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Probing for the Threshold Energy for Visual Transduction: Red-Shifted Visual Pigment Analogs from 3-Methoxy-3-Dehydroretinal and Related Compounds

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ABSTRACT

While azulenic retinal analogs failed to yield a red-shifted visual pigment analog, the 9-*cis* isomers of the push–pull polyenals 3-methoxy-3-dehydroretinal and 14F-3-methoxy-3-dehydroretinal yielded iodopsin pigment analogs with absorption maxima at, respectively, 663 and 720 nm. The former gave a relatively stable batho product (700 nm) and was able to activate transducin. A lower activity was observed for the latter. One possible explanation for the combined results is that the excitation energies of these red-shifted pigments are approaching the threshold energy for visual transduction (although at this time we cannot rigorously exclude a role of the added F-atom in reducing the transducin activity).

INTRODUCTION

The visual transduction process in photoreceptor cells begins with photon absorption by the visual pigment, which contains an 11-*cis* retinal or its derivative bound to a lysine residue of an apoprotein opsin through a protonated Schiff base linkage (1,2). Through *cis–trans* isomerization of the retinal chromophore the energy of the photon is converted into chemical energy (3). The stored energy is then utilized for conformational changes of the protein moiety to activate retinal G-protein, which in turn, generates an electrical response of the photoreceptor cells (4,5). Because the energy of the photon is inversely proportional to its wavelength, it is of interest to examine how far red-shifted a visual pigment can be absorbing and yet retain the characteristic photosignal transduction and, in so doing, to determine the threshold energy.

So far, the most red-shifted natural visual pigment (λ_{max} = 630 nm) has been identified in fish retinas, which has 3-dehydroretinal (DHR,† or vitamin A_2), **1**, as its chromophore (6). The red-cone pigment iodopsin (571 nm), on the other hand, is the most red-shifted natural visual pigment having the retinal chromophore. Understandably, because DHR has an extra double bond in its conjugated π system compared to retinal, the former exhibits an ~50 nm more red-shifted absorption maximum. Incorporation of DHR to the opsin moiety of iodopsin, however, forms iodopsin analog (cyanopsin) absorbing maximally at 624 nm (Fig. 1), comparable to that of the fish visual pigment (7).

Our strategy to produce pigments more red shifted than the native visual pigments is based on the available information on analogs derived from modified retinals (8). First, it is known that a more delocalized chromophore than the native ones causes a red shift. Hence, it will be of interest to introduce an electron-donating substituent at the opposite end of the protonated Schiff base (PSB) functionality to create a push–pull system so as to enhance delocalization of the positive charge. It is also known that the positioning of an electron-withdrawing substituent, such as an F atom, near the PSB end (*e.g.* 14F-retinal) induces a red shift (9). And, retinal analogs with the side chain anchored onto the highly colored nonalternant hydrocarbon azulene are known to give near-IR (NIR)-absorbing bacteriorhodopsin (bR) analogs (10). The possibility of introducing such structural features into retinal analogs for formation of visual pigment analogs (iodopsin as well as rhodopsin) has been explored. At the same time, we have carried out a parallel study examining the ability of any red-shifted pigment to induce G-protein activation. Results of these studies are reported in this paper.

MATERIALS AND METHODS

Synthesis of retinal analogs. 3-Methoxyretinal (**6**) was prepared by methylation of the reported 3-hydroxyretinal (11). The 9-*cis* and 11-*cis* isomers were isolated (HPLC) from the photomixtures. 9-*Cis* ($CDCl_3$): 7.20 (H-11, J = 11.4, 15.0), 6.65 (H-8, J = 15.9), 6.31 (H-12, J = 15.0), 6.27 (H-7, J = 15.9), 6.11 (H-10, J = 11.4), 5.79 ppm (H-14, J = 8.4 Hz); 11-*cis* ($CDCl_3$): 10.08 (H-15, J = 8.1), 6.72 (H-11, J = 13.8, 11.4), 6.55 (H-10, J = 11.5), 6.33 (H-7, J =

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†*Abbreviations:* bR, bacteriorhodopsin; conA, concanavalin A; DHR, 3-dehydroretinal; DTT, dithiothreitol; MeO, 3-methoxy; NIR, near infrared; PC, phosphatidylcholine; PSB, protonated Schiff base.

