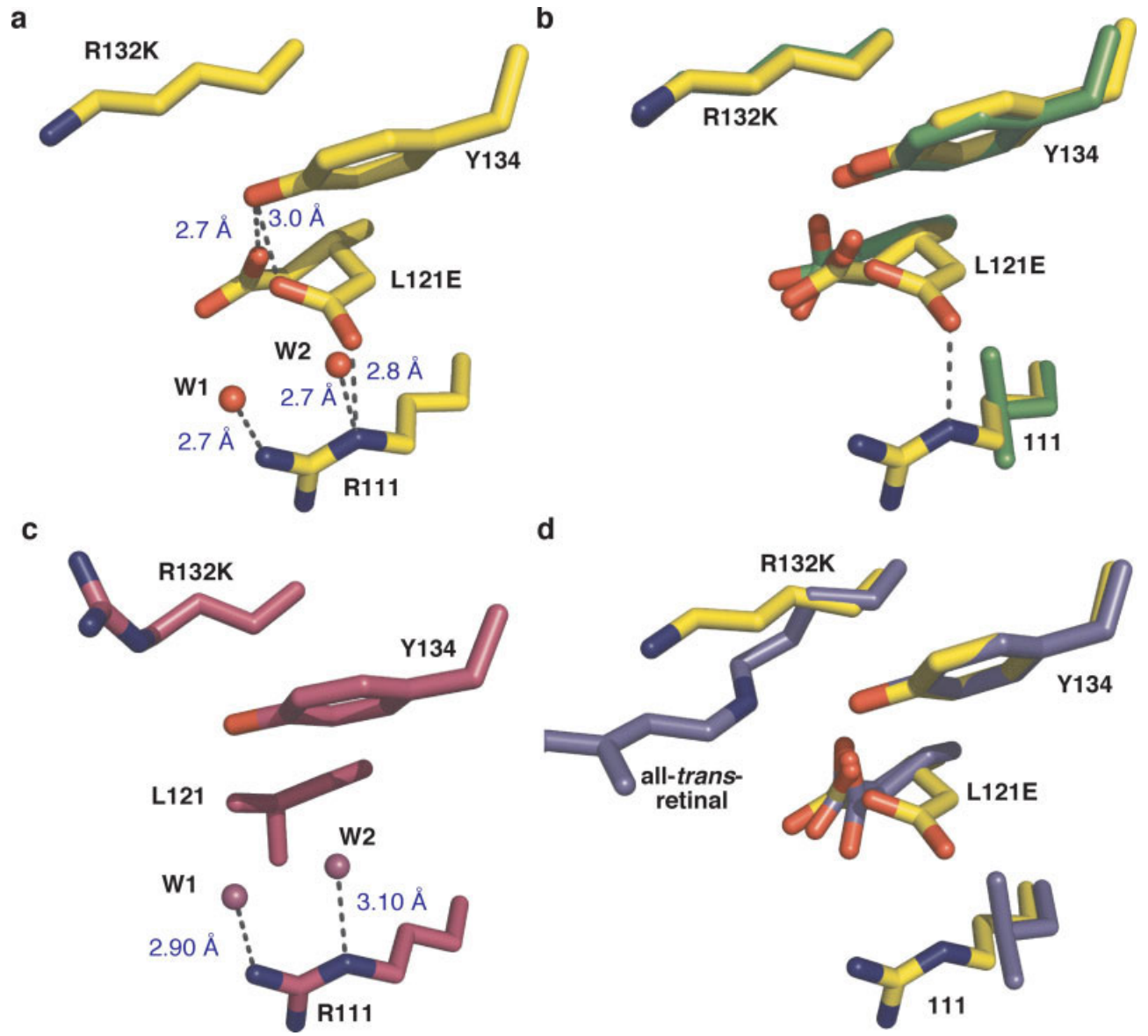

Figure 4

(**a**) Crystal structure of apo-R132K:L121E double mutant. The green spheres represent ordered water molecules (W1: 100% occupancy, W2: 50% occupancy) coordinated to Arg111. (**b**) Overlay of the crystal structures of apo-R132K:R111L:L121E (green carbon skeleton) and apo-R132K:L121E (yellow carbon skeleton) mutants. (**c**) Crystal structure of apo-WT-CRABPII. The two magenta spheres represent the two ordered water molecules coordinated to Arg111. (**d**) Overlay of the crystal structures of R132K:R111L:L121E-retinal complex (blue carbon skeleton) and apo-R132K:L121E (yellow carbon skeleton) mutants.

amine-nitrogen of Arg111, taking the place of an ordered water molecule present in the WT-CRABPII structure [W2, Fig. 4(c)].

