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Cyclodextrin retinylidene: A biomimetic kinetic trap model for rhodopsin

Kafui Kpegba, Matthew Murtha and Nasri Nesnas*

Department of Chemistry, Florida Institute of Technology, Melbourne, FL 32901, USA

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Abstract—All *trans* retinal was attached to both the primary face and the secondary face of β-cyclodextrin via a Schiff base linkage, analogous to that in rhodopsin. The new models were evaluated and compared with *n*-butylamine retinylidene Schiff base for their rates of hydrolysis, and factors that influence such rates. Competition studies using adamantane carboxylate demonstrated the kinetic trap theory by diminishing the binding of retinal in the cyclodextrin, thereby augmenting the rate of hydrolysis. NMR experiments indicate that the retinylidene is most probably bound in the form of a dimer.

Retinal, **1**, is the chromophore responsible for triggering the enzymatic cascade leading to vision in vertebrates. In its 11-*cis* geometry, **1b**, it is bound by the protein opsin, covalently linked to Lys296 via a Schiff base generating a retinylidene, which forms the photoactive photoreceptor, rhodopsin.[1–3] Upon absorption of light, the 11-*cis*-retinylidene undergoes an isomerization to the all *trans* retinylidene, which in turn triggers the activation of the G-protein. The active state of rhodopsin is referred to as the meta II or MII (see Fig. 1). In the vertebrate visual cycle, the all *trans* retinylidene is then hydrolyzed and is released out of the chromophore binding pocket. The all *trans* retinal is then shuttled through a series of retinol binding proteins (RBPs) that convert it back to the 11-*cis* geometry, thereby completing the visual cycle (Fig. 1).[3] The hydrolysis of the Schiff base is a crucial step and has not been fully explored mechanistically. Exploring the rate of hydrolysis is pivotal in understanding the abnormalities that affect the visual cycle, such as Stargardt disease.[4] It has been shown that some mutants of rhodopsin result in varying hydrolysis rates for the all *trans* retinal, often leading to adverse visual effects.[5,6] For instance, an accumulation of all *trans* retinal can lead to severe problems including a common form of age-related macular degeneration (AMD), in which two retinal molecules will condense with a phosphatidyl ethanolamine leading to a highly fluorescent pigment known as A2E.[7–10] The role of hydrogen bonding in Schiff base hydrolysis in rhodopsin has been carefully studied via conservative mutations of critical residues near the binding site.[11] We hereby present an alternative approach to understanding hydrolysis via a simple artificial biomimetic model using β-cyclodextrin[12] as the recognition site for retinal, for which it is known to bind in.[13,14]

β-Cyclodextrin (β-CD), **2**, was functionalized from both the primary and the secondary sides using standard techniques[12,15–20] to afford the primary 6-aminocyclodextrin and the secondary 3-aminocyclodextrin (see Fig. 2). The aminocyclodextrins were subsequently reacted with 1 equiv of retinal, **1**, in the presence of 0.9 equiv of acetic acid in DMSO, to generate **3** and **4** in 98% and 93% yields, respectively. The latter Schiff bases were isolated via lyophilization. The Schiff base of retinal with *n*-butylamine, **5**, was also generated and used as a control in the hydrolysis studies.

The new structures **3** and **4** were characterized by UV, ^{1}H NMR,[21] and FAB mass spectrometry. Their UV absorption in DMSO was at a λ_{max} of 370 and 372 nm, with molar absorptivities of 112,800 and 98,400 $mol^{-1}\,cm^{-1}$, respectively. The ^{1}H NMR in DMSO-d_6 revealed a distinctive peak for the aldimine proton (the former aldehyde proton), which was at δ 8.36 and at 8.45 ppm in structures **3** and **4**, respectively. FAB mass spectrometry revealed a mass of 1401.37 for the two structures corresponding to the [M+H] ion.