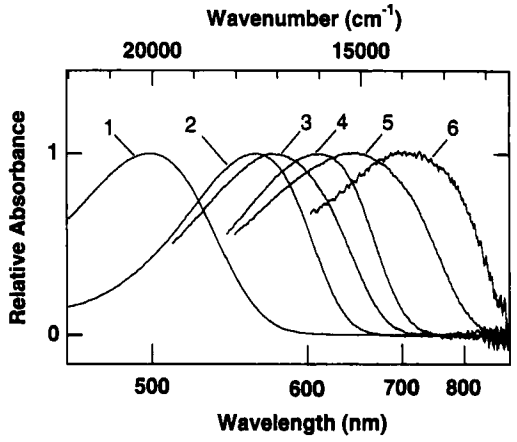


Figure 1. Absorption spectra of long-wavelength-sensitive visual pigments. Normalized absorption spectra of rhodopsin (curve 1), iodopsin (curve 2) and iodopsin analogs from 9-*cis* and 11-*cis* DHR (1) (curves 3 and 4, respectively) are shown with those from MeO-DHR– (4) and 14F-MeO-DHR– (5) iodopsins (curves 5 and 6, respectively).

15.8), 6.16, (H-8, J = 15.8), 6.10 (H-14, J = 8.4), 5.90 ppm (H-12, J = 13.8 Hz). The 9-CF_3-azulenylretinal **2** was prepared following procedures similar to other azulenic retinals (10). H-NMR ($CDCl_3$): 10.04 (H-14, J = 8.0), 7.19 (H-10, J = 10.9), 6.97 (H-11, 15.3, 10.9), 6.69 (H-12, J = 15.3), 6.00 ppm (H-14, J = 8.0 Hz). 3-Methoxy-3-dehydroretinal (**4**) and the 14F analog (**5**) were prepared *via* the sequences briefly described below.

3-Keto-α-C_{15}-nitrile **7** was prepared by reaction of 3-keto-α-ionone with the C_2-nitrile phosphonate in 86% yield. The ketonitrile **7** was then converted to 3-methoxy-3-dehydro-C_{15}-nitrile (**8**) by reaction with $HC(OMe)_3$ (64% yield). Standard reaction of the nitrile **8** with DIBAL-H yielded 3-methoxy-3-dehydro-C_{15}-aldehyde (**9**) in 62% yield.

3-Methoxy-3-dehydroretinonitrile (**10**) was prepared by reaction of the tetraenal **9** with tris-(CH_3CF_2)-2-methyl-3-cyano-2-propenylphosphonate in the presence of $KN(TMS)_2$ and 18-crown-6 (12). An early fraction from column chromatography of the product mixture was found to contain primarily the 11-*cis* isomer. Partial H-NMR ($CDCl_3$): 6.63 (H-11, J = 12.3, 11.6), 6.50 ppm (H-12, J = 12.3 Hz). The *trans* isomer was isolated in a later fraction. Partial H-NMR ($CDCl_3$): 6.95 (H-11, J = 15.3, 11.7), 6.73 ppm (H-12, J = 15.5 Hz). Reaction of a mixture of the retinonitrile **10** with DIBAL-H yielded a mixture of isomers of 3-methoxy-3-dehydroretinal from which the 9-*cis* isomer was isolated by HPLC separation. Partial H-NMR (CD_3CN): 9.98 (H-15, J = 7.8), 6.91 (H-11, J = 15.0, 11.4), 6.60 (H-8, J = 15.9), 6.16 (H-12, 15.0), 6.14 (H-7, J = 15.9), 6.03 (H-10, J = 11.4), 5.51 (H-14, J = 7.8 Hz), 4.77 ppm (H-4). However, under no circumstances was the 11-*cis* isomer isolated.

3-Methoxy-3-dehydro-C_{18}-ketone was obtained by condensation reaction of the tetraenal **9** with acetone (82%). The corresponding C_{20}-14F ester was obtained by reaction of the C_{18}-ketone with triethyl-2-fluoro-2-phosphonoacetate. The ester was subsequently converted to 3-methoxy-3-dehydro-14F-retinal **5** by sequential reactions with $LiAlH_4$ and MnO_2. The 9-*cis* isomer was isolated by HPLC. H-NMR (C_6D_6): 9.86 (H-15, J = 18.1), 7.08 (H-11, J = 14.5, 11.6), 6.50 (H-8, J = 15.9), 6.12 (H-7 and H-12), 5.96 (H-10, J = 11.5 Hz), 4.92 ppm (H-4).

