

## Mechanism of Transient Dark Activity of 13-Desmethylretinal/Rod Opsin Complex

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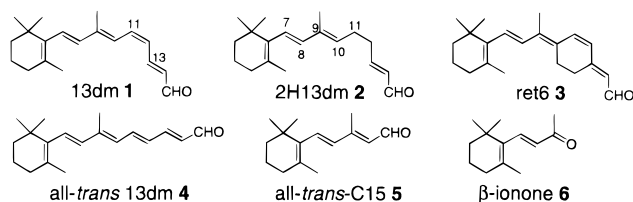
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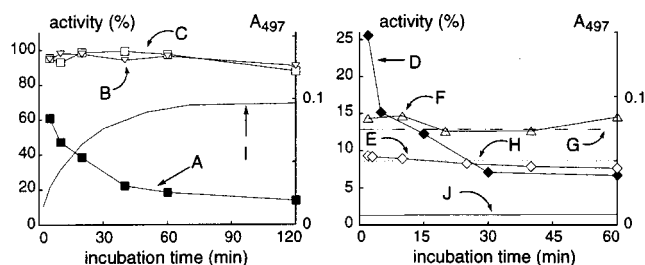
We report the studies employing 11-*cis*-13-desmethylretinal (13dm 1), its dihydro analogue 2H13dm 2, and 11-*cis* six-membered ring-locked analogue ret6 3, to clarify the mechanism of dark activity of the 13-desmethylretinal/rod opsin complex.

Rhodopsin (Rh) is composed of a retinal chromophore linked to Lys 296 of the apoprotein opsin via a protonated Schiff base (PSB).<sup>1</sup> Light-induced retinal 11-*cis* → *trans* isomerization triggers protein conformational changes leading to metarhodopsin II (Meta-II) which activates the G-protein transducin to initiate the enzymatic cascade of visual transduction.<sup>2</sup> Phosphorylation of Meta-II by rhodopsin kinase and subsequent binding of arrestin deactivate Meta-II.<sup>3</sup>

Recently it has been observed that opsin can be activated by retinal analogues including all-*trans*-retinal, all-*trans*-13dm 4, all-*trans*-C15 5, and  $\beta$ -ionone 6 in the absence of light.<sup>4</sup> Dark activity



of opsin/retinal complex has been proposed to be responsible for bleaching adaptation,<sup>4c–d,5</sup> whereas constitutive activation of opsin leads to certain night blindness and retinal degeneration diseases.<sup>5d,6</sup> Therefore, clarification of the mechanism of dark activity of opsin/retinal complex is essential for understanding the complicated



**Figure 1.** Traces A–H: Relative activities of incubation mixture measured by transducin assay. A. 13dm 1 and opsin; B. All-*trans*-retinal and opsin; C. All-*trans*-retinal and opsin pretreated with  $\beta$ -ionone; D. 2H13dm 2 and opsin; E. Ret6 3 and opsin; F. 13dm 1 and opsin pretreated with  $\beta$ -ionone; G.  $\beta$ -ionone and opsin; H. Opsin. Trace I: A<sub>497</sub> of 13dm 1 with opsin. Trace J: A<sub>497</sub> of 13dm 1 and opsin pretreated with  $\beta$ -ionone.

process of visual regulation. Following the 1980 report that 13dm 1 and opsin induced dark activation of phosphodiesterase,<sup>7</sup> a subsequent assay for rhodopsin kinase activity showed that the dark activity decayed with time, i.e., it is transient.<sup>4c</sup> In the following, the mechanism of this transient dark activity has been studied with analogues 1–3<sup>8a</sup> by performing UV/Vis/CD measurements and transducin assays,<sup>8b</sup> in which the relative activity of opsin/retinal complex is measured by counting the amount of GTP $\gamma$ <sup>35</sup>S bound to transducin.