Keywords: Cyclodextrin; Retinal; Cyclodextrin retinylidene; Schiff base hydrolysis; Kinetic trap; Rhodopsin mimic; Dimer.
* Corresponding author. Tel.: +1 321 674 8902; fax: +1 321 674 8951; e-mail: nesnas@fit.edu

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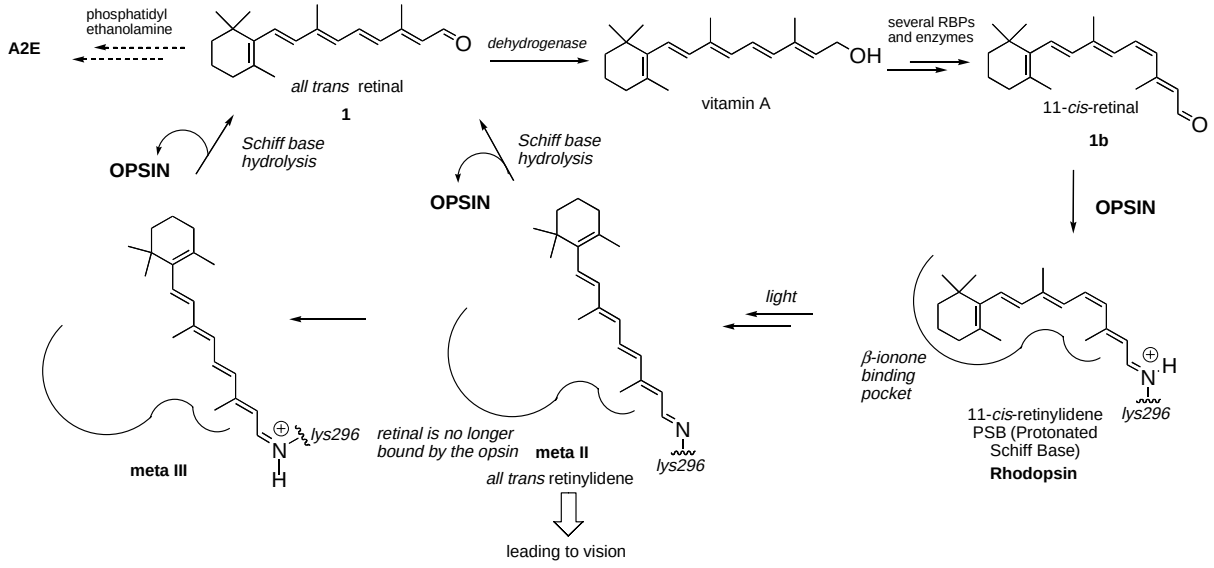

Figure 1. A simplified outline of the visual cycle.

