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OPSIN SHIFT IN AN ALDOLASE ANTIBODY

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Abstract, An antibody-retinal assembly that mimics the opsin shift (OS) of the naturally occurring visual pigments is reported. Both experiments and calculations show that the aldolase antibody 33F12 covalently binds all-*trans* retinal via a protonated Schiff base with a lysine residue. This chromophore, which exhibits a remarkable opsin red shift (140 nm), represents a useful model system for studying the factors that contribute to the OS.

The consequences of the interactions of retinal with the opsin protein to form a visual pigment are central to understanding the visual process.[1] This environmental effect of the host protein on the retinal chromophore is known as the opsin shift (OS). The absorbance maximum of visual pigments spans from the near UV at about 360 nm to the far visible red at about 620 nm. In contrast, a simple protonated Schiff base of retinal with *n*-butylamine absorbs at 440 nm. Experimental and theoretical considerations led to the external point charge model to account for the OS.[2] According to that model negative charges (or an equivalent of them) of amino acid side chains regulate the absorption maximum of the protonated Schiff base chromophore. Specific mutation[3] and NMR[4] experiments have shown that in rhodopsin, for example, that counteranion is Glu-113, with one of its carboxylate oxygen atoms being positioned approximately 3 Å away from the C-12 of the retinal molecule.

In vitro modeling of the OS is crucial not only for a better understanding of the visual pigments in vertebrates, arthropods and mollusks but also for the development of new photosensitive materials.[5] Many model studies have focused on regenerating the spectral properties of the vision pigments using the natural opsin proteins with retinal analogs.[6] Other models were based on retinal molecules in various environments, including silica gel,[7] micelles,[8] amphiphilic clay,[9] and even organic solvents at low temperatures.[10] Another model indicates that even weak electrostatic interaction between the positively charged chromophore and a remote negative charge can also lead to a significant red shift.[11]

Here we report on the first antibody-retinal assembly that mimics the OS of the visual pigments. Our calculations predicted and the experiments confirmed that a catalytic antibody covalently binds all-*trans* retinal, **1**, via a lysine residue and thus represents a useful protein model of the visual pigments.

Antibody 33F12, which was obtained via reactive immunization,[12] is characterized by a lysine residue (Lys-93) with perturbed acidity ($pK_a \sim 6$) embedded within a hydrophobic active site. This antibody catalyzes the aldol condensation as well as the retrograde aldol reaction under neutral pH with high efficiency and enantioselectivity over a broad range of ketone and aldehyde substrates.[13] The catalytic mechanism involves a reaction between the antibody's lysine residue and the donor carbonyl substrate to form first a protonated Schiff base and then an enamine intermediate. This mechanism is characteristic of the type-I aldolase enzymes.

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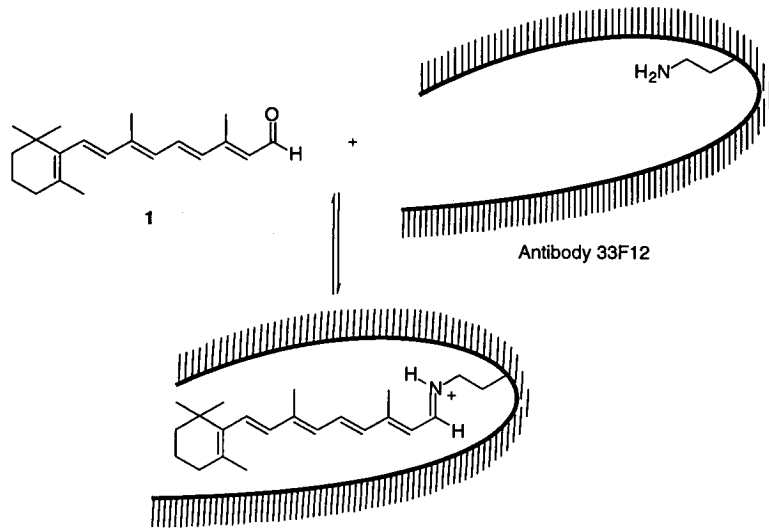


Scheme 1

Based on the crystallographic data of the Fab fragment of the antibody 33F12 (1axt in Brookheaven's PDB)[14] we built the three-dimensional model for the protonated Schiff base of **1** with Lys-93 (Scheme 1). The modified lysine residue was generated separately and merged within the protein's model. All water molecules were removed from the model prior to calculations. Minimization of the three dimensional model was achieved using the "Discover" program as implemented by the Biosym's InsightII package. A Morse potential was used for bond energies until a maximum derivative of 1.0 was accomplished. All heavy atoms were constrained except for those of the Trp-33, Glu-50, Lys-93 and the retinal moiety (all unconstrained residues belong to the heavy chain).

