J|A|C|S
ARTICLES
Published on Web 04/21/2007

Protein Design: Reengineering Cellular Retinoic Acid Binding Protein II into a Rhodopsin Protein Mimic

Chrysoula Vasileiou, Soheila Vaezeslami,† Rachael M. Crist,‡
Montserrat Rabago-Smith, James H. Geiger,* and Babak Borhan*

Contribution from the Department of Chemistry, Michigan State University, East Lansing, Michigan 48824

Received October 27, 2006; E-mail: geiger@cem.msu.edu; babak@chemistry.msu.edu

Abstract: Rational redesign of the binding pocket of Cellular Retinoic Acid Binding Protein II (CRABPII) has provided a mutant that can bind retinal as a protonated Schiff base, mimicking the binding observed in rhodopsin. The reengineering was accomplished through a series of choreographed manipulations to ultimately orient the reactive species (the ϵ-amino group of Lys132 and the carbonyl of retinal) in the proper geometry for imine formation. The guiding principle was to achieve the appropriate Bürgi−Dunitz trajectory for the reaction to ensue. Through crystallographic analysis of protein mutants incapable of forming the requisite Schiff base, a highly ordered water molecule was identified as a key culprit in orienting retinal in a nonconstructive manner. Removal of the ordered water, along with placing reinforcing mutations to favor the desired orientation of retinal, led to a triple mutant CRABPII protein capable of nanomolar binding of retinal as a protonated Schiff base. The high-resolution crystal structure of all-*trans*-retinal bound to the CRABPII triple mutant (1.2 Å resolution) unequivocally illustrates the imine formed between retinal and the protein.

Introduction

Protein design and engineering is becoming an area of rapidly expanding interest since it was first introduced in the 1980s.[1−5] The ability to design and produce a protein possessing a specific, desired function is a particularly powerful technique that will no doubt make valuable contributions to the chemical and biochemical industry, the field of medicine, and basic scientific research. Over the years, several successful examples have been reported, leading to an increase in the understanding of protein folding as well as structure/function relationships.[1−13] Both rational and random gene manipulation has been used to obtain a polypeptide sequence of desired conformation and function. Desired elements, such as novel metal binding sites, have been introduced into naturally occurring proteins in an attempt to form catalytically active structures.[6,14−31] A *de novo* protein design approach has also been used to produce small peptides and proteins that adopt predefined, even novel, secondary structures.[15,29] Our interest lies in using the rational protein

† Current address: Biochemistry Department, University of Wisconsin, Madison, WI 53706.
‡ Current address: NCI-Frederick, Frederick, MD 21702-1201.

(1) Minshull, J.; Ness, J.; Roy, A.; Fox, R.; Emig, R.; Govindarajan, S.; Mundorff, E.; Gustafsson, C. *FASEB J.* **2003**, *17*, A1300−A1301.
(2) Dahiyat, B. I.; Mayo, S. L. *Science* **1997**, *278*, 82−87.
(3) Degrado, W. F.; Wasserman, Z. R.; Lear, J. D. *Science* **1989**, *243*, 622−628.
(4) Estell, D. A.; Graycar, T. P.; Miller, J. V.; Powers, D. B.; Burnier, J. P.; Ng, P. G.; Wells, J. A. *Science* **1986**, *233*, 659−663.
(5) Ulmer, K. M. *Science* **1983**, *219*, 666−671.
(6) Park, H. S.; Nam, S. H.; Lee, J. K.; Yoon, C. N.; Mannervik, B.; Benkovic, S. J.; Kim, H. S. *Science* **2006**, *311*, 535−538.
(7) Fox, R.; Roy, A.; Govindarajan, S.; Minshull, J.; Gustafsson, C.; Jones, J. T.; Emig, R. *Protein Eng.* **2003**, *16*, 589−597.
(8) Landgraf, R.; Pan, C.; Sutton, C.; Pearson, L.; Sigman, D. S. *Protein Eng.* **1996**, *9*, 603−610.
(9) Kamtekar, S.; Schiffer, J. M.; Xiong, H. Y.; Babik, J. M.; Hecht, M. H. *Science* **1993**, *262*, 1680−1685.
(10) Hecht, M. H.; Richardson, J. S.; Richardson, D. C.; Ogden, R. C. *Science* **1990**, *249*, 884−891.
(11) Tang, L. X.; Pazmino, D. E. T.; Fraaije, M. W.; de Jong, R. M.; Dijkstra, B. W.; Janssen, D. B. *Biochemistry* **2005**, *44*, 6609−6618.
(12) Park, H. S.; Oh, K. H.; Kim, H. S. Improving the functional expression of *N*-carbamoylase by directed evolution using the green fluorescent protein fusion reporter system. *Methods in Enzymology*; Academic Press: 2004; Vol. 388, pp 187−195.
(13) Cheon, Y. H.; Park, H. S.; Kim, J. H.; Kim, Y.; Kim, H. S. *Biochemistry* **2004**, *43*, 7413−7420.
(14) Allert, M.; Hellinga, H. W. *Protein Sci.* **2004**, *13*, 159−160.
(15) Baltzer, L.; Nilsson, H.; Nilsson, J. *Chem. Rev.* **2001**, *101*, 3153−3163.
(16) Butterfoss, G. L.; Kuhlman, B. *Annu. Rev. Biophys. Biomol. Struct.* **2006**, *35*, 49−65.
(17) Coldren, C. D.; Hellinga, H. W.; Caradonna, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 6635−6640.
(18) DeGrado, W. F. *Nature* **2003**, *423*, 132−133.
(19) Dwyer, M. A.; Looger, L. L.; Hellinga, H. W. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 11255−11260.
(20) Dwyer, M. A.; Looger, L. L.; Hellinga, H. W. *Science* **2004**, *304*, 1967−1971.
(21) Fasan, R.; Dias, R. L. A.; Moehle, K.; Zerbe, O.; Vrijbloed, J. W.; Obrecht, D.; Robinson, J. A. *Angew. Chem., Int. Edit.* **2004**, *43*, 2109−2112.
(22) Hellinga, H. W. *Nat. Struct. Biol.* **1998**, *5*, 525−527.
(23) Koder, R. L.; Dutton, P. L. *Dalton Trans.* **2006**, 3045−3051.
(24) Looger, L. L.; Dwyer, M. A.; Smith, J. J.; Hellinga, H. W. *Nature* **2003**, *423*, 185−190.
(25) Meus, J.; Brylinski, M.; Piwowar, M.; Piwowar, P.; Wisniowski, Z.; Stefaniak, J.; Konieczny, L.; Surowka, G.; Roterman, I. *Med. Sci. Monitor* **2006**, *12*, BR208−BR214.
(26) Pinto, A. L.; Hellinga, H. W.; Caradonna, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 5562−5567.
(27) Sterner, R.; Schmid, F. X. *Science* **2004**, *304*, 1916−1917.
(28) Swift, J.; Wehbi, W. A.; Kelly, B. D.; Stowell, X. F.; Saven, J. G.; Dmochowski, I. J. *J. Am. Chem. Soc.* **2006**, *128*, 6611−6619.
(29) Wei, Y. N.; Liu, T.; Sazinsky, S. L.; Moffet, D. A.; Pelczer, I.; Hecht, M. H. *Protein Sci.* **2003**, *12*, 92−102.
(30) Wisz, M. S.; Garrett, C. Z.; Hellinga, H. W. *Biochemistry* **1998**, *37*, 8269−8277.
(31) Yang, W.; Jones, L. M.; Isley, L.; Ye, Y. M.; Lee, H. W.; Wilkins, A.; Liu, Z. R.; Hellinga, H. W.; Malchow, R.; Ghazi, M.; Yang, J. J. *J. Am. Chem. Soc.* **2003**, *125*, 6165−6171.

10.1021/ja067546r CCC: $37.00

engineering approach to convert a small cytosolic protein, Cellular Retinoic Acid Binding Protein II (CRABPII), into a system that can bind retinal as a Protonated Schiff Base (PSB) mimicking the binding observed in rhodopsin and other retinylidene forming proteins.