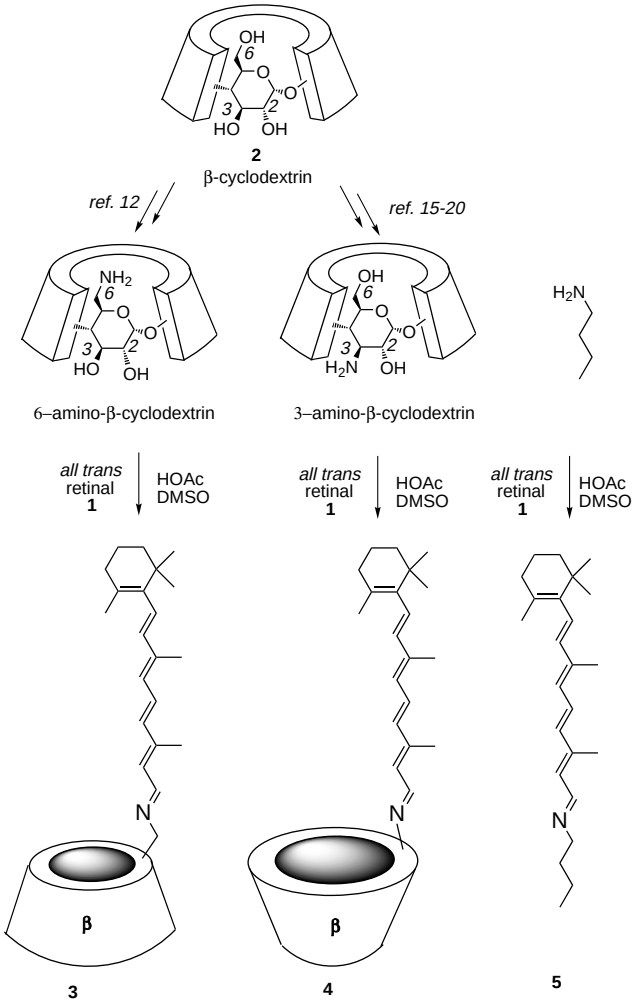

Figure 2. The synthesis of the three retinylidene Schiff bases.

Hydrolysis studies were carried out at 25 °C and in pH 4.0 buffer. Although the hydrolysis of the three Schiff bases was followed by ^{1}H NMR to prove the identity of the products, the rates were measured more precisely by following the decrease in absorption of the protonated Schiff bases at their corresponding λ_{max}, which was 442 nm for **3** and 447 nm for **4**, in pH 4 buffer. Since it is expected that retinal will bind into cyclodextrin[13,14] in water, analogous to retinal's binding into opsin, we sought to evaluate the effect of the binding of the hydrophobic retinoid on the rate of Schiff base hydrolysis. It is established that in rhodopsin, the binding that 11-*cis*-retinal has for opsin ensures its stability in the dark state, such that if hydrolysis of the 11-*cis* conformation occurs, the bound chromophore ligand simply reforms the Schiff base, hence the term kinetic trap.[5] However after isomerization of the 11-*cis*-retinal to the all *trans*, the β-ionone moiety of the all *trans* retinal no longer binds into the opsin binding pocket, thereby exposing the Schiff base to water molecules that will lead to its hydrolysis. Alternatively, the conformational change imposed by the isomerization of retinal could reorient the amino acid residues within the Schiff base binding site in such a manner as to orchestrate a hydrogen bonding network that will catalyze the hydrolysis of the Schiff base.[11] In our model studies, we envision compound **4** forming a dimer in water as depicted in Figure 3. The NMR data presented below support our reasoning behind it. Introduction of a strong hydrophobic β-cyclodextrin ligand, such as adamantane carboxylate which has a binding constant in β-cyclodextrin of 10^{4} M^{-1},[22] will undoubtedly break up the dimer 'releasing' the retinal from the hydrophobic cyclodextrin cavity. It is therefore expected that the rate of hydrolysis will be enhanced in the presence of adamantane carboxylate. In other words, in our model, adamantane carboxylate

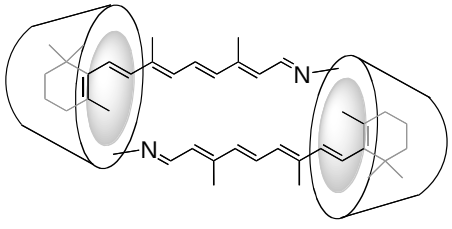

Figure 3. Proposed dimer of **4** formed in aqueous solutions.

competitively displaces the β-ionone from its binding pocket (the cyclodextrin cavity), which is a mimic for the light induced isomerization of the retinal, which releases the β-ionone from its binding pocket in rhodopsin.