Figure 1 shows the transducin assay and UV/Vis measurements. Opsin treated with 13dm 1 exhibited transient dark activity (trace A), consistent with previous kinase assay data.<sup>4c</sup> Decay of the activity was accompanied with the regeneration of 13-desmethyl rhodopsin (13dmRh) with a 497 nm  $\lambda_{\text{max}}$  (trace I), implying that an early active species is replaced by the inactive 13dmRh with the PSB linkage (497 nm). The residual activity after 120 min incubation could be due to the nonspecific binding of GTP $\gamma$ <sup>35</sup>S.

The retinal binding cavity contains a binding site (site I in Figure 3 below) for the cyclohexene ring of  $\beta$ -ionone 6,<sup>4d,9</sup> which activates opsin, but compared to 13dm 1, only weakly.<sup>4c–e</sup> Traces J and F show that  $\beta$ -ionone inhibits the binding and opsin activation of 13dm 1. Hence, for opsin activation, 13dm 1 needs to enter the protein cavity with the cyclohexene ring occupying the corresponding binding pocket. In contrast, traces B and C show all-*trans*-retinal exhibited sustained activity which was essentially unaffected by  $\beta$ -ionone, in agreement with the published data.<sup>4d</sup> 2H13dm 2<sup>10</sup> (prepared as in Scheme 1) and

(1) (a) Yoshizawa, T.; Wald, G. *Nature* **1963**, *197*, 1279–1286. (b) Wald, G. *Nature* **1968**, *219*, 800–807. (c) Unger, V. M.; Hargrave, P. A.; Baldwin, J. M.; Schertler, G. F. X. *Nature* **1997**, *389*, 203–206. (d) Schertler, G. F. X.; Villa, C.; Henderson, R. *Nature* **1993**, *362*, 770–772. (e) Tan, Q.; Lou, J.; Borhan, B.; Karnaukhova, E.; Berova, N.; Nakanishi, K. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2089–2093. (f) Zhang, H.; Lerro, K. A.; Yamamoto, T.; Lien, T. H.; Sastry, L.; Gawinowicz, M. A.; Nakanishi, K. *J. Am. Chem. Soc.* **1994**, *116*, 10165–10173.

(2) (a) König, B.; Arendt, A.; McDowell, J. H.; Kahlert, M.; Hargrave, P. A.; Hofmann, K. P. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6878–6882. (b) Stryer, L. *J. Biol. Chem.* **1991**, *266*, 10711–10714. (c) Khorana, H. G. *J. Biol. Chem.* **1992**, *267*, 1–4. (d) Nathans, J. *Biochemistry* **1992**, *31*, 4923–4931.

(3) (a) Bownds, D.; Dawes, J.; Miller, J.; M., S. *Nature New Biol.* **1972**, *237*, 125–127. (b) Palczewski, K.; Rispoli, G.; Detwiler, P. B. *Neuron* **1992**, *8*, 117–126. (c) Pulvermuller, A.; Palczewski, K.; Hofmann, K. P. *Biochemistry* **1993**, *32*, 14082–14088.

(4) (a) Kefalov, V. J.; Cornwall, M. C.; Crouch, R. K. *Invest. Ophthalmol. Visual Res.* **1998**, *39*, S1123. (b) Cornwall, M. C.; Corson, D. W.; Crouch, R. K.; Fain, G. L. *Invest. Ophthalmol. Visual Res.* **1996**, *37*, S239. (c) Buczylo, J.; Saari, J. C.; Crouch, R. K.; Palczewski, K. *J. Biol. Chem.* **1996**, *271*, 20621–20630. (d) Jäger, S.; Palczewski, K.; Hofmann, K. P. *Biochemistry* **1996**, *35*, 2901–2908. (e) Palczewski, K.; Jäger, S.; Buczylo, J.; Crouch, R. K.; Bredberg, D. L.; Hofmann, K. P.; Asson-Batres, M. A.; Saari, J. C. *Biochemistry* **1994**, *33*, 1–13750.