A second observation, after comparing the last two apo structures, relates to the presence of ordered water molecules within the binding cavity that, as we have previously reported, are considered important for the correct orientation of the chromophore and efficient SB formation. As can be seen in Figure 4(a) there are two ordered water molecules coordinated to Arg111 in the apo-R132K:L121E structure. The first water molecule (W1), associated with the imine nitrogen of Arg111 in all the CRABPII crystal structures, is also present here (2.7 Å, 100% occupancy) while the second water molecule (W2), associated with the secondary amine nitrogen of the guanidinium (2.7 Å), is only present 50% of the time. The overall picture is similar to what was previously observed for the apo-WT-CRABPII structure [Fig. 4(c)] where in the absence of Glu121 the two water molecules, W1 and W2, are always coordinated to Arg111 and are part of a

more extended ordered water network within the CRABPII cavity.[17,20] In the case of the apo-R132K:L121E mutant [Fig. 4(a)] the partial occupancy of W2 (50%) can also explain the two distinct conformations observed for Glu121. It appears that 50% of the time Arg111 is involved in a electrostatic interaction with Glu121 while the other 50% it is engaged in a hydrogen bond interaction with W2. It also appears that the conformation of Glu121 required for direct interaction with Arg111 pulls Glu121 away.

Finally, although we have not been successful in crystallizing the all-*trans*-retinal-bound form of R132K:L121E mutant we believe that, in compliance with the apo structure, the direct interaction between Glu121 and Arg111 is maintained in the holo structure as well. Keeping that in mind, when the crystal structures of apo-R132K:L121E and all-*trans*-retinal bound R132K:R111L:L121E are superimposed [Fig. 4(d)] one can notice that there are two orientations of Glu121 in R132K:R111L:L121E-retinal, both of which are pointing towards Tyr134 and the PSB and close to the non-Arg111-coordinating conformation of Glu121 in the apo-R132K:L121E structure. It appears that, even in the retinal-bound holo structure of R132K:R111L:L121E, Glu121 does not rotate away from Tyr134 and the PSB in the absence of Arg111. However, when Arg111 is present its direct interaction with Glu121 weakens the counter anion effect and results in a lower PSB pK_a value and more red-shifted UV–vis spectrum observed for the R132K:L121E mutant.

CONCLUSIONS

To summarize, we have identified three necessary conditions for PSB formation and have investigated the relative importance and ramifications of each: (1) The nucleophile provided by Lys132 must be properly positioned for attack. (2) The carbonyl must also be properly positioned and activated for attack. This is accomplished by either Tyr134 or Glu121, since they can both form a hydrogen bond with retinal's carbonyl to activate and position it in the correct conformation for the nucleophilic attack. It also requires the removal of the water-mediated interaction between Arg111 and the retinal carbonyl that positions it in the wrong conformation for attack. Though substitution at this position is important, replacement of Arg111 by different amino acids can be tolerated. However, any polar residue in this position can result in the incorrect carbonyl conformation via the water mediated interaction. (3) The PSB must be stabilized by a counter ion (Glu121). The counter ion must be deprotonated, which is enhanced by interaction with Tyr134, and must also be properly positioned and polarized, which can be sabotaged by interaction with Arg111 or even more detrimentally by Glu111.

