Institute (Grant CA 22215) is gratefully acknowledged.

Supplementary Material Available: Experimental details and ^1H NMR spectra (39 pages). Ordering information is given on any current masthead page.

Inactivation of Bovine Opsin by *all-trans*-Retinoyl Fluoride

Corinne G. Wong and Robert R. Rando*

Department of Pharmacology, Harvard Medical School Boston, Massachusetts 02115

Received June 1, 1982

The initial step in vertebrate visual transduction involves the absorption of a photon by rhodopsin with the subsequent isomerization of the chromophoric 11-*cis*-retinal Schiff's base to the all-trans isomer.[1] This photochemical isomerizatioon occurs within picoseconds and within seconds is followed by a series of conformational changes of the protein resulting in the eventual hydrolysis of the Schiff's base to *all-trans*-retinal and opsin.[2] The protein conformational changes, which begin the cascade of events leading to the hyperpolarization of the rod outer segment membranes, occur on the μs–ms time scale, and a great deal of attention has been focused on them.[3] An approach to studying the role of conformational changes of rhodopsin in visual transduction is to design specific, irreversible inactivators of opsin that could freeze it in either the activated or unactivated states. Although opsin is not generally perceived as being an enzyme, it nevertheless is the agent of nonphotochemical processes such as Schiff's base formation and hydrolysis (Scheme I). This being the case, it seemed likely that *all-trans*-retinoyl fluoride **1**, a close structural analogue of *all-trans*-retinal, would be a specific inactivator of opsin, because subsequent to the attack of the active-site lysine residue on the carbonyl group, fluoride ion would leave and result in the formation of a peptide bond rather than a Schiff's base (Scheme I). In this communication, we report the synthesis of *all-trans*-retinoyl fluoride and show that it specifically inactivates bovine opsin. To our knowledge, this is the first specific inactivator reported for opsin. In addition we are also introducing the substitution of acyl fluorides for aldehydes as potential active-site-directed irreversible inhibitors.

all-trans-Retinoyl fluoride **1** was prepared from *all-trans*-retinoic acid by using the fluoridating agent prepared from the condensation of hexafluoropropylene with diethylamine.[4] Purification of **1** as a light yellow powder was accomplished just prior to use via preparative silica plate chromatography in 7:3 diethyl ether/hexane as solvent. The structure proof of **1** rested on its quantitative conversions to *all-trans*-methyl retinoate by methanol and to *all-trans*-retinol by lithium aluminum hydride. In addition, the ^1H NMR, ^{13}C NMR, ^{19}F NMR infrared, and ultraviolet spectral properties of the compound were entirely in accord with the assigned structure. The compound, being a highly conjugated acyl fluoride, is not enormously reactive toward water. For example, incubation of **1** for 23 h in 10% water/isopropanol led only to the formation of approximately 38% *all-trans*-retinoic acid.

When digitonin-solubilized bovine opsin was treated with **1**, it was irreversibly inhibited as determined by both the rates and extent of rhodopsin regeneration upon addition of 11-*cis*-retinal. The data on this inactivation process are shown in Figure 1. Control bovine opsin (3.2 μM) treated with 11-*cis*-retinal rapidly