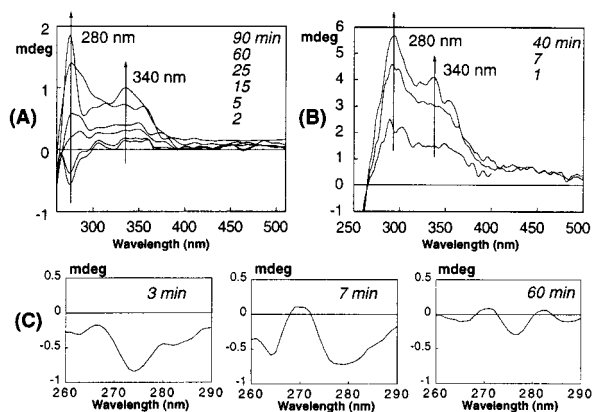
(5) (a) Cornwall, M. C.; Matthews, H. R.; Crouch, R. K.; Fain, G. L. *J. Gen. Physiol.* **1995**, *106*, 543–557. (b) Corson, D. W.; Cornwall, M. C.; MacNichol, E. F.; Tsang, S.; Derguini, F.; Crouch, R. K.; Nakanishi, K. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 6958–6962. (c) Jin, J.; Crouch, R. K.; Corson, D. W.; Katz, B. M.; MacNichol, E. F.; Cornwall, M. C. *Neuron* **1993**, *11*, 513–522. (d) Palczewski, K.; Saari, J. C. *Curr. Opin. Neurobiol.* **1997**, *7*, 500–504.

(6) (a) Rao, V. R.; Oprian, D. D. *Annu. Rev. Biophys. Biomol. Struct.* **1996**, *25*, 287–314. (b) Fahmy, K.; Zvyaga, T. A.; Sakmar, T. P.; Siebert, F. *Biochemistry* **1996**, *35*, 15065–15073.

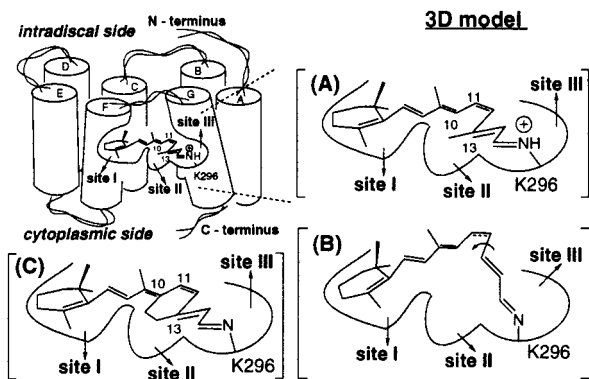
(7) Ebrey, T.; Tsuda, M.; Sassenrath, G.; West, J. L.; Waddell, W. H. *FEBS Lett.* **1980**, *116*, 217–219.

(8) (a) 13dm 1 was prepared according to: Broek, A. D.; Muradin-Szwejkowska, M.; Courtin, J. M. L.; Lugtenburg, J. J. R. *Neth. Chem. Soc.* **1983**, *102*, 46–51. (b) To 10 mM pH 7.4 Tris containing 4  $\mu$ M opsin or opsin pretreated with 20 equiv of  $\beta$ -ionone for 1 h was added 4 molar equiv retinal analogues in DMF (<2% v/v). According to the published procedures (Wessling-Resnick, M.; Johnson, G. L. *J. Biol. Chem.* **1987**, *262*, 12444–12447), a portion of the above mixture was transferred into the transducin assay solution in 10 mM pH 7.4 Tris, which then contained 1–10  $\mu$ M transducin, 200 nM opsin, and 10  $\mu$ M GTP $\gamma$ <sup>35</sup>S. After 5 min 70  $\mu$ L of the assay mixture was filtered through a nitrocellulose filter, which was dissolved in 10 mL of scintillation fluid Filtron-X, and its radioactivity, corresponding to the relative activity of opsin/retinal complex, was counted on a Beckman scintillation counter. CD and UV/Vis spectra were taken in 10 mM pH 7.4 Tris and 23 mM dodecyl maltoside.