Preparation of rhodopsin and iodopsin analogs. Opsin moieties of rhodopsin and iodopsin solubilized in buffer A (0.6% CHAPS, 0.8 mg/mL egg phosphatidylcholine [PC], 50 m*M* HEPES, 140 m*M* NaCl, 1 m*M* dithiothreitol [DTT], 200 m*M* methyl-α-D-mannopyranoside, pH 6.5 at 4°C) were prepared by the methods reported previously (13). The NO_3-bound forms of opsins were prepared by eluting the chloride-bound forms from the concanavalin A (conA)–Sepharose column with buffer A (0.6% CHAPS, 0.8 mg/mL egg PC, 50 m*M* HEPES, 140 m*M* $NaNO_3$, 1 m*M* DTT, 200 m*M* methyl-α-D-mannopyranoside, pH 6.5 at 4°C). To reconstitute a pigment analog having retinal analog as a chromophore, an excess amount (about 10 molar excess) of retinal analog was mixed with each opsin sample and incubated at 4°C until the reaction was complete (more than 10 h). The neutralized hydroxylamine solution (1 *M*, pH 6.5) was then added to the opsin solution to make a final concentration of 10 m*M*. Absorption spectra were measured at 4°C in the presence of 10 m*M* hydroxylamine. The rates of formation of iodopsin and rhodopsin pigment analogs were found to be slower than those of the parent systems (~1/40 and 1/10, respectively). But the eventual pigment yields were quantitative in both cases.

Spectroscopy. The system for recording absorption spectra was reported previously (14). Absorption spectra were recorded with a Shimadzu model MPS-2000 spectrophotometer interfaced with an NEC PC-9801 computer. The sample temperature was regulated to within 0.1°C by a thermostated cell holder equipped with the spectrophotometer. Irradiation of the sample was carried out with light from a 1 kW tungsten halogen lamp (Rikagaku Seiki). The wavelength of the irradiation light was selected with a glass cutoff filter (VY52, VR69; Toshiba) or an interference filter (KL63; Toshiba).

Chromophore extraction and HPLC analysis. The analog pigments were purified by means of conA–Sepharose column chromatography to remove unreacted retinal analogs as described previously (15). The experimental procedures for the chromophore extraction and HPLC analysis are identical to those reported previously (15).

Transducin activation assay. A filter-binding assay that monitors the light-dependent guanine-nucleotide exchange by transducin was performed. The reconstituted analog pigments were incorporated into egg PC liposome by dialysis against buffer B (50 m*M* HEPES, 140 m*M* NaCl, 3 m*M* $MgCl_2$, 1 m*M* DTT, pH 6.5 at 4°C). Transducin was prepared from fresh bovine retinas by the method previously reported (16). Reactions were initiated by irradiation of the reaction mixture (100 n*M* pigments, 500 n*M* transducin, 1.25 μ*M* [^{35}S]GTPgS in buffer B [20 μL]) with orange light (>500 nm) and terminated 3 min after irradiation by diluting into 250 μL buffer C (20 m*M* Tris-Cl, 100 m*M* NaCl, 25 m*M* $MgCl_2$, pH 7.4) with 10 μ*M* GTPgS. The solutions were immediately filtered through nitrocellulose membranes to separate the [^{35}S]GTPgS bound to transducin from the free [^{35}S]GTPgS. The membranes were then washed three times with 250 μL of buffer C. Dried membranes were assayed by liquid scintillation counting (LS6000IC, Beckman). Light-induced activation was calculated by subtracting the activity of the dark sample from that of the irradiated sample. Aliquots of irradiated pigments (200 μL) were extensively irradiated with yellow light (>500 nm) in the presence of hydroxylamine (10 m*M*), and the amounts of pigments photoconverted to the all-*trans* product upon first irradiation were estimated. The average and standard deviations were calculated from four independent experiments.