Scheme I. Inactivation of Opsin by 1

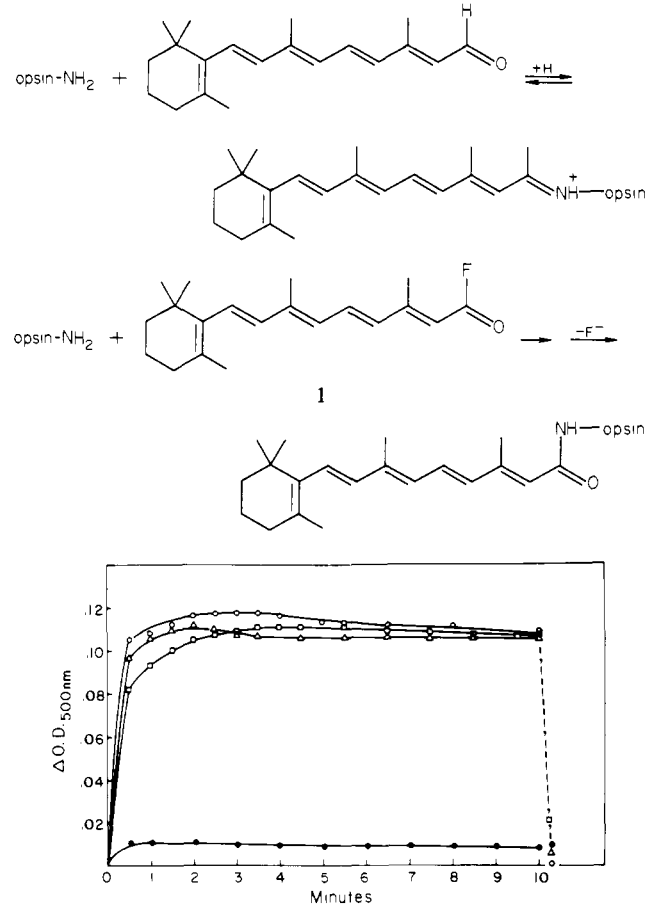


Figure 1. Inactivation of digitonin-solubilized bovine opsin by **1**. The assay for rhodopsin regeneration in 1% digitonin/66 mM KH_2PO_4, pH 6.5, after pretreatment at room temperature in the dark either with *all-trans*-retinoyl fluoride dissolved in isopropanol or with isopropanol as control followed the procedure of Hubbard et al.,[6] except that NH_2OH was added before regeneration with 11-*cis*-retinal.[8b,9] Final concentrations of *all-trans*-retinoyl fluoride, rhodopsin, hydroxylamine, and 11-*cis*-retinal are 105 μM, 3.2 μM, 66 mM, and 39 μM, respectively. (●) Retinoyl fluoride in isopropanol is incubated with bleached opsin in the dark at room temperature for 20 min before addition of NH_2OH and 11-*cis*-retinal. (□) As a control isopropanol is incubated with bleached opsin under the identical conditions as above. (△) As another control retinoyl fluoride in isopropanol is incubated with unbleached rhodopsin in the dark at room temperature for 20 min followed by addition of NH_2OH. Bleaching is followed by addition of 11-*cis*-retinal. (○) As a third control retinoyl fluoride in isopropanol is added to bleached opsin in 1% digitonin/66 mM KH_2PO_4, 66 mM NH_2OH, pH 6.5, and then incubated at room temperature in the dark for 20 min before the addition of 11-*cis*-retinal.

forms rhodopsin as measured by following the optical density increases at 500 nm (□). If the opsin (3.2 μM) is treated first with 105 μM **1** for 20 min, regeneration in the presence of 11-*cis*-retinal (39 μM) does not take place to any significant extent (○). Even after an overnight incubation with 11-*cis*-retinal, no specific regeneration was observed. Two controls are noteworthy in Figure 1. First, incubation of unbleached rhodopsin with 105 μM **1** for 20 min followed by the addition of hydroxylamine and subsequent bleaching resulted in full regeneration upon the introduction of 11-*cis*-retinal (△). Second, when bovine opsin was pretreated with hydroxylamine prior to the addition of **1**, no inactivation occurred presumably because **1** is converted to the hydroxymate under these conditions (○). It should be noted that the inactivation of opsin appears specific for **1**, since another potential acylating agent, *all-trans*-methyl retinoate, at concentrations up to 440 μM had no effect, presumably because it is not active-site directed. Furthermore, we have found that **1** is also not an inactivator of horse liver alcohol dehydrogenase even though

(1) Wald, G. *Science (Washington, D.C.)* **1968**, *162*, 230–239.
(2) Hayward, G.; Carlsen, W.; Siegman, A.; Stryer, L. *Science (Washington, D.C.)* **1981**, *211*, 942–944.
(3) (a) Hubbell, W. L.; Bownds, M. D. *Ann. Rev. Neurosci.* **1979**, *2*, 17–34. (b) Hagins, W. A. *Ann. Rev. Biophys. Bioeng.* **1972**, *1*, 131–158.
(4) Takaoka, A.; Iwakiri, H.; Ishikawa, N. *Bull. Chem. Soc. Jpn.* **1979**, *52*, 3377–3380.