(9) Matsumoto, H.; Yoshizawa, T. *Nature* **1975**, *258*, 523–526.

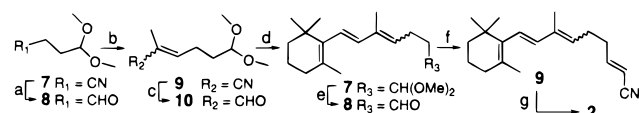


**Figure 2.** Time-dependent CD spectra of opsin incubated with retinal analogues. (A) 13dm **1** (0 °C). (B) Ret6 **3** (rt). (C) 2H13dm **2** (rt).



**Figure 3.** Proposed model of activity of opsin/13dm **1** complex. 3D model shows seven  $\alpha$ -helices surrounding the retinal binding cavity including sites I–III. (A) The activation is transient since PSB formation at site III leads to inactivation of the protein. (B) Prior to PSB formation, as the retinal cyclohexene ring occupies site I and the aldehyde approaches Lys 296 at site III without PSB formation, 13dm analogues **1** and **2** are able to adjust the polyene conformation at site II to transiently adopt a shape close to Meta-II rhodopsin. (C) Ret6 **3** is inactive since the ring-fixed polyene, unlike the 13dm analogues, cannot achieve the Meta-II-like conformation.

#### Scheme 1<sup>a</sup>



<sup>a</sup> Conditions: (a) DIBAL-H,  $\text{CH}_2\text{Cl}_2$ ,  $-78^\circ\text{C}$ , 78%; (b) diethyl (1-cyanoethyl)phosphonate, NaH, THF, 76% overall; (c) DIBAL-H,  $\text{CH}_2\text{Cl}_2$ ,  $-78^\circ\text{C}$ , 80%; (d)  $\beta$ -onyltriphenylphosphonium bromide, BuLi, THF, 65%; (e) amberlyst acidic resin, acetone, 95%; (f) diethyl cyanomethyl phosphonate, NaH, THF, quant.; (g) DIBAL-H,  $\text{CH}_2\text{Cl}_2$ ,  $-78^\circ\text{C}$ , silica gel chromatography to separate 1:1 isomers, 79% overall.

ret6 **3**<sup>11</sup> formed pigments with opsin, with  $\lambda_{\text{max}}$  at 270 and 508 nm, respectively. As shown in Figure 1, **2** exhibited transient activity similar to 13dm **1** (traces A, D), while **3** was inactive (trace E).

The conformational changes of retinal analogues and opsin during activation were studied by CD (Figure 2). For 13dm **1** and 2H13dm **2** which display transient activity (Figure 1, traces A, D), the CD shown in Figure 2A,C both exhibit a negative

Cotton effect around 280 nm that decays with loss in activity. In contrast, opsin incubated with ret6 **3** (Figure 2B) showed neither activity (Figure 1, trace E) nor a negative 280 nm CD Cotton effect (Figure 2B). It should be noted that in the case of native Rh, the physiologically active Meta-II has a negative 280 nm CD band as opposed to the positive 280 nm CD of Rh.<sup>12</sup> Hence, it is possible that the transient dark active species of opsin incorporating 13dm **1** or 2H13dm **2** adopts a Meta-II-like conformation.

On the basis of these data, we propose the following rationalization for the *transient dark activation* of opsin by 13dm **1** and 2H13dm **2** (Figure 3; the 3D structure is based on photoaffinity cross-linking<sup>16</sup>). The opsin cavity incorporating the retinals is divided into three binding domains, site I for the cyclohexene ring, site II for the polyene chain, and site III for PSB.

(i) Occupation of site I by the cyclohexene moiety is required for opsin activation.

(ii) Prior to pigment formation, 11-*cis*-retinals transiently activate the protein during the binding process. Rhodopsin kinase assay study<sup>4c</sup> showed that 13dm **1** exhibits no transient dark activation when Lys 296 is dimethylated. Thus, approach of the retinal aldehyde close to Lys 296 in site III or formation of a Schiff base linkage is necessary for opsin activation.