RESULTS AND DISCUSSION

New retinal and their visual pigment analogs

Following the successful preparation of NIR-absorbing bR analogs using retinal analogs containing the highly colored azulene chromophore (8), we first prepared the azulene analogs **2** for visual pigment studies. Its 9-*cis* geometry was attained through the *cis*-directing effect of the CF_3 group. However, analog **2** was found not to yield a visual pigment analog upon incubation with bovine opsin or the more reactive chicken red opsin. The negative result parallels that of the naphthalene-trienal, **3** (M. Denny and A. E. Asato, unpublished). Apparently the planar bicyclic aromatic chromophores are not compatible with the binding site of the visual proteins. We therefore turned to derivatives of 3-dehydroretinal (**1**).

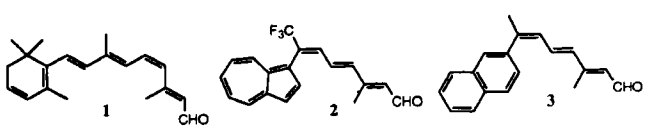
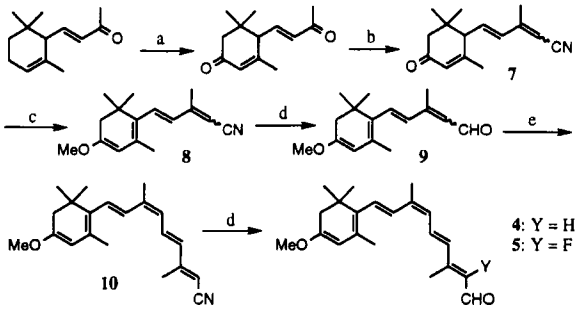


Table 1. Molecular properties of iodopsin analogs

	λ_{max} (Cl^-) (nm)	λ_{max} (NO_3^-) (nm)	*K* (m*M*)	GTPgS binding*
A_1 11-*cis*	571	538	51.0	1
9-*cis*	534	493	184.4	0.97
3MeO-R, **6** (A_1) 11-*cis*	536	—	—	†
9-*cis*	529	—	—	†
DHR (A_2) 11-*cis*	624	588	33.7	0.84
9-*cis*	585	533	161.9	0.81
MeO-DHR, **4** 9-*cis*	663	578	223.5	1.02
14F-MeO-DHR, **5** 9-*cis*	720	611	ND‡	0.10

*Relative activity to original (A_1, 11-*cis*) iodopsin.
†Pigment yield too low (<5%) for this essay.
‡Not determined.

3-Methoxy-3-dehydroretinal, **4** (MeO-DHR), was prepared in a nonstereoselective manner following the synthetic sequence shown below. The 9-*cis* isomer was isolated by preparative HPLC. Photoisomerization failed to produce any detectable amounts of the 11-*cis* isomer. A separate attempt to synthesize the isomer through stereoselective construction of the hindered 11-*cis* geometry using a modified C_5-phosphonate (12) led to the corresponding 11-*cis* isomer of the nitrile **10**. However, its conversion to the aldehyde following typical reaction conditions for such functional group manipulation resulted in the isolation of the all-*trans* and 13-*cis* isomers only. Apparently, the 11-*cis* isomer of such a push–pull polyene is too unstable for isolation. Therefore, the current study is limited to the 9-*cis* isomer of **4** and 14-fluoro-3-methoxy-3-dehydroretinal, **5** (14F-MeO-DHR) that was also prepared following the established procedure for introducing the 14F substituent, *i.e.* the corresponding C_{18}-ketone with the C_2-fluorophosphonoacetate (17). For comparison, the 9-*cis* and 11-*cis* isomers of 3-methoxyretinal, **6** (MeO-R) and DHR, **1**, were also prepared.



a. t-butyl chromate. b. $(EtO)_2P(O)CH_2CN$ + NaH. c. MeOH, $HC(MeO)_3/H^+$.
d. DIBAL-H. e. $(EtO)_2P(O)CH_2(CH_3)C{=}CHCN$ + NaH