The presence of Schiff base (SB) intermediates has been verified in a plethora of enzymatic reaction mechanisms that include several, seemingly very different naturally occurring systems such as decarboxylases,[32,33] aldolases,[34,35] hydroxylases,[36] and transaminases.[37] In addition, a number of artificial enzymatic systems have been developed that also employ the use of SB chemistry. These include Baltzer's four-helix bundles[14,38−40] and Benner's and Allemann's oxaldies[41−43] that efficiently perform decarboxylations, small di- and tri-peptides that catalyze direct asymmetric aldol reactions as reported by Cordova and co-workers,[44] and Distefano's pyridoxamines used for transaminations.[45−48] The latter system is of particular interest to us since it involves the design and engineering of specific enzymatic activity in the otherwise enzymatically inert proteinic scaffold of a fatty acid binding protein that shares a common fold with CRABPII.[49]

However, among the SB forming systems the most well studied are the various opsins. These include the proteins involved in vision (rhodopsin, color opsins)[50−53] and the other opsin systems present in bacteria [bacteriorhodopsin (proton pump),[54,55] halorhodopsin (halogen pump),[56] and sensory rhodopsins (phototaxis)[57,58]]. The formation of a PSB between a nucleophilic Lys residue in the protein's interior and one of the isomeric forms of vitamin A aldehyde (retinal) is the initiation step for a cascade of reactions that ultimately lead to function for all the various opsins. Furthermore, the opsins are unusual because the spectroscopic characteristics elicited from the different systems bound to similar chromophores are unique. In fact, most retinal-SB forming opsins result in significantly red-shifted pigments, and this shift has been the focus of intense study for the past 50 years.[59−64] In particular, the mechanism of wavelength regulation in rhodopsin and the color opsins that leads to color vision has been the subject of much work, through both modeling studies[59,60,65,66] as well as mutagenesis of rod rhodopsin and subsequent spectroscopic analysis.[64,67−70]

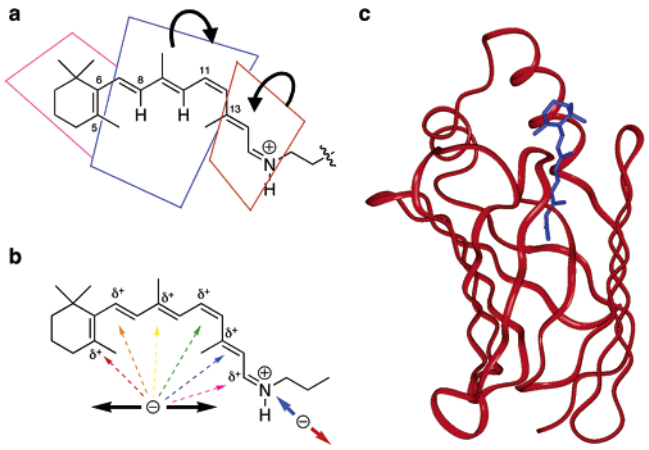

Figure 1. (a) Twisting about the intervening singles bonds leads to disrupted conjugation and a possible mode of wavelength regulation. (b) Varying positions of either the counteranion or a "point charge" along the backbone of the polyene may also result in differing degrees of conjugation that results in wavelength regulation. (c) Crystal structure of the CRABPII with retinoic acid bound.

Several stereoelectronic factors have been postulated to affect the conjugation along the chromophore's long chain and therefore result in wavelength regulation.[71] These include rotations about the polyene's intervening single bonds, placing of either point charges or dipoles at different positions along the backbone of the polyene, and varying the distance between the PSB and its counteranion (Figure 1a and 1b).[59,60,65,72−76] Yet, difficulties in producing mutants of recombinant membrane

(32) Highbarger, L. A.; Gerlt, J. A.; Kenyon, G. L. *Biochemistry* **1996**, *35*, 41−46.
(33) Lee, B.; Suh, S. W. *J. Mol. Biol.* **2004**, *340*, 1−7.
(34) Fullerton, S. W. B.; Griffiths, J. S.; Merkel, A. B.; Cheriyan, M.; Wymer, N. J.; Hutchins, M. J.; Fierke, C. A.; Toone, E. J.; Naismith, J. H. *Bioorg. Med. Chem.* **2006**, *14*, 3002−3010.
(35) Schneider, G.; Sprenger, G. A. *Methods Enzymol.* **2002**, 354, 197−201.
(36) Morais, M. C.; Zhang, G. F.; Zhang, W. H.; Olsen, D. B.; Dunaway-Mariano, D.; Allen, K. N. *J. Biol. Chem.* **2004**, *279*, 9353−9361.
(37) Shen, B. W.; Hennig, M.; Hohenester, E.; Jansonius, J. N.; Schirmer, T. *J. Mol. Biol.* **1998**, *277*, 81−102.
(38) Allert, M.; Baltzer, L. *Chem.—Eur. J.* **2002**, *8*, 2549−2560.
(39) Allert, M.; Kjellstrand, M.; Broo, K.; Nilsson, A.; Baltzer, L. *J. Chem. Soc., Perkin Trans. 2* **1998**, 2271−2274.
(40) Anderson, L. K.; Caspersson, M.; Baltzer, L. *Chem.—Eur. J.* **2002**, *8*, 3687−3697.
(41) Johnsson, K.; Allemann, R. K.; Widmer, H.; Benner, S. A. *Nature* **1993**, *365*, 530−532.
(42) Taylor, S. E.; Rutherford, T. J.; Allemann, R. K. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2631−2635.
(43) Taylor, S. E.; Rutherford, T. J.; Allemann, R. K. *J. Chem. Soc., Perkin Trans. 2* **2002**, 751−755.
(44) Dziedzic, P.; Zou, W. B.; Hafren, J.; Cordova, A. *Org. Biomol. Chem.* **2006**, *4*, 38−40.
(45) Davies, R. R.; Distefano, M. D. *J. Am. Chem. Soc.* **1997**, *119*, 11643−11652.
(46) Haring, D.; Distefano, M. D. *Bioconjugate Chem.* **2001**, *12*, 385−390.
(47) Haring, D.; Kuang, H.; Qi, D. F.; Distefano, M. D. *J. Mol. Catal. B: Enzym.* **2001**, *11*, 967−970.
(48) Tann, C. M.; Qi, D. F.; Distefano, M. D. *Curr. Opin. Chem. Biol.* **2001**, *5*, 696−704.
(49) Ory, J. J.; Mazhary, A.; Kuang, H.; Davies, R. R.; Distefano, M. D.; Banaszak, L. J. *Protein Eng.* **1998**, *11*, 253−261.
(50) Lythgoe, J. N. *Handbook of sensory physiology*; Springer: New York 1972; Vol. 7, pp 604−624.
(51) Merbs, S. L.; Nathans, J. *Nature* **1992**, *356*, 433−435.
(52) Nathans, J. *Biochemistry* **1992**, *31*, 4923−4931.
(53) Palczewski, K.; Kumasaka, T.; Hori, T.; Behnke, C. A.; Motoshima, H.; Fox, B. A.; Le Trong, I.; Teller, D. C.; Okada, T.; Stenkamp, R. E.; Yamamoto, M.; Miyano, M. *Science* **2000**, *289*, 739−745.
(54) Hoffmann, M.; Wanko, M.; Strodel, P.; Konig, P. H.; Frauenheim, T.; Schulten, K.; Thiel, W.; Tajkhorshid, E.; Elstner, M. *J. Am. Chem. Soc.* **2006**, *128*, 10808−10818.
(55) Luecke, H.; Schobert, B.; Richter, H. T.; Cartailler, J. P.; Lanyi, J. K. *J. Mol. Biol.* **1999**, *291*, 899−911.
(56) Kolbe, M.; Besir, H.; Essen, L. O.; Oesterhelt, D. *Science* **2000**, *288*, 1390−1396.
(57) Luecke, H.; Schobert, B.; Lanyi, J. K.; Spudich, E. N.; Spudich, J. L. *Science* **2001**, *293*, 1499−1503.
(58) Royant, A.; Nollert, P.; Edman, K.; Neutze, R.; Landau, E. M.; Pebay-Peyroula, E.; Navarro, J. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 10131−10136.
(59) Beppu, Y. *J. Phys. Soc. Jpn.* **1997**, *66*, 3303−3309.
(60) Beppu, Y.; Kakitani, T. *Photochem. Photobiol.* **1994**, *59*, 660−669.
(61) Blatz, P. E.; Liebman, P. A. *Exp. Eye Res.* **1973**, *17*, 573−580.
(62) Motto, M. G.; Sheves, M.; Tsujimoto, K.; Baloghnair, V.; Nakanishi, K. *J. Am. Chem. Soc.* **1980**, *102*, 7947−7949.
(63) Nakanishi, K.; Berova, N.; Fishkin, N.; Fujioka, N. *J. Chin. Chem. Soc.* **2002**, *49*, 443−451.
(64) Nathans, J. *Biochemistry* **1990**, *29*, 9746−9752.
(65) Yamamoto, S.; Wasada, H.; Kakitani, T.; Yamato, T. *THEOCHEM* **2001**, *543*, 79−87.
(66) Andersen, L. H.; Nielsen, I. B.; Kristensen, M. B.; El Ghazaly, M. O. A.; Haacke, S.; Nielsen, M. B.; Petersen, M. A. *J. Am. Chem. Soc.* **2005**, *127*, 12347−12350.
(67) Asenjo, A. B.; Rim, J.; Oprian, D. D. *Neuron* **1994**, *12*, 1131−1138.
(68) Govardhan, C. P.; Oprian, D. D. *J. Biol. Chem.* **1994**, *269*, 6524−6527.
(69) Sakmar, T. P.; Franke, R. R.; Khorana, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 3079−3083.
(70) Yokoyama, S. *Mol. Biol. Evol.* **1995**, *12*, 53−61.
(71) Kochendoerfer, G. G.; Lin, S. W.; Sakmar, T. P.; Mathies, R. A. *Trends Biochem. Sci.* **1999**, *24*, 300−305.
(72) Han, M.; Dedecker, B. S.; Smith, S. O. *Biophys. J.* **1993**, *65*, 899−906.
(73) Hays, T. R.; Lin, S. H.; Eyring, H. *Proc. Natl. Acad. Sci. USA* **1980**, *77*, 6314−6318.
(74) Lin, S. W.; Kochendoerfer, G. G.; Carroll, H. S.; Wang, D.; Mathies, R. A.; Sakmar, T. P. *J. Biol. Chem.* **1998**, *273*, 24583−24591.
(75) Singh, A. K.; Majumdar, N. *J. Photochem. Photobiol., B* **1997**, *39*, 135−139.