(iii) Protonation of the Schiff base (Figure 3A) inactivates the protein through salt bridge formation between PSB and its counteranion Glu 113.<sup>6a,13</sup>

(iv) During the process of “anchoring” of retinal in sites I (as in i) and III (as in ii), the polyene chain adopts a shape which gives rise to a transient Meta-II-like conformation in opsin.

In the case of 13dm **1** and 2H13dm **2**, as the chromophore enters the opsin binding site with its cyclohexene ring in site I and the unprotonated Schiff base linkage in site III, the retinal polyene enters site II and adjusts the torsions around C10–C13 to attain the conformation responsible for protein activation (Figure 3B). The ring-locked ret6 **3** cannot adopt this conformation and is thus inactive (Figure 3C). No dark activity has been reported for 11-*cis*-retinal;<sup>4c</sup> a possible explanation is that since the binding of 11-*cis*-retinal is 10 times faster than 13dm **1**, the retinal and protein rapidly attain the inactive state of rhodopsin. Experiments designed to slow this binding process may reveal transient activation. Note that traces B and C in Figure 1, which denote *sustained* dark activity by all-*trans*-retinal, are different from the present *transient* dark activity. It is regarded that in the case of sustained activity, the chromophore lies outside the binding site;<sup>4d</sup> however, further studies are necessary.

Finally, the opsin used for the experiments was from a rod outer segment preparation<sup>14</sup> and contains mainly the rod opsin. In isolated salamander rods, a similar response is noted, i.e., activation without light by  $\beta$ -ionone **6**<sup>4a</sup> and 13dm **1**.<sup>4b</sup> However, in isolated salamander cones, the opposite result is obtained as  $\beta$ -ionone deactivated the basal activity of the photoreceptor, acting as an inverse agonist.<sup>5c</sup> Therefore, the interaction of the opsin with the ligand appears to be dependent on the structure of the opsin and may explain some of the differences in the properties of rods and cones.

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(11) Prepared according to: de Grip, W. J.; van Oostrum, J.; Bovee-Geurts, P. H. M.; van der Steen, R.; van Amsterdam, L. J. P.; Groesbeek, M.; Lugtenburg, J. *Eur. J. Biochem.* **1990**, *191*, 211–220.

(12) Okada, T.; Matsuda, T.; Kandori, H.; Fukada, Y.; Yoshizawa, T.; Shichida, Y. *Biochemistry* **1994**, *33*, 4940–4946.

(13) (a) Cohen, G. B.; Oprian, D. D.; Robinson, P. R. *Biochemistry* **1992**, *31*, 12592–12601. (b) Sakmar, T. P.; Franke, R. R.; Khorana, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 8309–8313.

(14) Opsin prepared according to: Papermaster, D. S. *Methods Enzymol.* **1982**, *81*, 49–52.

(10) <sup>1</sup>H NMR ( $\text{CDCl}_3$ , 400 MHz, ppm) for **2**:  $\delta$  9.54 (1H, d,  $J = 7.9$  Hz), 6.89 (1H, dt,  $J_1 = 15.6$  Hz,  $J_2 = 12.9$  Hz), 6.18 (1H, ddt,  $J_1 = 15.6$  Hz,  $J_2 = 7.9$  Hz,  $J_3 = 1.4$  Hz), 6.07 (1H, d,  $J = 16.6$  Hz), 6.01 (1H, d,  $J = 16.2$  Hz), 5.40 (1H, t,  $J = 6.6$  Hz), 2.47 (2H, m), 2.42 (2H, m), 2.02 (2H, t,  $J = 6.3$  Hz), 1.83 (3H, s), 1.71 (3H, s), 1.64 (2H, m), 1.50 (2H, m), 1.04 (6H, s). nOe was observed between 9-Me/7-H, 9-Me/11-H, and 8-H/10-H. HRMS: Calculated 272.2140 for  $\text{C}_{19}\text{H}_{28}\text{O}$ , observed 272.2137.