Rhodopsin and iodopsin analogs derived from these retinal analogs were prepared by incubation of each analog with bovine opsin or chicken red-cone opsin (13). The rhodopsin analog of 3-MeO-DHR was formed in the same manner as rhodopsin. It shows a slightly red-shifted absorption maxima at 530 nm. But the iodopsin analog of the same pigment shows a much red-shifted maximum at 663 nm. The 14F-3-MeO-DHR was found to react with opsin at too slow a rate, while with iodopsin at a detectable rate giving a 720 pigment

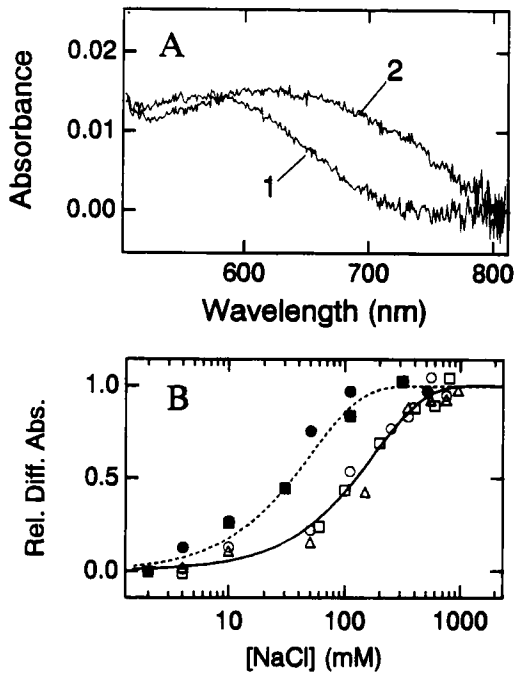


Figure 2. Effect of anion substitution on the absorption spectra of iodopsin analogs. (A) Absorption spectra of the NO_3- (curve 1) and Cl- (curve 2) bound forms of MeO-DHR–iodopsin were measured at 4°C. (B) An NaCl solution (4 *M*) was added to the NO_3-bound form of iodopsin analogs and spectra were measured. Changes in the absorbance at maxima were plotted against calculated NaCl concentration. Smooth and dotted lines indicate the exponential fitting curve of 9-*cis* (open square) and 11-*cis* (closed square) iodopsins, respectively. The 9-*cis*-DHR (open circle), 11-*cis*-DHR (closed circle) and MeO-DHR (open triangle) iodopsins show similar anion-exchange profiles to the original iodopsins.

at low yield (~10%). Isomers of MeO-R, **6**, were found to give pigments at low yields (<10%). The absorption maxima of the resultant rhodopsin and iodopsin pigment analogs are listed in Table 1 and partly shown in Fig. 1.

The data show that red-shift attenuation of the absorption maxima was successfully achieved in the iodopsin analogs of **4** and **5**. In the 9-*cis* series, a 51 nm red shift was observed in the DHR analog that was further augmented in MeO-DHR (**4**) and 14F-MeO-DHR (**5**) by an additional 78 and 135 nm shift, respectively. The enhanced red shift in analog **5**, 720 nm, is a combined effect of the extended π system, the ground-state destabilizing effect of the electron-withdrawing F atom and the excited-state stabilizing effect of the F substituent in the chromophore and to the enhanced protein–chromophore interaction. It is by far the most red-shifted visual pigment analog reported.

In natural iodopsin, the maximum wavelength and protein–substrate interactions have been known to be affected by chloride binding. When chloride is replaced with nitrate, a characteristic blue shift is observed, but this does not destabilize the pigment (18). We found that these new analogs exhibit the same anion-dependent absorption characteristics. As shown in Table 1 and Fig. 2, chloride replacement with nitrate in the DHR, MeO-DHR or 14F-MeO-DHR analogs was accompanied with a blue shift, albeit with concomitant lowering of extinction coefficients. A plot of relative absorbance *versus* NaCl and $NaNO_3$ concentrations (Fig. 2B)

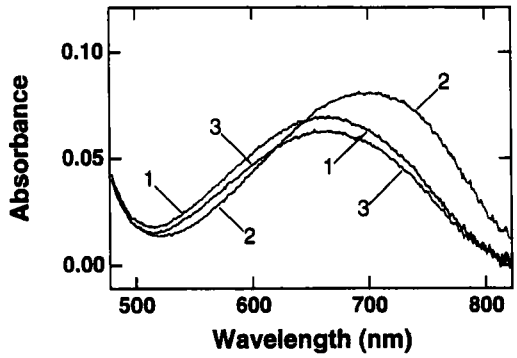


Figure 3. Photochemical and thermal reactions of the MeO-DHR–iodopsin analog. Absorption spectra of the MeO-DHR–iodopsin were measured at 0°C before (curve 1) and after (curve 2) irradiation with orange light (630 nm) for 10 min in the presence of 10 m*M* hydroxylamine. Curve 3 shows the spectrum recorded 1 h after the irradiation.

