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Engineering a Rhodopsin Protein Mimic

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Human eyes contain four different types of rhodopsin (Rh, red, rod, green, and blue);[1] however, all vertebrate pigments employ the same substrate, 11-*cis*-retinal, bound as a protonated Schiff base (PSB) through an active site Lys residue.[2] Typical N-retinylidene-*n*-alkylamines absorb at 365 nm in methanol (Figure 1). In their protonated state (same as the bound state in Rh), there is a bathochromic shift to 440 nm.[3] Yet, absorption maxima associated with different rhodopsin pigments range from ~400 to ~600 nm, resulting in the wavelength regulation necessary for color vision.[2] This difference, observed in varying degrees in all visual pigments, is referred to as the "opsin shift" and is attributed to the unique interactions of each protein with the retinylidene chromophore.[4]

Several hypotheses have been proposed regarding the mechanism of wavelength regulation in Rh, most of which focus on altering the degree of retinal's conjugation by different means.[5] Protonation of the retinylidene Schiff base leads to a 80 nm red-shift.[3] Therefore, the additional bathochromic shift observed in visual pigments must arise through other means. Rotations about the polyene's intervening single bonds can result in varied degrees of orbital overlap.[6] Stereoelectronic factors, such as varying the distance of the PSB counteranion and placement of either point charges or dipoles at different positions along the backbone of the polyene, have also been suggested to modulate the degree of cationic conjugation along the polyene.[7] However, the challenges in biophysical studies of membrane-bound proteins and the lack of 3D structures for the colored pigments have hampered efforts to fully test these theories. These issues have motivated us to engineer a protein mimic of Rh that will not suffer from the limitations associated with such proteins. The engineered protein must serve as a platform for biophysical studies geared toward understanding the origins of wavelength regulation through designing specific protein/retinal interactions of interest. Here we report the rational redesign of cellular retinoic acid binding protein II (CRABPII) into a retinal-PSB-forming protein to mimic Rh.[8]

Human CRABPII is a small (~16 kDa), cytosolic protein, amenable to biochemical manipulations.[9] It belongs to the intracellular lipid binding (iLBP) family of proteins, which have a β-barrel fold in common and are structurally robust, especially with regards to changes in amino acid sequence.[10] Although they are relatively small proteins, their structural motif leads to a large and solvent-protected binding pocket. CRABPII has been overexpressed in *E. coli*, and its X-ray crystal structure has been determined.[11] The binding of retinoic acid (RA) occurs primarily through direct hydrogen bonding with Arg132 and Tyr134, located 2.7 and 2.6 Å away, respectively, from the chromophore's carboxylate (Figure 1a). The binding is also supported by a water-mediated hydrogen bond (Wat16) between RA and Arg 111, found ~4.3 Å away. RA binds the protein with high affinity (K_d = 2 nM),[12] while all-*trans*-retinal (RT) exhibits an affinity 3 orders of magnitude lower (K_d = 6600 nM).

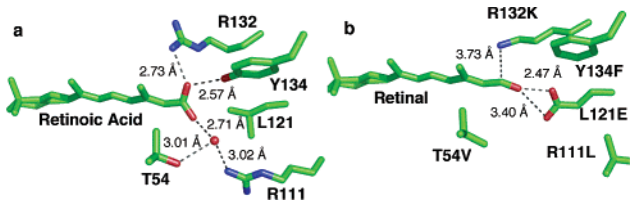

Figure 1. (a) Crystal structure of CRAPBII bound to retinoic acid. (b) Energy-minimized model of the CRABPII KFLEV pentamutant bound to retinal.

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 ± 36	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] Deconvolution of overlapping UV−vis spectra is detailed in 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.

To engineer a Rh protein mimic, three basic elements were deemed necessary: (1) presence of an active site Lys residue for SB formation; (2) protonation of the SB that initiates the bathochromic shift; and (3) a carboxylate counteranion, such as Glu113 in bovine Rh, that stabilizes the PSB. Molecular modeling led to the R132K mutant (Arg132 is the residue closest to the carboxylate of RA) as the site for incorporation of Lys. The R132K mutant revealed some loss of binding for RA (K_d = 65 ± 14 nM) and a slight increase for RT binding as measured by fluorescence quenching (Table 1). The λ_{max} did not change significantly when RT was incubated with R132K mutant (379 nm); therefore, formation of a PSB, which would have led to a bathochromic shift of the chromophore's λ_{max}, was not observed by UV spectroscopy; however, a small amount of covalently bound RT was detected by MALDI-TOF after reductive amination was performed.