Additional evidence for the binding of the retinoid into the β-cyclodextrin (β-CD) cavity comes from NMR anisotropy experiments carried out in D_2O, using CH_3CN as an internal standard for accurate calibration of chemical shifts. The binding of adamantane carboxylate into β-cyclodextrin resulted in an upfield shift of the β-CD H-2, H-4, H-3, and H-5 by ca. 0.056 ppm and a downfield shift of 0.101 ppm for the 3° protons of $AdCO_2^-$. The well-resolved anomeric proton (H-1) of β-CD is quite informative to follow as it exhibited an upfield shift of 0.049 ppm. The anomeric proton of structure **4** was also upfield shifted, albeit to a lesser extent, by ca. 0.041 ppm. This could be a result of the smaller bulk of the β-ionone unit relative to the adamantane structure. Introduction of the $AdCO_2^-$ resulted in a further upfield shift of the anomeric protons by about 0.035 ppm, indicating displacement of the retinoid from the β-CD cavity. The upfield shift observed upon addition of $AdCO_2^-$ to **4** is considerably smaller than the 0.056 ppm upfield shift observed when an equal concentration of the $AdCO_2^-$ was introduced in β-CD. This indicated that the β-cyclodextrin cavity in **4** was already occupied by a hydrophobic moiety, and that the smaller 0.035 ppm upfield shift is a result of the adamantane being a stronger recognition element thereby displacing the retinoid out of the cavity. The adamantane 3° protons experienced nearly the same exact 0.100 ppm downfield shift upon binding into the β-CD cavity of **4** as they have done so in β-cyclodextrin. Therefore, these studies point to the direction that the β-CD cavity had a hydrophobic moiety bound in it, namely the retinoid, that was displaced by the stronger ligand $AdCO_2^-$, which resulted in the release of the retinoid. Unfortunately, the β-ionone proton peaks were not clearly resolved and we were unable to accurately monitor their shifts. We were challenged by the rapid hydrolysis of **4**, which made the NMR measurements somewhat cumbersome.

The results of hydrolysis in the absence and presence of adamantane carboxylate at pH 4.0 are summarized in Table 1. The rate constants were obtained by measuring the initial rates of the various substrates at identical concentrations. As expected, the presence of adamantane carboxylate enhanced the hydrolysis rate by displacing the bound retinal exposing it to the solvent. The rate enhancement was nearly twofold in the case of the secondary face attached retinylidene **4** and about 50% in the case of the primary face attached retinylidene **3**. It is likely that the secondary side of β-cyclodextrin, possessing the larger opening, will bind the retinal (in particular the β-ionone portion) better than the primary side. Thus, the retinal bound to the secondary side of β-cyclodextrin, being bound stronger in a dimeric form (Fig. 3) than its primary counterpart, experiences a greater rate enhancement upon release of the retinal via competition with excess adamantane carboxylate.

Table 1. Observed rate constants[a] for hydrolysis of the Schiff bases at pH 4 ($s^{-1} \times 10^{-3}$)

Compound[b]	In the absence of $AdCO_2^-$	In the presence[c] of $AdCO_2^-$
3	8.58	12.7
4	2.02	3.84
5	68.6	28.7

[a] The results were reproducible within a 5% error.
[b] The concentrations of Schiff bases were 1 μM.
[c] $AdCO_2^-$ is adamantane carboxylate. The concentration used was 100-fold that of compounds **3**–**5**.

The faster rate observed for the hydrolysis of the Schiff base of the 1° face versus 2° face of cyclodextrin is a combination of the binding effect of the different sides of cyclodextrin as well as the difference in pK_a of imines (Schiff bases). The imine attached to the secondary face of cyclodextrin is situated closer to the anomeric carbon of the cyclodextrin rendering the electron density more withdrawn than in the case of the imine of the primary face. This is evidenced by the difference in ^1H NMR chemical shifts of the aldimine protons, which is 0.09 ppm downfield in structure **4** relative to **3**, a result of a more electron-withdrawing environment.[21] Therefore, according to the mechanism[23] presented in Figure 4, a greater percentage of Schiff base **3** will be protonated shifting the equilibrium in favor of the attack of the water molecule on the protonated Schiff base.

The *n*-butyl Schiff base, **5**, is nearly an order of magnitude faster due to its exposure to solvent. The addition of adamantane carboxylate resulted in lowering the rate of hydrolysis, possibility due to the potentially intimate ion-pair that renders the protonated Schiff more stable, and hence more resistant to hydrolysis. It is known that Glu113 in rhodopsin aids in the stabilization of the protonated Schiff base.[5] In the case of **3** and **4**, the binding of the hydrophobic adamantane ring into the β-CD cavity is more favorable than the formation of an ion-pair.