bound proteins such as opsins, coupled with the lack of X-ray crystal structures of the colored opsins have hindered efforts to elucidate the molecular mechanism of color vision. In an effort to probe the specific interactions that lead to the wavelength regulation of bound retinal, we have initiated a program to redesign proteins to mimic retinal binding in rhodopsin. These engineered proteins will serve as a platform for creating and studying specific protein/retinal interactions of interest. The protein targeted for reengineering should satisfy the following criteria: (1) It should have its three-dimensional structure known so that changes to the sequence can be planned rationally. (2) It must bind retinal as a PSB. (3) The PSB must be stabilized by a counteranion such as Glu (mimicking the same arrangement in rhodopsin). Our research was thus focused on the parameters that enable the formation of a PSB within a proteinic environment in an effort to mimic the iminium bond formation that is observed in visual pigments.

We have recently reported the reengineering of CRABPII (Figure 1c), a small cytosolic protein amenable to both crystallography and biochemical manipulations, into a retinal binding protein that can form a PSB.[77] The design features that led to this first protein mimic were mainly based on hydrophobic tuning of the active site. However, subsequent data from high-resolution structural studies have led to the conclusion that the original design principles were overly simplistic. What was previously not appreciated was the importance of precisely defining a favorable reaction geometry that promoted the chemical reaction required for Schiff base formation. We now report a complete restructuring of the design principle that takes into account the geometric disposition of reactive groups in the active site, leading to engineered proteins with superior PSB characteristics. We have also, for the first time, determined the high-resolution structure of the reengineered protein bound to retinal via a Schiff base, which is critical for verifying the design principles that were established by this study.

Results and Discussion

Our initial reengineering of CRABPII into a PSB forming protein was accomplished through a series of mutations to yield a nucleophilic active site Lys residue by manipulating its pK_a. This was in large part accomplished through hydrophobic tuning of the binding cleft surrounding the Lys ϵ-amino group (increase of hydrophobicity through changing polar to nonpolar amino acids), which should lead to the lowering of the amine pK_a.[78] The result was the R132K:Y134F:R111L:L121E:T54V (KFLEV) penta mutant (Figure 2). The Lys residue, necessary for Schiff base formation was introduced at position 132 (R132K). The location was determined via *in silico* mutations of various amino acid residues. The Lys substitution of Arg132 provided reasonable distances to the putative position of the bound retinal's carbonyl group to facilitate Schiff base formation. In order to increase the nucleophilicity of Lys132 for successful SB formation (by increasing the hydrophobicity of the pocket), three mutations were performed: Y134F, R111L, and T54V. Finally, a critical Glu residue was positioned in close proximity to the location of the anticipated imine (L121E), which would act as

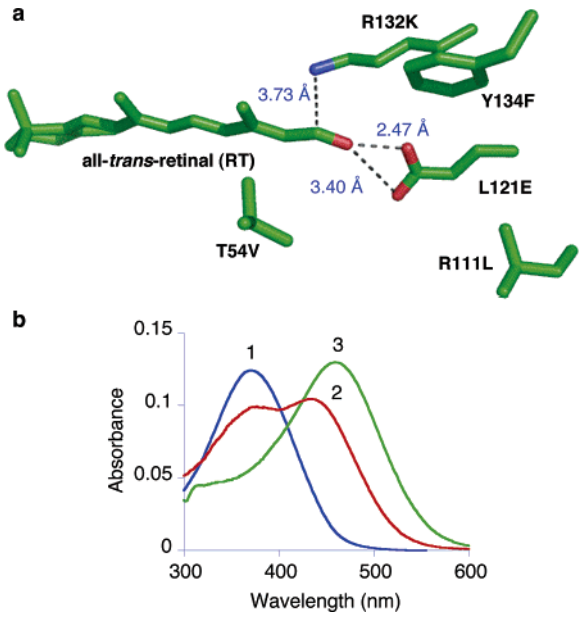

Figure 2. (a) Energy minimized model of CRABPII KFLEV penta mutant bound to all-*trans*-retinal prior to Schiff base formation. (b) UV−vis of retinal complexed with WT-CRABPII (**1**, 377 nm) and penta mutant (**2**, **3**). Clear bathochromic shift upon PSB formation leads to absorption at 446 nm at pH = 7.2 (**2**). Upon acidification the trace completely shifts to 459 nm (**3**, pH = 4.0).

a counteranion to stabilize the protonated SB. These changes led to the KFLEV penta mutant, which exhibited strong binding of all-*trans*-retinal (RT) as a PSB ($K_d = 2.7 \pm 7$ nM). However, the apparent pK_a of the PSB was rather low (~6.5), and therefore, at the working physiological pH a mixture of protonated and nonprotonated SB was present. Repeated attempts to crystallize the KFLEV mutant bound to all-*trans*-retinal were not successful.

With a CRABPII mutant that could bind retinal as a PSB in hand, we then attempted to optimize the system to increase the pK_a value of the PSB formed. The protein mutants are characterized using a battery of spectroscopic and chemical techniques. Binding constants (K_d's) are measured via fluorescence quenching of the three Trp residues in the protein. The wild-type CRABPII has a K_d of 6600 ± 360 nM for RT. We deemed any K_d below 200 nM as sufficient for our purposes. UV/vis spectroscopy provides details regarding the protonation of the Schiff base. Retinal and its Schiff base absorb at ~370 ± 10 nm, and thus it is not possible to discriminate between them. However, protonation of the Schiff base (PSB) leads to a bathochromic shift with an absorption of >420 nm. The pK_a of the imine is determined by incubation of the protein/retinal complex (preformed) in buffers of different pH's. Schiff base formation (both protonated and nonprotonated forms) can be probed by reductive amination. MALDI-TOF analysis of the protein/retinal complex, which has been treated with $NaCNBH_3$, provides masses that either correspond to the mass of the protein (no apparent Schiff base) or exhibit protein mass adducts corresponding to the addition of one retinal molecule ($[M + 268]^+$, evidence of SB).