The model predicts an excellent fit of the retinal substrate within the antibody's binding site. Most importantly, it predicts that one of the carboxylate oxygen atoms of Glu-50 is positioned 6.2 Å from C-12 of the retinal molecule (Fig. 1). Intermolecular energy was considered in order to assess the stabilization of the Lys-retinal protonated Schiff base within the protein's cavity using an 8 Å cutoff.[15] The conformational energies (intramolecular) of the isolated Lys-retinal residue were also obtained. Since both the Lys-retinal residue and the surrounding protein environment are hydrophobic, the dispersion forces dominate the intermolecular energy (non-bond). The calculated model supports the formation of a protonated Schiff base between Lys-93 of antibody 33F12 and retinal. Moreover, considering the hydrophobic environment within the binding site and the weak interactions between the chromophore and the relatively distant Glu-50 carboxylate anion, we expect the 33F12-retinal system to exhibit a significant OS.

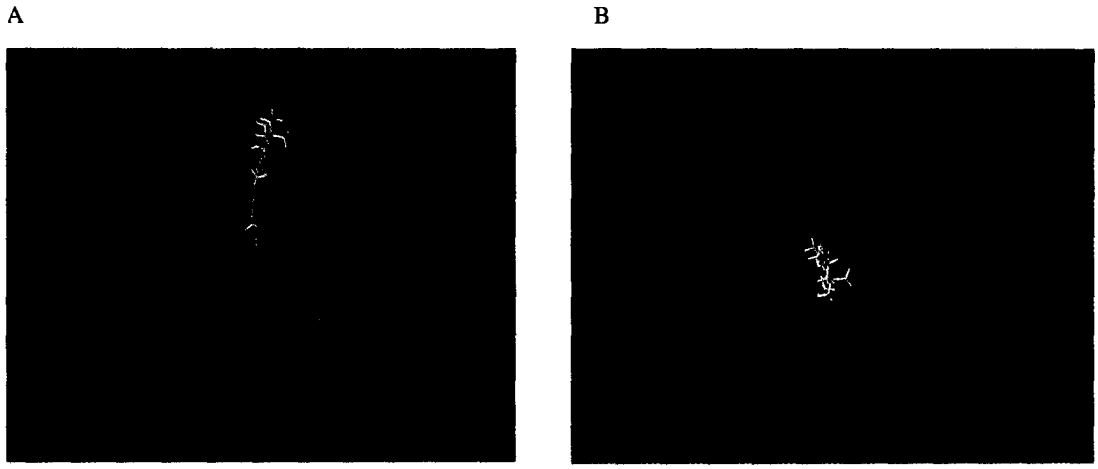
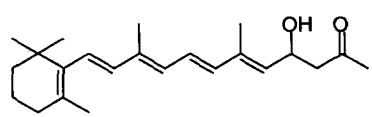


Figure 1 Calculated model of the protonated Schiff base **1**. (A) Lateral view. (B) Top view. Heavy chain: cyan ribbon. Light chain: magenta ribbon. Glu-50: red tubes. Lys-93: green tubes. Retinal moiety: yellow tubes. Residues in the binding site are wireframe depicted.

A simple experiment in which the antibody 33F12 catalyzed the aldol condensation between **1** and acetone[16] verified that a retinal molecule could fit into the antibody's binding site. Formation of the aldol product **2** was confirmed by HPLC using an authentic sample of the β-hydroxyketone product.[17]



2

Additional evidence for the formation of a retinal Schiff base with the lysine residue was obtained by chemical reduction followed by mass spectral analysis.[18] The mass spectrum (MALDI) of antibody 33F12 exhibits two peaks at 150013 (M^+) and 75000 (M^{+2}).[19] A solution of this antibody (10 mg/mL, 0.18 mL) was mixed with a solution of **1** (1.74 mM in CH_3CN, 0.02 mL). The mixture was kept in the dark at room temperature for 6 h and then treated with excess $NaBH_3CN$, and eluted through a sephadex column using ultra-pure water. The mass spectrum (MALDI) of the resultant modified protein exhibited two peaks at 150378 (M^+) and 75281 (M^{+2}). Considering that this MALDI analysis has a 0.1% rate of accuracy this observation supports the formation of a retinal derivative covalently bound to antibody 33F12.