0002-7863/82/1504-7374$01.25/0

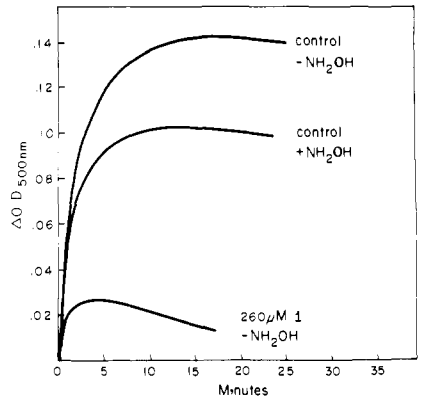


Figure 2. Inactivation of membrane-bound bovine opsin by **1**. Bovine rod outer segments were obtained from frozen retinas (George Hormel Co.) by the published procedure.[8] Control without NH_2OH: To a 500-μL sample containing 100 mM Tris·HCl, pH 7.2, 100 mM $MgCl_2$, 10% sucrose, 4% isopropanol, and bleached rod outer segments incubated in the light for 20 min at room temperature was added 20 μL of egg phosphatidyl choline based liposomes (2 μmol/mL containing 10% 11-*cis*-retinal). Control with NH_2OH: To a 500-mL sample as above containing 40 mM NH_2OH and incubated under the same conditions as above was added 20 μL of the 11-*cis*-retinal bearing liposomes. Inhibitor **1** without NH_2OH: To a 480-μL sample without NH_2OH was added 20 μL of retinoyl fluoride (264 μM) dissolved in isopropanol. The mixture was incubated in the light for 20 min, and then 20 μL of liposomes containing 11-*cis*-retinal was added.

the enzyme utilizes *all-trans*-retinal as a substrate.[5] These experiments taken together are consistent with a specific, active-site mode of inactivation. Inactivation studies with radiolabeled **1** will ascertain the stoichiometry and position of labeling.

Many of the studies of interest using **1** will of necessity take place in the intact rod outer segments. It was therefore of interest to determine whether **1** would inactivate membrane bound opsin. To this end, freshly isolated bovine rod outer segments[6] were bleached and treated with **1**. The results given in Figure 2 show that essentially complete inactivation is achieved in the intact system. Bleached rod outer segments treated with 11-*cis*-retinal show a rapid increase in absorbance as monitored at 500 nm ($-NH_2OH$). Part of this absorbance increase is caused by nonspecific Schiff's base formation between 11-*cis*-retinal and phosphatidyl ethanolamine.[6] This nonspecific absorbance increase is eliminated by including hydroxylamine in the buffer ($+NH_2OH$).[6] Note that treatment of the bleached rod outer segments with **1** in the absence of hydroxylamine leads to about the same absorbance increase as the difference between the two controls. Pretreatment of the rod outer segments with **1** followed by 11-*cis*-retinal and hydroxylamine does not afford a statistically significant absorbance increase at 500 nm. In addition to demonstrating that **1** inactivates opsin in its natural membraneous environment, the data in Figure 2 also suggest that random acylation of amino groups does not occur to a significant extent.

The inactivation of opsin by **1** and isomers of **1** opens up many intriguing questions concerned with the molecular mechanisms of visual transduction. For example, it will be of interest to determine whether the opsins so modified are still photochemically active, whether they will be active in the visual transduction process, and whether this activity is dependent on the stereochemistry of the labeling agent such as 11-*cis*-retinoyl fluoride and 9-*cis*-retinoyl fluoride. On a structural level the active-site sequence of opsin and related molecules can be determined unambiguously.

Acknowledgment. We thank Dr. William Scott of Hoffmann-LaRoche Inc. for the generous gifts of 11-*cis*-retinal. These studies were funded by National Institute of Health Grant EY03624. C.G.W. was funded by National Institute of Health Training Grant 5T32MH1427507.

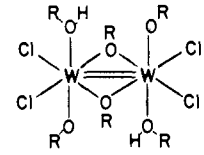
Ditungsten(IV) Alkoxides as Reagents for Carbon–Carbon Bond Formation via the Reductive Coupling of Ketones

F. Albert Cotton,*[1a] David DeMarco,[1b] Larry R. Falvello,[1a] and Richard A. Walton*[1b]

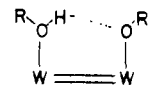
Department of Chemistry, Texas A&M University
College Station, Texas 77843
Department of Chemistry, Purdue University
West Lafayette, Indiana 47907

Received August 13, 1982

The ditungsten(IV) alkoxides $W_2Cl_4(\mu\text{-}OR)_2(OR)_2(ROH)_2$ (**1**)



1



2

possess a tungsten–tungsten double bond characterized by the $\sigma^2\pi^2$ ground-state electronic configuration[2-4] and a strong hydrogen bond (**2**) between syn-axial alcohol and alkoxide ligands.[3,4] These molecules undergo facile oxidation to their singly bonded ditungsten(V) congeners, $W_2Cl_4(\mu\text{-}OR)_2(OR)_4$.[3-5] In contrast to most alkoxide complexes of lower-valent molybdenum and tungsten, **1** is stable in air for prolonged periods. In the course of studying the lability of the alcohol ligands of **1** to various nucleophiles we have found that complexes of type **1** bring about the reductive coupling of ketones and lead to metal-containing products in which the resulting organic molecules bridge the ditungsten center.