Table 1 lists the pertinent data for a number of mutants that were investigated initially to arrive at the KFLEV penta mutant.[77] Entries 1−7 show the progression of the UV/vis data toward more red-shifted chromophores and increased binding as apparent from the K_d values. Entry 8 is the "Lys control" to

(76) Sugihara, M.; Buss, V.; Entel, P.; Elstner, M.; Frauenheim, T. *Biochemistry* **2002**, *41*, 15259−15266.
(77) Crist, R. M.; Vasileiou, C.; Rabago-Smith, M.; Geiger, J. H.; Borhan, B. *J. Am. Chem. Soc.* **2006**, *128*, 4522−4523.
(78) Harris, T. K.; Turner, G. J. *IUBMB Life* **2002**, *53*, 85−98.

Table 1. K_d and λ_{max} for Retinal Bound to CRABPII Mutants

	CRABPII protein	K_d (nM)	λ_{max}[a]	red. am.[b]
1	WT-CRABPII	6600 ± 360	377	no
2	R132K	280 ± 17	379	yes
3	R132K:Y134F	120 ± 5	404	no
4	R132K:Y134F:R111L	160 ± 7	400	yes
5	R132K:Y134F:T54V	81 ± 14	417	yes
6	R132K:Y134F:R111L:L121E	200 ± 8	446	yes
7	R132K:Y134F:R111L:T54V:L121E	2.7 ± 7	446	yes
8	Y134F:R111L:L121E	570 ± 32	381	no
9	R132K:Y134F:R111L:T54V:L121Q	600 ± 66	371	yes

[a] Deconvolusion of overlapping UV−vis spectra is detailed in the Supporting Information. [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.

demonstrate that in fact it is the engineered Lys residue and not the other 13 Lys residues in the protein that are responsible for SB formation. The R132K:Y134F:R111L:L121Q:T54V (KFLQV) penta mutant (entry 9) illustrates the importance of the Glu counteranion in KFLEV, as apparent from the loss of protonation of the SB (note that it is still an SB as indicated from reductive amination).

Our attention was turned onto the R132K:Y134F double mutant due to its inability to produce a reductively aminated adduct upon incubation with all-*trans*-retinal and $NaCNBH_3$ (Table 1, entry 3). Curiously, addition of RT to the R132K:Y134F double mutant results in a ∼25 nm red shift (λ_{max} = 404 nm) as compared with the R132K single mutant (λ_{max} = 379 nm), thus suggesting the possible formation of a PSB. However, unlike the R132K mutant (Table 1, entry 2), the R132K:Y134F double mutant failed to reveal the $[M + 268]^+$ adduct peak in the MALDI-TOF spectrum of the reductively aminated R132K:Y134F/RT complex. This seemed to indicate that the Y134F mutation might actually be detrimental to SB formation, which is inconsistent with our assumptions regarding the importance of hydrophobic tuning. However, additional mutations, such as R132K:Y134F:R111L and R132K:Y134F:T54V, restore the protein's ability to form a stable SB. The positive reductive amination results were maintained throughout the rest of the mutants that included the engineered Lys132 residue.

The results detailed above led us not only to completely re-evaluate the function of Tyr134 and the effects of the Y134F mutation but also to reconsider the elements necessary for the reengineering of the protein such that a Schiff base is formed within the protein cavity. In particular, questions regarding the orientation of reactive groups (the ϵ-amino group of Lys132 and the carbonyl group of retinal) and activation of the aldehyde to initiate imine formation became paramount in the design phase. In an attempt to more clearly visualize the reactive species in the binding pocket of CRABPII mutants, a number of the protein mutants were crystallized and analyzed in detail. Most importantly, the structures of the R132K:Y134F double mutant bound to both all-*trans*-retinoic acid (RA) and RT were determined. The latter two crystal structures were instrumental in understanding the necessary elements to design a protein capable of binding retinal as a Schiff base since the R132K:Y134F double mutant had failed to form the requisite imine.

Figure 3 depicts the crystal structures of WT-CRABPII bound to RA (a and b)[79,80] and the double mutant R132K:Y134F bound to either RA (c and d) or RT (e and f), both determined at 1.7 Å resolution (PDB ID codes are 2G78 and 2G79, respectively). The crystal structure of the R132K:Y134F double mutant bound to RA reveals binding of the chromophore (Figure 3c) very similar to that of the WT-CRABPII/RA (Figure 3a),[79] although two major binding partners (Arg132, Tyr134) have been removed (overlay depicted in Figure 3g).

The crystal structure of the RT bound R132K:Y134F double mutant (Figure 3e) unambiguously verifies the reductive amination studies that indicated the lack of imine formation. All-*trans*-retinal is located within the binding pocket at a position almost identical to that of the natural substrate (all-*trans*-retinoic acid, overlay depicted in Figure 3h) and does not form a covalent bond with any of the neighboring residues. The chromophore's binding is supported by a series of hydrogen bonding interactions with the engineered Lys132 (3.67 Å) and an ordered water molecule (W1, Figure 3e).[81] A closer look reveals that there are two orientations of the bound chromophore, one with the carbonyl oxygen pointing toward Lys132 (30% occupancy) and the other orientation with the carbonyl oxygen pointing away from the Lys residue (70% occupancy, Figure 3e). The three crystal structures depicted in Figure 3 were central in proposing a hypothesis to address the lack of reactivity observed in the R132K:Y134F double mutant, which consequently led us to a rational redesign of a rhodopsin protein mimic.

The trajectory of reacting nucleophiles with respect to the carbonyl functionality is a critical parameter for efficient nucleophilic attack. Bürgi and Dunitz, using an elegant crystallographic study of amine nucleophiles attacking electrophilic carbonyls, have shown that nucleophiles approach carbonyls at an optimal angle of 107°.[82,83] This probably stems from a compromise between the perpendicular approach to the carbonyl, maximizing overlap of the nucleophile HOMO with π^*, and the electronic repulsion between the nucleophile and the π-electrons. Achieving the necessary disposition of reactive groups became the guiding principle throughout the reengineering of CRABPII, namely, orienting the bound RT with respect to the active site Lys with a favorable Bürgi−Dunitz trajectory. A second look at the three crystal structures in Figure 3 reveals a nonoptimum trajectory between the reactive amine group and the carbonyl of the retinal bound to the R132K:Y134F double mutant. In fact, the orientation of the aldehydic carbonyl in the two crystal forms (Figure 3f) closely resembles the same orientation found for the carboxylate oxygens of the RA bound structure in both the R132K:Y134F double mutant (Figure 3d) and the WT-CRABPII structures (Figure 3b), all of which are 40°−50° away from the desired trajectory.

In trying to understand why the carbonyl of retinal does not rotate to adopt a favorable conformation, the role of a single highly ordered water molecule was questioned. The three crystal structures depicted in Figure 3, and in fact the crystal structures of all mutants that contain Arg111 determined to date (data not shown), have in common a highly ordered water molecule (W1,

(79) Kleywegt, G. J.; Bergfors, T.; Senn, H.; Lemotte, P.; Gsell, B.; Shudo, K.; Jones, T. A. *Structure* **1994**, *2*, 1241−1258.
(80) Vaezeslami, S.; Mathes, E.; Vasileiou, C.; Borhan, B.; Geiger, J. H. *J. Mol. Biol.* **2006**, *363*, 687−701.
(81) The active site water residue in all crystal structures in this manuscript are labeled as W1 to indicate the fact that they are all in the same position. The actual water numbering of W1 in the PDB files for each mutant is as follows: wild type CRABPII bound to RA (1CBS, W309), R132K:Y134F-CRABPII bound to RA (2G78, W11), R132K:Y134F-CRABPII bound to RT (2G79, W10).
(82) Burgi, H. B.; Dunitz, J. D. *Acc. Chem. Res.* **1983**, *16*, 153−161.
(83) Burgi, H. B.; Dunitz, J. D.; Shefter, E. *J. Am. Chem. Soc.* **1973**, *95*, 5065−5067.