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REFERENCES

1. Bolen DW, Rose GD. Structure and energetics of the hydrogen-bonded backbone in protein folding. Annu Rev Biochem 2008; 77:339–362.
2. Borgia A, Williams PM, Clarke J. Single-molecule studies of protein folding. Annu Rev Biochem 2008;77:101–125.
3. Chatani E, Hayashi R. Functional and structural roles of constituent amino acid residues of bovine pancreatic ribonuclease A. J Biosci Bioeng 2001;92:98–107.
4. Daniel RM, Dunn RV, Finney JL, Smith JC. The role of dynamics in enzyme activity. Annu Rev Biophys Biomol Struct 2003;32:69–92.
5. Fersht AR. From the first protein structures to our current knowledge of protein folding: delights and scepticisms. Nat Rev Mol Cell Biol 2008;9:650–654.
6. Guillen Schlippe YV, Hedstrom L. A twisted base? The role of arginine in enzyme-catalyzed proton abstractions. Arch Biochem Biophys 2005;433:266–278.
7. Lee JC. Modulation of allostery of pyruvate kinase by shifting of an ensemble of microstates. Acta Biochim Biophys Sinica 2008;40:663–669.
8. Nagano N, Orengo CA, Thornton JM. One fold with many functions: the evolutionary relationships between TIM barrel families based on their sequences, structures and functions. J Mol Biol 2002; 321:741–765.
9. Whitten ST, Garcia-Moreno EB, Hilser VJ. Local conformational fluctuations can modulate the coupling between proton binding and global structural transitions in proteins. Proc Natl Acad Sci USA 2005;102:4282–4287.
10. Yang XY, Wang M, Fitzgerald MC. Analysis of protein folding and function using backbone modified proteins. Bioorg Chem 2004; 32:438–449.
11. Dolle P, Ruberte E, Leroy P, Morrisskay G, Chambon P. Retinoic Acid receptors and cellular retinoid binding-proteins.I. A systematic study of their differential pattern of transcription during mouse organogenesis. Development 1990;110:1133–1151.
12. Gaub MP, Lutz Y, Ghyselinck NB, Scheuer I, Pfister V, Chambon P, Rochette-Egly C. Nuclear detection of cellular retinoic acid binding proteins I and II with new antibodies. J Histochem Cytochem 1998;46:1103–1111.
13. Napoli JL. Retinoic acid biosynthesis and metabolism. FASEB J 1996;10:993–1001.
14. Napoli JL. Interactions of retinoid binding proteins and enzymes in retinoid metabolism. Biochim Biophys Acta 1999;1440:139–162.
15. Ong DE, Newcomer ME, Chytil F. Cellular retinoid binding proteins. In: Sporn MB, Roberts AB, DeWitt S, Goodman DS, editors. The retinoids: Biology, Chemistry and Medicine, 2nd edition. New York: Raven Press; 1994. pp 283–318.
16. Kleywegt GJ, Bergfors T, Senn H, Lemotte P, Gsell B, Shudo K, Jones TA. Crystal-structures of cellular retinoic acid-binding protein-I and protein-II in complex with all-trans-retinoic acid and a synthetic retinoid. Structure 1994;2:1241–1258.
17. Vaezeslami S, Mathes E, Vasileiou C, Borhan B, Geiger JH. The structure of apo-wild-type cellular retinoic acid binding protein II at 1.4 A and its relationship to ligand binding and nuclear translocation. J Mol Biol 2006;363:687–701.
18. Wang LC, Li Y, Yan HG. Structure-function relationships of cellular retinoic acid-binding proteins—quantitative analysis of the ligand binding properties of the wild-type proteins and site-directed mutants. J Biol Chem 1997;272:1541–1547.