The above-results set the stage for the crucial examination of the OS induced by the antibody. Thus, a series of experiments were carried out using stock solutions of 33F12 (10 mg/mL) in phosphate buffer (50 mM, pH 7.4, 100 mM NaCl) and a solution of **1** (1.7 10^{-3} M, stored in the dark at 4 °C) in acetonitrile. Another

solution of a non-relevant protein was prepared for the control experiments. Thus, bovine serum albumin (BSA, 10 mg/mL) was dissolved in the same phosphate buffer solution. The protein stock solution and the solution of **1** were mixed in a 9:1 ratio, respectively and their UV-vis absorbance was measured. The BSA-retinal mixture absorbed at λ_{max}= 440 nm and did not show any further significant spectral changes over the course of the 6 h. at room temperature. Conversely, the mixture of antibody 33F12 and **1** displayed a new absorption signal at λ_{max}= 510 nm (Fig. 2). This signal reached maximum intensity within 6 h. Other cases of slow reactivity of retinal with amines to form Schiff bases have also been reported.[6g,20] This observed OS was effectively suppressed by acetylacetone (equimolar quantities with respect to **1**), a known inhibitor of 33F12, suggesting that retinal only binds to Lys-93.

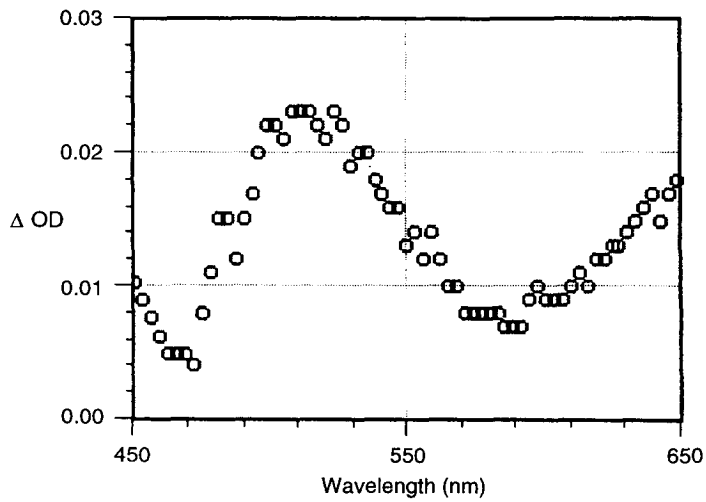


Figure 2. Difference UV-vis spectrum of the 33F12 - retinal sample.

The pH dependence of the OS of various protein retinal systems provides useful information about the immediate electrostatic environment within the host proteins. A typical natural photoreceptor, such as the sensory rhodopsin I (*Halobacterium halobium*), exhibits an OS within a broad pH range between 3.5 and 8.0.[23] This range is much narrower, between pH 6.0 and 7.5, for the antibody 33F12 retinal system (Fig. 3). The lower limit of this range may reflect the pK_a value of the antibody's Glu-50 residue, and the upper limit may reflect the pK_a of the retinal's protonated Schiff base.

In conclusion, both experiments and calculations show that antibody 33F12 binds all-*trans* retinal via a protonated Schiff base. This system exhibits a remarkable opsin red shift (140 nm) and thus mimics the typical shifts observed with the naturally occurring visual pigments. Since catalytic antibodies can be modified by either chemical[21] or by site-directed mutagenesis methods,[22] antibody 33F12 represents an excellent system for