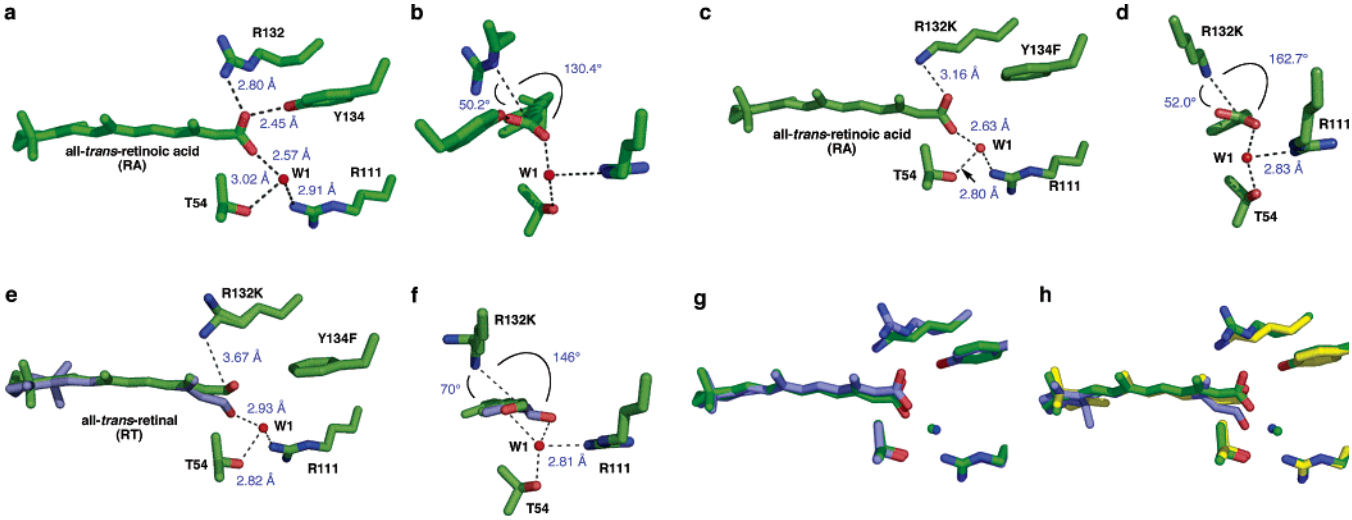

Figure 3. (a, b) Crystal structure of WT-CRABPII bound to all-*trans*-retinoic acid (PDB ID 1CBS). (c, d) Crystal structure of CRABPII-R132K:Y134F double mutant bound to RA. (e, f) Crystal structure of CRABPII-R132K:Y134F bound to RT. The retinal and Lys132 were found in two crystallographic forms (overlaid structures). (g) Overlay of WT-CRABPII (blue carbon skeleton) and R132K:Y134F double mutant (green carbon skeleton) bound to all-*trans*-retinoic acid. (h) Overlay of R132K:Y134F double mutant bound to all-*trans*-retinoic acid (blue carbon skeleton) and R132K:Y134F double mutant bound to all-*trans*-retinal (blue and yellow carbon skeleton).

Figure 3). W1 in the RA bound WT-CRABPII structure is tightly hydrogen bonded to Arg111 and the carboxylate of RA (Figure 3a). The same binding motif is found for the RA bound R132K:Y134F double mutant (Figure 3c). Interestingly, the RT bound R132K:Y134F double mutant illustrates the same W1 hydrogen bonding with the carbonyl oxygen of retinal (70% occupancy was calculated for the crystal structure with the carbonyl oxygen H-bonded to W1, Figure 3e). At this juncture, we hypothesized that the strong hydrogen bond to W1 prevents the carbonyl from adopting a favorable trajectory to allow SB bond formation. In particular, it is clear from the latter crystal structures that the plane of the carbonyl has to be rotated along the long axis of the chromophore in order to achieve a favorable trajectory for nucleophilic attack (~40° CCW considering the chromophore with the carbonyl oxygen pointing away from the Lys residue). It was thus deemed critical to remove the highly ordered W1, so that the carbonyl group is freed to adopt the necessary conformation for SB formation.

The rational engineering of a second generation of retinal SB-forming proteins was therefore based on the following suppositions. Removal of unfavorable interactions leading to the undesired orientation of the aldehyde was coupled with building favorable and reinforcing interactions that would promote the chromophore's rotation of ~40° about its long axis. The position of the active site Lys residue was preserved from the original modeling work (R132K). Removing either or both Arg111 and Thr54 should remove W1 from play. Mutation of Arg111 to Leu was planned accordingly. In addition it seemed plausible from modeling studies that Tyr134 may contribute to the activation of the carbonyl group through hydrogen bonding interactions (2.95 Å, Figure 4), which would explain why its mutation to Phe was detrimental to SB formation. Tyr134 was thus preserved in the new series.

Next, the placement of a counteranion to stabilize the PSB was considered. *In silico* mutation of Leu121 into a Glu residue provided the best results for placing a counteranion that would be 3.5−4.5 Å away from the putative PSB (Figure 4). Also, Glu121 would be positioned midway between Lys132 and Arg111 (Figure 4) so that its interaction with the retinal's

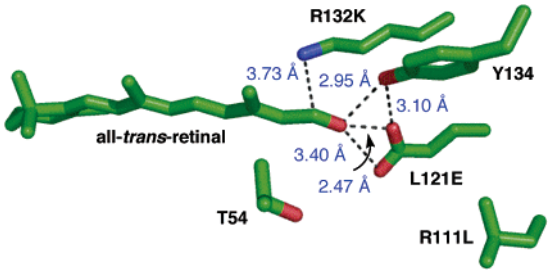

Figure 4. Model of KLE-CRABPII triple mutant bound to all-*trans*-retinal.

carbonyl oxygen through hydrogen bonding (3.40 Å, 2.47 Å, Figure 4) could aid in rotating the plane of the carbonyl in a more favorable arrangement to achieve the necessary Bürgi−Dunitz trajectory (all evidence suggests that Glu121 is protonated inside the hydrophobic pocket of CRABPII at pH = 7.3).[84] Furthermore, because of the proximity of Glu121 to Tyr134 (3.10 Å, Figure 4) a favorable hydrogen bond between the two was anticipated. This could enhance Schiff base formation due to increased activation of retinal's carbonyl group as a result of the amplified acidity of Glu121. Glu121 may assume a dual role as proton donor for the reaction and as counteranion for the resultant PSB. The latter suppositions led to the R132K:L121E:R111L triple mutant (KLE-CRABPII).

RT binds KLE-CRABPII with high affinity (1.4 nM vs 6.5 μM for WT CRABPII), concomitant with loss of binding affinity for RA (426 nM vs 2 nM for wild type CRABPII) (Figure 5a). The binding of RT also led to a red-shifted absorption at 449 nm (Figure 5b), strongly suggestive of PSB formation. This was further verified through MALDI-TOF analysis of the reductively aminated protein−retinal complex that showed an $[M + 268]^+$ peak corresponding to the increased mass due to covalent binding of one retinal molecule (Figure 5c and 5d).

The KLE-CRABPII triple mutant was cocrystallized with RT, and its structure was refined to 1.2 Å resolution (the cocrys-

(84) Crystal structure of L121E mutants bound to retinoic acid (unpublished results) exhibit a bis-carboxylic acid dimer, formed between the Glu121 and retinoic acid. The geometry and distance between the tightly hydrogen bonded carboxylic acids is suggestive of the fact that Glu121 is protonated.