Table 2. Molar composition of MeO-DHR isomers extracted from rhodopsin and iodopsin analogs*

	11-*cis*	All-*trans*	9-*cis*
MeO-DHR–rhodopsin			
Dark	ND†	10.2 (0.6)	81.5 (4.4)
Irradiation at 4°C	ND†	77.8 (1.6)	4.2 (1.5)
Irradiation at −196°C	1.3 (0.4)	11.5 (1.5)	76.9 (8.8)
MeO-DHR–iodopsin			
Dark	3.5 (2.7)	18.3 (3.7)	71.2 (6.6)
Irradiation at 4°C	15.8 (2.2)	34.0 (2.7)	36.9 (4.0)
Dark after irradiation	4.3 (2.3)	18.9 (1.7)	69.2 (3.4)

*Molar composition is denoted by percentages that were calculated from the peak areas of *syn* forms (7–15 min) monitored at 400 nm assuming that all the isomers have the same extinction coefficients. Values are average of at least three independent experiments and standard deviations are shown in parentheses.
†Not detected.

demonstrated that these artificial pigments possess relative affinities to the anions, chloride and nitrate, similar to the natural pigments. The calculated dissociation constant, *K* (shown in Table 1, third column), correlates with the ratio of chloride-bound and -depleted iodopsin, for the analogs are consistent with those of the parent 11-*cis* and 9-*cis* iodopsin. The results are highly suggestive that the same binding sites are involved in these iodopsin analogs.

Photoreaction of the red-shifted pigments

The structural changes in the current set of retinal analogs are the presence of the 3,4 double bond, the addition of the MeO group or the introduction of the 14F substituent. Previous analog studies showed that introduction of the 3,4 double bond (7) or the relatively small F substituent (16) does not interfere with pigment formation. The current result with isomers of MeO-R, **6** (low yield, Table 1), suggests possible steric interference near this site. However, the additional change of unsaturation at the 3,4 bond in MeO-DHR brought the yield back to the respectable ~50%. Furthermore the resultant analog was found to undergo a normal photobleaching sequence (*i.e. via* batho, lumi and M1, M2 intermediates). Apparently, reorientation of the methoxy group on an sp^2-C has diminished the steric interference. Therefore, by design, the current set of analogs involves relatively small structural changes; hence, it is hoped that there is only minimal alteration in protein–chromophore interactions in the resultant pigment analogs.

The photoreaction of the chloride-bound 9-*cis*-MeO-DHR (**3**) iodopsin analog (curve 1, Fig. 3) was studied first in more detail. Irradiation with 630 nm light at 4°C in the presence of hydroxylamine formed a photoproduct (curve 2), absorbing maximally at about 700 nm. Conversion to the batho product was complete after 10 min of irradiation, suggesting that the photosensitivity of the pigment is comparable to that of the original iodopsin. Upon standing at 4°C in the dark, spectral changes were observed, akin to a reversion to the original species (curve 3). This dark reversion process was complete within 20 min. These features are different from those of the parent iodopsin, irradiation of which under the same conditions led to bleaching. An identical stable red-shifted photoproduct can be produced from the parent iodopsin; but it is stable only at lower temperatures (−196°C), and upon warming to −140°C, iodopsin was regenerated (15).

To confirm that the 700 pigment was due to photoisomerization of the polyene chain, we carried out chromophore extraction experiments. Results in Table 2 show that the major new isomer present in the irradiated sample was the all-*trans* isomer (by comparison of HPLC retention time). A minor isomer was also present, which, by analogy with the photochemistry of rhodopsin and other visual pigments (15), has tentatively been assigned to be the missing 11-*cis* isomer.