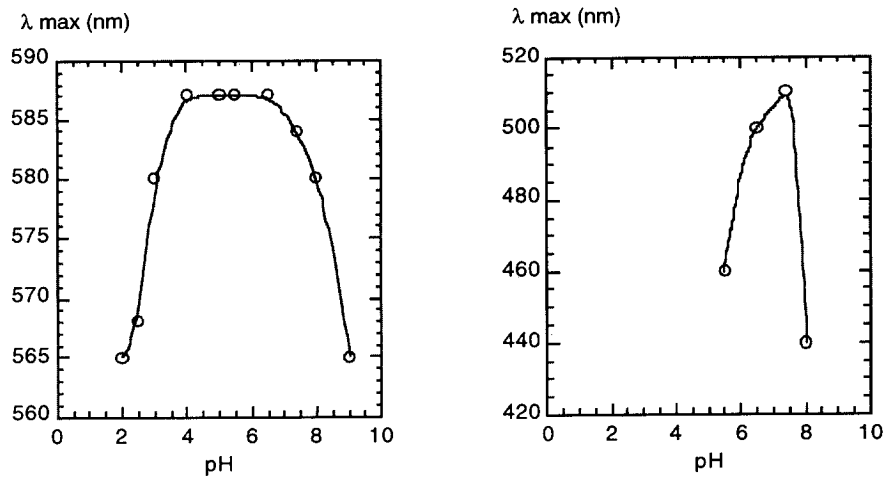


Figure 3. Effect of the pH on the λ_{max} of sensory rhodopsin I (left, extracted from reference 23) and antibody 33F12–retinal sample (right).

studying the factors that contribute to the OS. These methods enable fine-tuning of the induced OS in retinal or in other related chromophores, which will lead not only to better understanding of the vision chemistry but also to the design of novel photosensitive materials. Work along these lines is currently underway.

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Incumbent of the Benno Gitter & Ilana Ben-Ami chair of Biotechnology, Technion.

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15. Calculations were carried out for vacuum with a dielectric constant of 1.0. In the solvated system, the dielectric constant varies from approximately 3 for the interior of the protein to 80, the dielectric constant of water. Therefore, it is likely that the actual electrostatic contribution (Coulomb) is much smaller than the calculated value. Calculated interaction energies for antibody 33F12-retinal: Intermolecular= 55.04 Kcal/mol (Non Bond=6.02 Kcal/mol, Coulomb= 49.02 Kcal/mol); Intramolecular= 118.20 Kcal/mol (Non Bond= 39.95 Kcal/mol, Coulomb= 78.25 Kcal/mol).
16. A mixture of **1** (5 μL, acetone solution) with antibody 33F12 (90 μL, 10 mg/mL) in PBS (50 mM phosphate, 100 mM NaCl, pH 7.4, 90 μL) was incubated at 25 °C for 19 h. HPLC analysis (reverse-phase C-18 column, isocratic program 75/25; CH_3CN/H_2O, 1 mL/min, monitored at λ= 330 nm) showed **1** (t_R: 14.99 min) and the aldol product **2** (5%, t_R: 9.30 min, identified by coinjection with authentic sample of **2**).[17] In a control experiment a mixture of **1** (10^{-2} M in acetone, 5 μL), acetylacetone (acac, 10^{-2} M in acetone, 5 μL), antibody 33F12 (90 μL, 10 mg/mL) and the same buffer solution (PBS, pH 7.4, 90 μL) was incubated at 25 °C for 19 h. HPLC analysis showed only intact **1**. The same result was obtained in two other control experiments with buffered solution containing either **1** and acetone or **1**, acetone and acac.
17. Diisopropylamine (0.4 mmol) in dry CH_3CN (5 mL) was cooled to 0 °C. A solution of *n*-butyllithium (0.4 mmol) in hexane was added, the mixture was stirred for 20 min under argon then cooled to –78 °C. Acetone (0.4 mmol) was added and the mixture was stirred at –78 °C for 1 h. A solution of **1** (3.6 mmol) in dry THF (2 mL) was slowly added, the mixture was stirred for 1 h at the same temperature, warmed to room temperature, washed with brine and extracted with ether. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (silica gel, DCM:MeOH 95:5) yielding pure **2**. ^{1}H NMR (300 MHz, $CDCL_3$, rt) δ 1.00 (s, 6H), 1.24 (s, 3H), 1.26 (s, 3H), 1.53 (m, br, 4H), 2.01 (m, br, 2H), 2.15 (s, 3H), 2.28 (s, 3H), 2.61 (s, br, 2H), 4.10 (m, 1H), 6.15 (m, 5H), 6.35 (d, J = 20Hz, 1H) ppm. MS (ESI): 365 $(M+Na)^+$, 341 $(M-H)^+$.
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19. A solution of antibody 33F12 in PBS (50 mM, pH 7.4) was passed through a sephadex G-25M pre-packed column and the protein was eluted with ultra-pure water and submitted to MALDI.
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