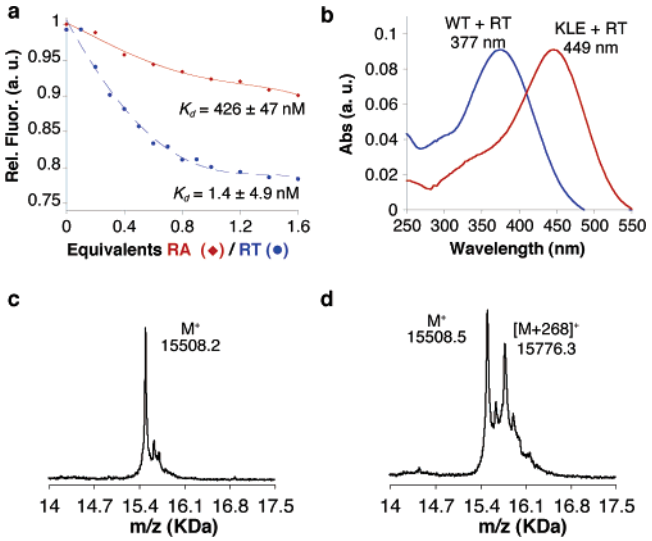

Figure 5. (a) Fluorescence titrations of the KLE-CRABPII triple mutant with retinoic acid (K_d = 426 ± 47 nM) and retinal (K_d = 1.4 ± 4.9 nM). (b) UV−vis spectrum of RT bound to WT-CRABPII and KLE-CRABPII triple mutant. Retinal bound with WT is a noncovalent interaction with λ_{max} = 377 nm. The UV of retinal with KLE-CRABPII triple mutant reveals a 449 nm peak, indicating formation of a PSB. (c) MALDI-TOF of KLE-CRABPII (15508.2 Da). (d) Reductive amination of retinal with the KLE-CRABPII triple mutant reveals a mixture of the apoprotein (15508.5 Da) as well as the covalent adduct of the protein with RT (15776.3 Da, $[M + 268]^+$).

tallization required dark conditions at 4 °C). The overall structure of KLE-CRABPII bound to all-*trans*-retinal remained very similar to that of WT-CRABPII bound to retinoic acid (Figure 6a). The very high-resolution data allowed us to visualize the details of the structure with exceptional clarity (Figure 6b and 6c). The structure clearly shows RT occupying the RA binding site with the ionone ring pointing toward the mouth of the binding cavity and the aldehyde end of the molecule buried deeply in the interior of the protein. The Schiff base between Lys132 and RT is also clearly seen and adopts a *cis*-imine conformation (Figure 6b, 6c). A closer look at the binding site reveals the close contacts between Tyr134, Glu121, and the iminium nitrogen (Figure 6b). Comparison of the active site of the triple mutant (Figure 6b) with the active site of rhodopsin (Figure 6d) reveals a similar arrangement of the counteranion and the PSB, although retinal in rhodopsin has a *trans*-imine conformation.[85]

There are significant differences in the trajectory of the chromophore (RT) in the binding pocket of KLE-CRABPII relative to RA as seen in the WT-CRABPII/RA complex (Figure 7). The tightly hydrogen-bonded W1 with Arg111 in the WT structure is moved out of play in the KLE-CRABPII triple mutant, thus freeing the carbonyl of RT to assume the necessary orientation for nucleophilic attack (presumably through H-bonding interactions with Tyr134 and/or Glu121). Similar to RA, RT is relatively planar across its conjugated length, but its trajectory through the binding site is substantially different, culminating in a 1.9 Å shift in the position of C15 relative to RA-bound CRABPII (Figure 7a). Three motions lead to this difference. The ionone ring is translated by about 0.5 Å and rotated by ∼21° about an axis that intersects C5−C6 (Figure 7b). At the same time, the C5−C6-C7−C8 dihedral angle is

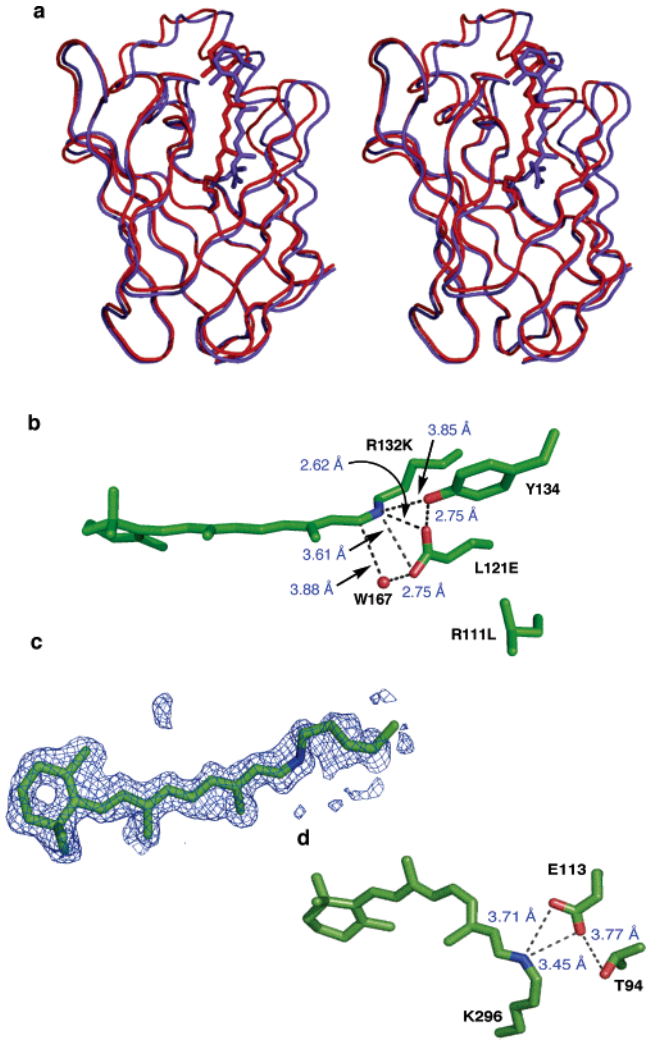

Figure 6. (a) Stereoview of the overlay of WT-CRABPII bound to RA (blue) and KLE-CRABPII bound to RT (red). (b) RT is bound to KLE-CRABPII via a Schiff base with Lys132. (c) Fo−Fc omit map of retinal bound to KLE-CRABPII as a Schiff base. Maps are contoured at 2.2 σ. (d) Crystal structure of the binding site of bovine rhodopsin bound to 11-*cis*-retinal (PDB ID 1U19).

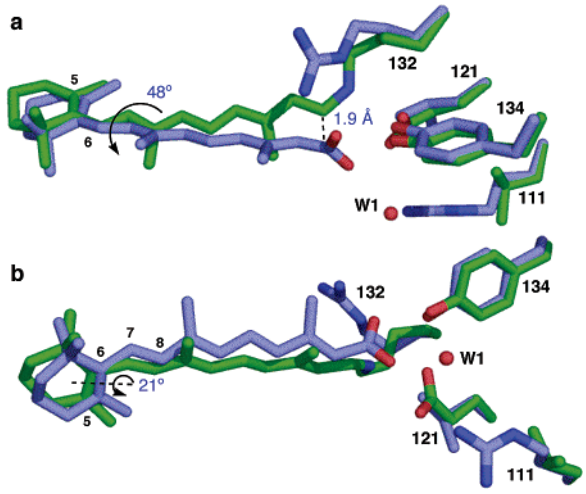

Figure 7. Different views of the overlay of RT bound to KLE-CRABPII (green carbon atoms) and RA bound to WT-CRABPII (blue carbon atoms).

rotated by about 48° to reposition the chromophore in the binding pocket (Figure 7a). The result is a large rotation of the

(85) Okada, T.; Sugihara, M.; Bondar, A. N.; Elstner, M.; Entel, P.; Buss, V. *J. Mol. Biol.* **2004**, *342*, 571−583.