Nucleophilically active Lys residues, such as those found in Rh, bacteriorhodopsin, and the aldolase antibody, are imbedded in deep hydrophobic pockets, which in effect reduce the pK_a of the ϵ-amino group.[12] Therefore, we reengineered the CRABPII binding pocket to render it more hydrophobic. In particular, the crystal structure of CRABPII indicated that the hydroxyl functionality of Tyr134 directly pointed to the putative position of Lys132. A structurally conservative replacement with Phe would lead to increased hydrophobicity. At the same time, to remove the ordered water molecule present in the vicinity of the retinoid binding site (Wat16, Figure 1a) while increasing the hydrophobicity of the pocket,

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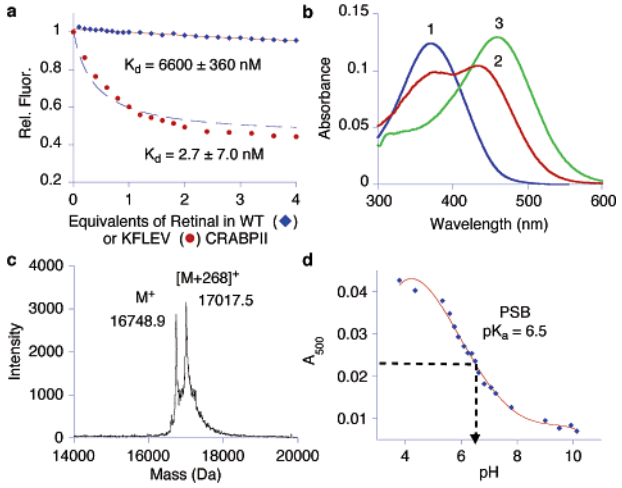

Figure 2. (a) Fluorescence quenching experiments with WT-CRABPII and KFLEV upon addition of RT. (b) UV−vis of retinal complexed with WT-CRABPII (**1**, 377 nm) and KFLEV (**2**, **3**). Clear bathochromic shift upon PSB formation leads to absorption at 446 nm at pH = 7.2 (**2**). Upon acidification, the trace completely shifts to 459 nm (**3**, pH = 4.0). (c) MALDI-TOF analysis of the reductively aminated KFLEV/retinal complex. The peak at 17017.5 Da is due to addition of one molecule of retinal to the protein $[M + RT - H_2O]^+$. (d) The pK_a of the PSB determined via titration of the KFLEV/retinal complex.

Arg111 and Thr54 were also identified as targets for modification. Following this logic, mutants 3−5 (Table 1) were prepared and evaluated for RT binding. Although substitution of Tyr134 with Phe resulted in significantly improved retinal binding as evident by the K_d values, it appears that removal of Arg111 and/or Thr54 was crucial for SB formation as evident from the reductive amination results (Table 1, entries 4 and 5). In addition, the hydrophobic tuning of the binding pocket resulted in formation of a red-shifted species, as detected by UV−vis, with a λ_{max} varying from 400 to 417 nm, a 20−40 nm red-shifting as compared to the WT-RT spectrum. However, the amount of detected PSB was very small, indicating that the pK_a value is lower than that of the working pH.

To stabilize the PSB and to mimic the binding pocket of rhodopsin,[13] a carboxylate residue that would act as a counteranion was incorporated. Further molecular modeling led to position 121 as the site for installation of a Glu residue. Two different mutants (Table 1, entries 6 and 7) were prepared, and the RT binding was evaluated. The UV−vis of the R132K:Y134F:R111L:L121E mutant revealed a species absorbing at 446 nm, nearly a 70 nm red-shift as compared to the WT protein bound to RT. Additional mutation of Thr54 to Val results in the R132K:Y134F:R111L:L121E:T54V (KFLEV) mutant, which maintains a red-shifted UV−vis (λ_{max} = 446 nm) and exhibits high affinity binding to RT (K_d = 2.7 nM) (Figure 2a,b). In addition, the covalent bond between RT and KFLEV was verified by MALDI-TOF analysis after reductive amination (Figure 2c).

Two control mutants were important to demonstrate the function of the engineered active site residues. The Y134F:L121E:R111L triple mutant (entry 8) was prepared to demonstrate that Lys132, and not any of the other 13 Lys in the protein, is responsible for PSB formation. The latter mutant, lacking the engineered Lys132 (retaining the original Arg132) failed to produce a red-shifted chromophore upon addition of RT (λ_{max} = 381 nm). It also did not yield a mass corresponding to conjugation of the protein with RT upon MALDI-TOF analysis. The R132K:Y134F:L121Q:R111L:T54V mutant (entry 9) was made to assess the role of the Glu counteranion (replacing Glu with Gln at position 121). Interestingly, this mutant bound RT over 200-fold worse (600 nM), but yet did yield a SB forming protein ($[M + 268]^+$). However, the protein complex with RT did not red-shift (λ_{max} = 371 nm), suggesting that the Glu counteranion is necessary for protonation of the SB. Similar behavior has been observed in Rh mutants since replacement of Glu113 (the counteranion in bovine Rh) with Gln leads to blue-shifting of the pigment from 500 to ∼380 nm.[14]

Acid−base titration of the KFLEV PSB of the retinal-bound KFLEV mutant could be easily followed via UV−vis spectroscopy (PSB λ_{max} = 459 nm, SB λ_{max} = 360 nm). As illustrated in Figure 2d, the pK_a of the retinylidene SB is estimated to be 6.5. This is lower than the pK_a reported for visual pigments, which are typically in the range of 9.3 to >16.[15] The relatively low pK_a value explains the presence of overlapping absorptions (Figure 2b, red curve), which correspond to protonated and unprotonated imine species at pH 7.

In summary, we have redesigned the binding site of CRABPII to mimic the same binding mode found in rhodopsin. This should allow for a novel strategy to further studies into the nature of wavelength regulation in rhodopsin pigments. Future modifications aim to increase the pK_a of the PSB.

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Supporting Information Available: Experimental procedures for expression, purification and spectral characterization of proteins 1−9. This material is available free of charge via the Internet at http://pubs.acs.org.

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