Solutions of $W_2Cl_4(\mu\text{-}OEt)_2(OEt)_2(EtOH)_2$ in acetone in the presence of a small quantity of acetic acid turned from green to red over a period of 6 h, and a red crystalline product, **3**, separated.[8] Cyclic voltammograms and electronic absorption spectra of dilute CH_2Cl_2 solutions of **3** were characteristic of ditungsten(V) complexes of the type $W_2Cl_4(\mu\text{-}OR)_2(OR)_4$.[3-5,6,7]

An X-ray crystallographic study[9] showed that **3** has the structure shown in Figure 1. The molecule has a center of inversion, and the central $W_2Cl_4(\mu\text{-}OEt)_2O_4$ portion closely resembles $W_2Cl_4(\mu\text{-}OEt)_2(OEt)_4$,[5] with a W–W distance of 2.701

(1) (a) Texas A&M University; (b) Purdue University.
(2) Cotton, F. A.; Walton, R. A. "Multiple Bonds Between Metal Atoms"; Wiley: New York, 1982.
(3) Anderson, L. B.; Cotton, F. A.; DeMarco, D.; Fang, A.; Ilsley, W. H.; Kolthammer, B. W. S.; Walton, R. A. *J. Am. Chem. Soc.* **1981**, *103*, 5078.
(4) Cotton, F. A.; DeMarco, D.; Falvello, L. R.; Fredrich, M. F.; Walton, R. A. *J. Am. Chem. Soc.*, in press.
(5) Cotton, F. A.; DeMarco, D.; Kolthammer, B. W. S.; Walton, R. A. *Inorg. Chem.* **1981**, *20*, 3048.
(6) Reagan, W. J.; Brubaker, C. H., Jr. *Inorg. Chem.* **1970**, *9*, 827.
(7) In a 0.2 M tetra-*n*-butylammonium hexafluorophosphate CH_2Cl_2 solution **3** exhibits two one-electron reductions, one possessing an $E_{1/2}$ value of −0.75 V vs. SCE and the other characterized by $E_{p,c} = -1.30$ V and $E_{p,a} = -1.10$ V with $i_{p,c} \gg i_{p,a}$. In CH_2Cl_2 electronic absorption band maxima λ_{max} are at 503 (sh) and 446 nm.
(8) In a typical reaction for the preparation of these complexes, $W_2Cl_4(\mu\text{-}OEt)_2(OEt)_2(EtOH)_2$ (0.1 g, 0.128 mmol) was treated with acetone (1.0 mL) and acetic acid (0.02 mL, 0.347 mmol) and the resulting mixture set aside under a N_2(g) atmosphere for 6 h. The resulting red crystalline product was filtered off, washed with acetone and vacuum dried, yield ca. 30%. Anal. Calcd for $C_{16}H_{34}Cl_4O_6W_2$: C, 23.08; H, 4.08. Found: C, 22.76; H, 4.04. When the reaction filtrate was set aside, a blue amorphous solid precipitated after several additional hours. The latter material was insoluble in all solvents, and its IR spectrum was indicative of a tungsten blue species.

(5) Bliss, A. F. *Arch. Biochem. Biophys.* **1951**, *31*, 197-204.
(6) Hubbard, R.; Brown, P. K.; Bownds, D. *Methods Enzymol.* **1971**, *18*, 615-653.
(7) DeGrip, W. J.; Daemen, F. J. M.; Bonting, S. L. *Methods Enzymol.* **1980**, *67*, 301-320.
(8) (a) McCaslin, D. R.; Tanford, C. *Biochemistry* **1981**, *20*, 5212-5221. (b) McCaslin, D. R.; Tanford, C. *Ibid.* **1981**, *20*, 5207-5212.
(9) Hong, K.; Hubbell, W. L. *Biochemistry* **1973**, *12*, 4517-4523.

0002-7863/82/1504-7375$01.25/0