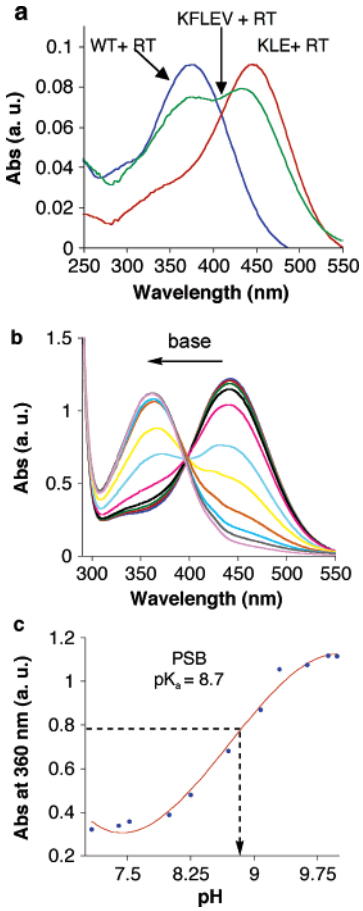

Figure 8. (a) UV−vis spectra of RT bound to WT-CRABPII (λ_{max} = 377 nm, blue trace), KLE-CRABPII (λ_{max} = 449 nm, red trace), and KFLEV-CRABPII (green trace) at pH = 7.3. (b) Base titration of the KLE-CRABPII/retinal complex. Upon addition of base the PSB peak at 446 nm blue shifts to 364 nm. The shift is reversible upon acidification. (c) The calculated pK_a value for the KLE-CRABPII/retinal PSB is 8.7.

entire chromophore (RT) in the KLE-CRABPII structure relative to the WT-CRABPII/RA complex. All other dihedral angles, including those that define the ionone ring pucker, are identical in the two molecules. Thus all the changes in the molecule's conformation occur at the end of the molecule located at the mouth of the binding cleft and serve to rotate the aldehydic carbonyl of RT as required (~48° relative to the chromophore bound to the R132K:Y134F double mutant) for reaction with Lys132, based on the requirements for reaction described above. No significant collisions occur between the protein and RT, emphasizing that the CRABPII binding cavity is large relative to the size of the ligand, allowing for significant motion within.

Figure 8a depicts the UV/vis spectra of RT bound to WT-CRABPII, the KFLEV penta mutant, and the KLE triple mutant at pH = 7.3. Acid−base titration of the PSB formed between the KLE-CRABPII mutant and all-*trans*-retinal (Figure 8b and 8c) allowed us to calculate a pK_a value of 8.7. The pK_a of this triple mutant is higher than the pK_a of a retinylidene Schiff base in solution (~7.2),[86] thus maintaining a PSB at physiological pH, and approaches the pK_a of known rhodopsins (9.3− ~16).[87−91] The deprotonated SB appears to absorb at 364 nm, very close to the reported value of nonprotonated *N*-retinylidene-*n*-alkylamines (λ_{max} = 365 nm).[92]

Table 2. K_d and λ_{max} for Retinal Bound to CRABPII Mutants

	CRABPII protein	K_d (nM)	λ_{max}[a]	red. am.[b]
1	R132K:R111L:L121E (KLE)	1.36 ± 4.9	449	yes
2	R132L:R111L:L121E	25 ± 10	376	no
3	R132K:R111L:L121Q	1526 ± 83	377	yes
4	R132K:R111L	567 ± 36	408	yes
5	R132K:R111L:T54V:L121E	1.99 ± 4.1	449	yes
6	R132K:L121E	104 ± 11	457	yes
7	R132K:Y134F:L121E	160 ± 9.8	381	yes

[a] Deconvolusion of overlapping UV−vis spectra is detailed in the Supporting Information. [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.

Two control mutants were important to further demonstrate the function of the engineered active site residues. The R132L:L121E:R111L triple mutant was prepared to prove that Lys132 and not any of the other 13 Lys residues of the native protein is responsible for PSB formation. The latter triple mutant failed to produce a red-shifted chromophore upon addition of RT (λ_{max} = 376 nm, Table 2, entry 2). It also did not yield a mass corresponding to conjugation of the protein with retinal upon MALDI-TOF analysis. The R132K:R111L:L121Q mutant was made to assess the role of the Glu counteranion. Interestingly, this mutant bound RT over 1000-fold worse (1526 nM), but yet did yield a Schiff base forming protein ($[M + 267]^+$ present in the MALDI-TOF spectrum). However, the protein complex with retinal did not red-shift (λ_{max} = 377 nm, Table 2, entry 3), suggesting that the carboxylate counteranion is necessary for protonation of the Schiff base. Similar behavior has been observed in rhodopsin mutants in which the replacement of Glu113 (the counteranion in bovine rhodopsin) with Gln leads to blue-shifting of the pigment from 500 nm to ~380 nm.[64,93] Similarly, the R132K:R111L double mutant, which lacks the Glu121 counteranion, does not form the requisite PSB (Table 2, entry 4), although as expected it forms a SB upon addition of RT.

Overall, restoring Tyr134 results in improvement of both retinal binding and PSB formation, as evident from the fluorescence quenching and UV−vis data summarized in Table 2. In the absence of Tyr134 (Y134F series of proteins, Table 1) both R111L and T54V mutations are crucial for retinal binding and efficient PSB formation. As previously discussed, these mutations are believed to assist the correct positioning of the chromophore within the binding pocket for optimal nucleophilic attack by Lys132, by removal of an associated water molecule. This postulate agrees with the data obtained for the Y134F series of proteins. However, in the presence of both R111L and Tyr134 the additional mutation of T54V does not appear to be crucial for either SB formation or protonation. In particular, the R132K:R111L:L121E:T54V tetra mutant (Table 2, entry 5) does not exhibit any improved retinal binding

(86) Rousso, I.; Friedman, N.; Sheves, M.; Ottolenghi, M. *Biochemistry* **1995**, *34*, 12059−12065.
(87) Druckmann, S.; Ottolenghi, M.; Pande, A.; Pande, J.; Callender, R. H. *Biochemistry* **1982**, *21*, 4953−4959.
(88) Koutalos, Y. *Biophys. J.* **1992**, *61*, 272−275.
(89) Koutalos, Y.; Ebrey, T. G.; Gilson, H. R.; Honig, B. *Biophys. J.* **1990**, *58*, 493−501.
(90) Liang, J.; Steinberg, G.; Livnah, N.; Sheves, M.; Ebrey, T. G.; Tsuda, M. *Biophys. J.* **1994**, *67*, 848−854.
(91) Steinberg, G.; Ottolenghi, M.; Sheves, M. *Biophys. J.* **1993**, *64*, 1499−1502.
(92) Blatz, P. E.; Baumgartner, N.; Balasubramaniyan, V.; Balasubramaniyan, P.; Stedman, E. *Photochem. Photobiol.* **1971**, *14*, 531−549.
(93) Sakmar, T. P.; Franke, R. R.; Khorana, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 8309−8313.

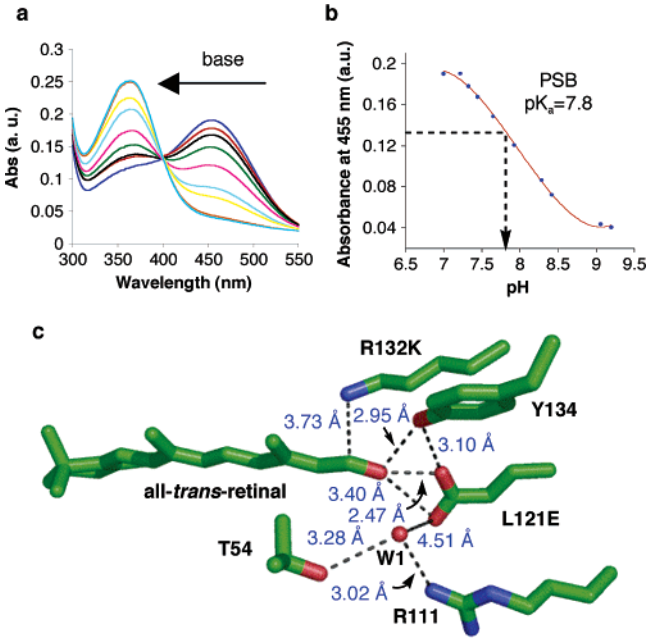

Figure 9. (a) Base titration of the KE-CRABPII/retinal complex. Upon addition of base the PSB peak at 457 nm blue shifts to 364 nm. The shift is reversible upon acidification. (b) The calculated pK_a value for the KE-CRABPII/retinal PSB is 7.8. (c) Model of CRABPII R132K:L121E mutant bound to all-*trans*-retinal.

characteristics (K_d = 1.99 ± 4.1 nM, λ_{max} = 449 nm) as compared to the KLE triple mutant (Table 2, entry 1; K_d = 1.36 ± 4.9, λ_{max} = 449 nm). The additional hydrophobic mutation (T54V) that was essential for stable PSB formation in the absence of Tyr134 (Y134F series, Table 1) is not as significant when Tyr134 is restored. The presence of Tyr134 appears to be important, especially if the ordered water molecule (W1) is not removed. This is probably due to the fact that Tyr134 can compete with W1 in hydrogen bonding with the bound chromophore and thus orient the carbonyl in a favorable trajectory. The absence of Tyr134 leads to W1 as the major contributor to the orientation of the carbonyl group of the bound chromophore.