19. Vaezeslami S, Jia X, Vasileiou C, Borhan B, Geiger JH. structural analysis of site-directed mutants of cellular retinoic acid binding protein ii addresses the relationship between structural integrity and ligand binding. Acta Crystallogr Sect D 2008;64:1228–1239.
20. Vasileiou C, Lee KSS, Crist RM, Vaezeslami S, Goins SM, Geiger JH, Borhan B. Dissection of the critical binding determinants of cellular retinoic acid binding protein II by mutagenesis and fluorescence binding assay. Proteins: Structure, Function and Bioinformatics 2009;76:281–290.
21. Vasileiou C, Vaezeslami S, Crist RM, Rabago-Smith M, Geiger JH, Borhan B. Protein design: re-engineering cellular retinoic acid binding protein II into a rhodopsin protein mimic. J Am Chem Soc 2007;129:6140–6148.
22. Crist RM, Vasileiou C, Rabago-Smith M, Geiger JH, Borhan B. Engineering a rhodopsin protein mimic. J Am Chem Soc 2006;128:4522–4523.
23. Blatz PE, Baumgart N, Balasubramaniyan V, Balasubramaniyan P, Stedman E. Wavelength regulation in visual pigment chromophore. Large induced bathocromic shifts in retinol and related polyenes. Photochem Photobiol 1971;14:531–549.
24. Blatz PE, Liebman PA. Wavelength regulation in visual pigments. Exp Eye Res 1973;17:573–580.
25. Gat Y, Sheves M. A mechanism for controlling the pK_a of the retinal protonated schiff-base in retinal proteins—a study with model compounds. J Am Chem Soc 1993;115:3772–3773.
26. Honig B, Dinur U, Nakanishi K, Baloghnair V, Gawinowicz MA, Arnaboldi M, Motto MG. external point-charge model for wavelength regulation in visual pigments. J Am Chem Soc 1979;101: 7084–7086.
27. Kim JE, Tauber MJ, Mathies RA. Wavelength dependent cis-trans isomerization in vision. Biochemistry 2001;40:13774–13778.
28. Kochendoerfer GG, Lin SW, Sakmar TP, Mathies RA. How color visual pigments are tuned. Trends Biochem Sci 1999;24:300–305.
29. Lythgoe JN. The adaptation of visual pigments to the photic environment. In Handbook of sensory physiology, Vol. 7/1, Photochemistry of vision, Dartnall HJA editor, New York: Springer, 1972. pp 604–624.
30. Merbs SL, Nathans J. Absorption-spectra of human cone pigments. Nature 1992;356:433–435.
31. Motto MG, Sheves M, Tsujimoto K, Baloghnair V, Nakanishi K. Opsin shifts in bovine rhodopsin and bacteriorhodopsin—comparison of 2 external point-charge models. J Am Chem Soc 1980;102: 7947–7949.
32. Nathans J, Thomas D, Hogness DS. Molecular-genetics of human color-vision—the genes encoding blue, green, and red pigments. Science 1986;232:193–202.
33. Oprian DD, Asenjo AB, Lee N, Pelletier SL. Design, chemical synthesis, and expression of genes for the 3 human color-vision pigments. Biochemistry 1991;30:11367–11372.
34. Gill SC, Vonhippel PH. Calculation of protein extinction coefficients from amino-acid sequence data. Anal Biochem 1989;182:319–326.
35. Otwinowski Z, Minor W. Processing of X-ray diffraction data collected in oscillation mode. Macromol Crystallogr A 1997;276:307–326.
36. Vagin A, Teplyakov A. MOLREP: an automated program for molecular replacement. J Appl Crystallogr 1997;30:1022–1025.
37. Bailey S. The CCP4 Suite—programs for protein crystallography. Acta Crystallogr Sect D 1994;50:760–763.
38. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. Acta Crystallogr Sect D 2004;60:2126–2132.
39. Murshudov GN, Vagin AA, Dodson EJ. Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr Sect D 1997;53:240–255.
40. Murshudov GN, Vagin AA, Lebedev A, Wilson KS, Dodson EJ. Efficient anisotropic refinement of macromolecular structures using FFT. Acta Crystallogr Sect D 1999;55:247–255.
41. Pannu NS, Murshudov GN, Dodson EJ, Read RJ. Incorporation of prior phase information strengthens maximum-likelihood structure refinement. Acta Crystallogr Sect D 1998;54:1285–1294.
42. Winn MD, Isupov MN, Murshudov GN. Use of TLS parameters to model anisotropic displacements in macromolecular refinement. Acta Crystallogr Sect D 2001;57:122–133.
43. Davis AM, Teague SJ. Hydrogen bonding, hydrophobic interactions, and failure of the rigid receptor hypothesis. Angew Chem Int Ed 1999;38:737–749.
44. Ermondi G, Caron G. Recognition forces in ligand-protein complexes: blending information from different sources. Biochem Pharmacol 2006;72:1633–1645.
45. Fersht AR, Shi JP, Knilljones J, Lowe DM, Wilkinson AJ, Blow DM, Brick P, Carter P, Waye MMY, Winter G. Hydrogen-bonding and biological specificity analyzed by protein engineering. Nature 1985;314:235–238.
46. Baasov T, Sheves M. Alteration of pK_a of the bacteriorhodopsin protonated Schiff-base—a study with model compounds. Biochemistry 1986;25:5249–5258.
47. Luecke H, Schobert B, Richter HT, Cartailler JP, Lanyi JK. Structure of bacteriorhodopsin at 1.55 angstrom resolution. J Mol Biol 1999;291:899–911.
48. Scheiner S, Duan XF. Effect of intermolecular orientation upon proton-transfer within a polarizable medium. Biophys J 1991; 60:874–883.
49. Scheiner S, Hillenbrand EA. Modification of pK values caused by change in H-bond geometry. Proc Natl Acad Sci USA 1985; 82:2741–2745.
50. Sheves M, Albeck A, Friedman N, Ottolenghi M. Controlling the pK_a of the bacteriorhodopsin Schiff-base by use of artificial retinal analogs. Proc Natl Acad Sci USA 1986;83:3262–3266.