The plot of the relative absorbance of pigments *versus* time of irradiation at 630 nm (Fig. 4) shows the apparent lack of photobleaching of the two MeO-DHR pigment analogs in spite of the above-mentioned efficient photoreaction of MeO-DHR–iodopsin. The unusual nonbleachable characteristics of the batho product and its efficient reversion to

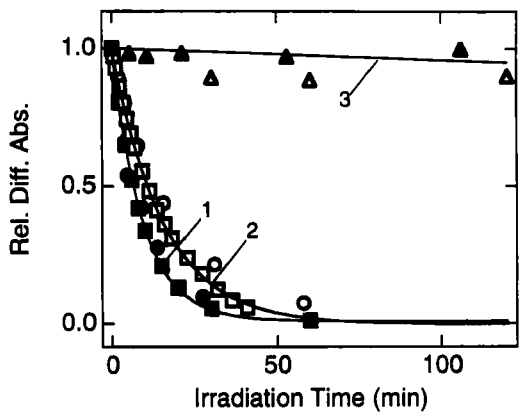


Figure 4. Photobleaching kinetics of the red-shifted pigments. Iodopsin analogues were irradiated with 630 nm light at 4°C. Changes in absorbance at the maxima monitored 20 min after irradiation were plotted against irradiation time. The 9-*cis*- (open square), 11-*cis*- (closed square), 9-*cis*-DHR– (open circle), 11-*cis*-DHR– (closed circle) MeO-DHR– (open triangle) and 14F-MeO-DHR– (closed triangle) iodopsins. Lines indicate exponential fitting curve of 11-*cis*- (curve 1), 9-*cis*- (curve 2) and MeO-DHR– (curve 3) iodopsins, respectively.

the original pigment is likely the principal contributing factor to this apparent anomaly in relative photosensitivity.

The apparent lack of photobleaching also shows insensitivity of the batho product of MeO-DHR–iodopsin toward hydroxylamine, added prior to irradiation of the pigments. It is well known that the meta intermediates of rhodopsin and iodopsin are unstable toward hydroxylamine, reflecting conformational changes of the nearby protein moieties upon photoisomerization of the chromophore that are responsible for the transducin activation. Therefore, it is of interest to examine whether or not the MeO-DHR–iodopsin and 14F-MeO-DHR–iodopsin have the ability to activate transducin. The experimental results clearly showed that MeO-DHR–iodopsin exhibits transducin activity comparable to that of the original iodopsin (also approximately the same as those of 9-*cis* iodopsin, 9-*cis* and 11-*cis* DHR–iodopsin, Table 2) while that of 14F-MeO-DHR–iodopsin is considerably less active (~1/4 of the others).

This observation suggests that the 700 nm batho product of MeO-DHR–iodopsin is capable of activating transducin, whereas for rhodopsin and iodopsin, the deprotonated and thus blue-shifted intermediates instead activate transducin. These facts also suggest that deprotonation of the Schiff base chromophore is not necessary for transducin activation. In this sense, the activation mechanism of MeO-DHR–iodopsin is similar to those of invertebrate visual pigments, although a detailed mechanism for the conformational change of the protein moiety is not clear yet.

There are several possible reasons for the lower activity of the 14-F-MeO-DHR pigment. One possibility is the lower amount of the active intermediate produced by this most red-shifted pigment. We note that while the energy available (720 nm, ~40 kcal/mol) is definitely more than the estimated strain energy of bathorhodopsin (30–35 kcal/mol) (19,20), the energy of the relaxed excited species may not be sufficient to overcome efficiently any added activation energy necessary for the isomerization or the transducin activation process. Unfortunately, due to the very low yield of formation of the red-shifted pigment (5–10%), it is not possible to carry out detailed photochemical experiments to identify the cause for the low transducin activity.

In summary, we have prepared a set of new red-shifted pigment analogs. A relatively stable batho product was produced from such a long-wavelength-absorbing pigment. The reduced transducin activity of the most red-shifted 14F-MeO-DHR–iodopsin could suggest a close approach of its excitation energy to the threshold energy necessary for visual transduction although further experiments are necessary to rule out rigorously other possible explanations such as altered protein–substrate interactions at different stages of the visual transduction process.

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