To further support the latter conclusion, double mutant R132K:L121E (KE, Table 2, entry 6) was prepared (note that both Tyr134 and Arg111 are present in this mutant). The R132K:L121E double mutant exhibits good retinal binding affinity (K_d = 104 ± 11 nM), with an absorption maximum at 457 nm, an ~8 nm red shift as compared to the KLE mutant (λ_{max} = 449 nm). However, the pK_a of the PSB formed is reduced by 0.9 units (Figure 9a and b; pK_a(KE) = 7.8, pK_a-(KLE) = 8.7). This result indicates that the presence of the structured water molecule associated with Arg111 and Thr54 can affect the acidity of the engineered Glu121. As can be seen in Figure 9c *in silico* mutagenesis suggests interaction between Glu121 and W1 (4.51 Å), which could decrease its acidity and, therefore, its efficiency as a PSB counteranion. In the absence of Tyr134 (mutant R132K:Y134F:L121E, Table 2, entry 7) the affinity for retinal is further reduced (K_d = 160 ± 9.8 nM) and no apparent PSB is formed (λ_{max} = 381 nm). At the same time, absence of the counteranion compromises the protein's ability to produce a PSB as demonstrated by the R132K:R111L double mutant (K_d = 567 ± 36 nM, λ_{max} = 408 nm, Table 2, entry 4).

Table 3. Ability of Some CRABPII Mutants to Bind Retinal

	CRABPII protein	SB[a]	PSB[b]	PSB pK^a
1	R132K	yes	no	nd
2	R132K:Y134F	no	no	nd
3	R132K:Y134F:R111L	yes	no	nd
4	R132K:R111L	yes	no	nd
5	R132K:R111L:L121E	yes	yes	8.7
6	R132K:Y134F:L121E	yes	no	nd
7	R132K:L121E	yes	yes	7.8
8	R132K:Y134F:R111L:L121E	yes	yes	6.5

[a] SB formation as determined by positive reductive amination results. [b]PSB formation as determined by UV−vis (λ_{max} > 420 nm).

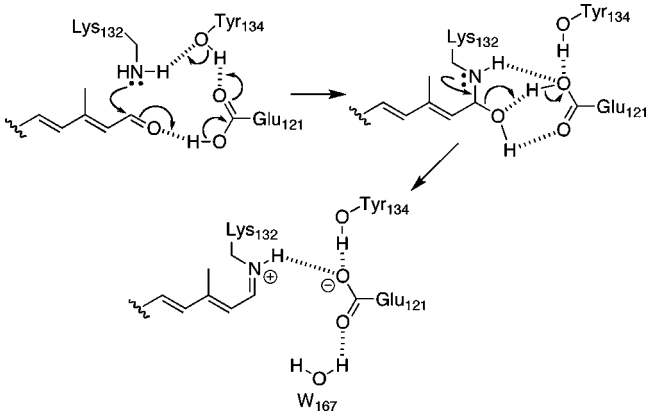

Figure 10. Suggested mechanism for the formation of a protonated Schiff base between the R132K:R111L:L121E CRABPII triple mutant and all-*trans*-retinal.

Table 3 summarizes the SB and PSB forming ability of some of the major CRABPII mutants discussed so far based on their positive reductive amination and UV−vis absorption results accordingly. Glu121 is necessary for both PSB formation and stabilization (Table 3, entries 5, 7, 8) yet not essential for SB formation (Table 3, entries 1, 3, 4). Tyr134 can assist both PSB formation and stabilization (Table 3, entry 6 vs entry 7) and, in the absence of both Glu121 and Leu111, is essential for SB formation (Table 3, entry 1 vs entry 2). Finally, the R111L mutation is very important for both SB and PSB formation and stabilization, especially in the absence of Tyr134 (Table 3, entry 5 vs entry 7).

Based on the above observations, we suggest a mechanism for PSB formation between all-*trans*-retinal and the KLE-CRABPII triple mutant (Figure 10). Tyr134 is important for orienting and possibly activating retinal's carbonyl for efficient nucleophilic attack by Lys132. Its hydrogen bond to Glu121 could lead to enhanced polarization of the carboxylic acid and, consequently, further activation of retinal. The latter arrangement should lead to the generation of an intermediate hemiaminal. Subsequent proton transfer activates the hydroxyl, leading to expulsion of water. The resultant PSB is stabilized by Glu121's carboxylate. The crystal structure of the RT bound KLE-CRABPII triple mutant (Figure 6b) reveals the presence of a tightly hydrogen-bonded water molecule (W167), which is 2.75 Å away from Glu121 and 3.88 Å away from the iminium carbon. One could speculate that W167 is the expelled water that originates from the carbonyl oxygen in retinal (note that the iminium carbon was originally the carbonyl carbon). The proposed mechanism can explain the observed spectroscopic data but by no means is the only one that could be suggested. Nevertheless, a number of important criteria for PSB formation

in a protein can be realized. First, a suitably positioned Lys residue would also require an optimal orientation with respect to the electrophilic carbonyl. As opposed to solution reactions that can eventually adopt the correct orientation in a random fashion, in the pocket of a protein it is the appropriately placed interactions that will govern the orientation of the molecule. In our case, crystallographic data were invaluable in realizing the necessary parameters for correctly orienting the retinal molecule. Second, the activation of the carbonyl through hydrogen bonding is crucial. The mutants that provided adequate hydrogen bonding, with the correct orientation of the carbonyl relative to the nucleophilic Lys residue, yielded the highest binding affinities. At the same time stabilization of the iminium through close electrostatic interaction with a suitable partner is required to maintain the PSB. What seems to be less critical is the need for a hydrophobic pocket in proximity of the nucleophilic Lys residue. Further mechanistic studies of this system using NMR techniques are underway.

Overall, three crucial mutations in the binding site of CRABPII were sufficient to lead to a surprisingly efficient protein capable of binding retinal as a PSB, resulting in a 72 nm bathochromic shift in absorption. The design followed basic principles in organic chemistry and protein engineering and has led to a soluble, stable, and convenient macromolecule that mimics the binding of retinal in rhodopsin. The crystal-structure-based design applied provided invaluable information on the structural and electronic elements essential for the SB formation. The engineered protein can now be used to further probe the nature of protein-mediated wavelength regulation when bound to retinal. Alternatively, similar systems can be used with other bathochromic chromophores, potentially providing additional means of protein tagging.

Acknowledgment. We are grateful for financial support through the NIH (GM54082 to B.B.). We are grateful to the beamline staff at COM-CAT 32-ID and SBC-CAT 19-ID at the APS, Argonne National Laboratory. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-ENG-38. Use of the beamline 32-ID with beamline management and support provided by the IMCA-CAT staff at the Advanced Photon Source was supported by the companies of the Industrial Macromolecular Crystallography Association through a contract with the Center for Advanced Radiation Sources at the University of Chicago. Use of the Argonne National Laboratory Structural Biology Center beamlines at the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Energy Research, under Contract No. W-31-109-ENG-38.

Supporting Information Available: Experimental procedures for expression, purification, and spectral characterization of all the mentioned proteins. Crystallization conditions for all CRABPII-mutants and their retinoid complexes that were crystallized. This material is available free of charge via the Internet at http://pubs.acs.org.

JA067546R