The rates of hydrolysis of three Schiff bases, **3**–**5** were studied in both the presence and the absence of the hydrophobic moiety adamantane carboxylate. The disruption of the retinal binding in the hydrophobic cavity of β-cyclodextrin, most probably in the form of a dimer, resulted in increased rates of hydrolysis. This mimics the

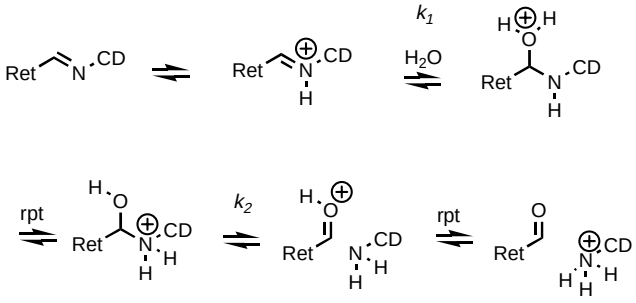

Figure 4. Mechanism of the Schiff base hydrolysis, (CD = β-cyclodextrin).

biological rhodopsin model in which the rate of hydrolysis of the retinal is enhanced after its light induced isomerization from 11-*cis* to all *trans*, which releases the β-ionone ring from its hydrophobic binding pocket. This indicates that our biomimetic model is in accordance with the kinetic trap theory.

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21. NMR data. Schiff base **3**: ^{1}H NMR (360 MHz, DMSO-d_6) δ = 8.36 (d, J = 9.5 Hz, 1H, aldimine: 6′-CD–CH$_2$–N=C–*H*), 6.87 (dd, J = 15.1, 11.3 Hz, 1H, ret-H-11), 6.46 (d, J = 15.3 Hz, 1H, ret-H-12), 6.02–6.37 (m, 4H, ret-H-7, 8, 10, 14), 5.6–6.0 (m, 14H, 2°-CD-OH), 4.7–5.0 (m, 7H, CD anomeric), 4.35–4.65 (m, 6H, 1°-CD-OH), 3.5–4.0 (m, 28H, CD), 3.1–3.5 (m, 14+, CD + DMSO), 2.05 (s, 3H, ret-H-13′), 1.9–2.0 (m, 2H, ret-H-4), 1.95 (s, 3H, ret-H-9′), 1.71 (s, 3H, ret-H-5′), 1.55 (m, 2H, ret-H-3), 1.42 (m, 2H, ret-H-2), 1.00 (2s, 6H, ret-H-1′,1′). Schiff base **4**: ^{1}H NMR (360 MHz, DMSO-d_6) δ = 8.45 (d, J = 9.8 Hz, 1H, aldimine: 3′–CD–*N*=C–*H*), 6.97 (dd, J = 15.1, 11.3 Hz, 1H, ret-H-11), 6.50 (d, J = 15.3 Hz, 1H, ret-H-12), 6.16–6.35 (m, 3H, ret-H-7, 8, 10), 6.10 (d, J = 9.9 Hz, 1H, ret-H-14), 5.6–6.0 (m, 13H, 2°-CD-OH), 4.7–5.0 (m, 7H, CD anomeric), 4.35–4.65 (m, 7H, 1°-CD-OH), 3.5–4.0 (m, 28H, CD), 3.1–3.5 (m, 14+, CD + DMSO), 2.14 (s, 3H, ret-H-13′), 1.95-2.10 (m, 2H, ret-H-4), 2.00 (s, 3H, ret-H-9′), 1.71 (s, 3H, ret-H-5′), 1.59 (m, 2H, ret-H-3), 1.45 (m, 2H, ret-H-2), 1.02 (2s, 6H, ret-H-1′,1′).
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