Rod Outer Segment Retinol Dehydrogenase: Substrate Specificity and Role in Phototransduction†

Krzysztof Palczewski,‡,§ Stefan Jäger,⊥ Janina Buczyłko,‡ Rosalie K. Crouch,*,|| D. Lucille Bredberg,‡ Klaus Peter Hofmann,⊥ Mary Ann Asson-Batres,‡ and John C. Saari‡,▽

Departments of Ophthalmology, Pharmacology, and Biochemistry, University of Washington, Seattle, Washington, Institut für Biophysik and Strahlenbiologie, Universität Freiburg, 79104 Freiburg, Federal Republic of Germany, and Departments of Biochemistry and Ophthalmology, Medical University of South Carolina, Charleston, South Carolina 29425-2501

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ABSTRACT: The reaction catalyzed by *all-trans*-retinol dehydrogenase of rod outer segments completes the quenching of photoactivated rhodopsin and initiates the cycle of reactions leading to regeneration of visual pigment. The goal of this study was to determine the kinetic parameters of the dehydrogenase at physiological levels of bleaching, to investigate its specificity, and to determine its possible role in modulating phototransduction. Reduction of *all-trans*-retinal could be measured after bleaching <0.15% rhodopsin. Kinetic parameters for the forward reaction determined with endogenous *all-trans*-retinal were $K_m = 1.1$ μM; $V_{max} = 7$ nmol/min/mg rhodopsin. The low enzymatic activity suggests that at high bleach rates, *all-trans*-retinal could accumulate, increasing the steady state level of bleaching intermediates or promoting formation of pseudophotoproducts. Active pseudophotoproducts, which stimulate G_t activation and opsin phosphorylation by rhodopsin kinase, are formed with opsin and *all-trans*-retinal as well as retinal analogues lacking the 13 methyl or the terminal two carbons of the polyene chain. Addition of *all-trans*-retinol, NADP, and [^{32}P]ATP to rod outer segments increased rhodopsin phosphorylation. Kinetic parameters for the reverse reaction determined with exogenous *all-trans*-retinol were $K_m = 10$ μM; $V_{max} = 11$ nmol/min/mg rhodopsin. Our results support the hypothesis that *all-trans*-retinol dehydrogenase could influence the phototransduction cascade, including activities of G_t, rhodopsin kinase, and binding of arrestin, by impeding the recycling of rhodopsin at high bleach levels.

In vertebrate rod photoreceptor cells, light photoisomerizes rhodopsin's chromophore, 11-*cis*-retinal, to the *all-trans* isomer inducing conformational changes in the protein that result in the formation of a long-lived, activated rhodopsin intermediate, metarhodopsin II (Meta II).[1] Each excited receptor activates hundreds of G_t molecules during its lifetime, initating a cascade of enzymatic reactions that results in suppression of the dark current through cyclic GMP-gated cation channels [reviewed by Hargrave and McDowell (1992) and Lagnado and Baylor (1992)]. This process of activation and amplification is quenched by rhodopsin kinase (RK), which catalyzes the phosphorylation of Meta II (Kühn & Dreyer, 1972; Bownds et al., 1972; Frank et al., 1973; Pulvermüller et al., 1994), and arrestin, which binds to the phosphorylated receptor, blocking continued activation of G_t (Wilden et al., 1986, Palczewski et al., 1992). Hydrolysis of the Schiff base linking *all-trans*-retinal to opsin and reduction of *all-trans*-retinal to *all-trans*-retinol in rod outer segments (ROS) by *all-trans*-retinol dehydrogenase (*t*-RDH) (Zimmerman et al., 1975; Ishiguro et al., 1991) completes the quenching process, since the phosphorylated apo-protein is incapable of binding arrestin, G_t, or RK.

all-trans-Retinol diffuses across the ROS plasma membrane into the interphotoreceptor space where it binds to interphotoreceptor retinoid binding protein. The mechanism of its eventual movement to the retinal pigment epithelium (RPE) remains the subject of inquiry, but could involve aqueous diffusion of retinol between binding sites (Ho et al., 1989). The complete regeneration of rhodopsin requires dephosphorylation of rhodopsin or opsin by protein phosphatase 2A (Palczewski et al., 1989; Fowles et al., 1989), import of 11-*cis*-retinal from the RPE where it is synthesized, and reassociation of opsin with the chromophore [reviewed in Rando (1991) and Saari (1994)].

The important role for *t*-RDH in controlling the levels of *all-trans*-retinal is highlighted by its known biological activities. First, *all-trans*-retinal associates with opsin to form pseudophotoproducts capable of activating the phototransduction cascade (Fukada & Yoshizawa, 1981; Sakmar et al., 1989; Hofmann et al., 1992); second, regulation of

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‡ Department of Ophthalmology, University of Washington.
§ Department of Pharmacology, University of Washington.
⊥ Universität Freiburg.
|| Medical University of South Carolina.
▽ Department of Biochemistry, University of Washington.

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[1] Abbreviations: G_t, transducin; Meta II, metarhodopsin II; ROS, rod outer segments; RPE, retinal pigment epithelium; RK, rhodopsin kinase; *t*-RDH, *all-trans*-retinol dehydrogenase. Common names (underlined) and respective IUP names of retinoid compounds: *all-trans*-9-desmethylretinal, 9-(2′,2′,6′-trimethylcyclohex-1′-enyl)-3-methylnona-2,4,6,8-*trans*-tetraenal; *all-trans*-13-desmethylretinal, 9-(2′,2′,6′-trimethylcyclohex-1′-enyl)-7-methylnona-2,4,6,8-*trans*-tetraenal; *all-trans*-9,13-desmethylretinal, 9-(2′,2′,6′-trimethylcyclohex-1′-enyl)nona-2,4,6,8-*trans*-tetraenal; 11-*cis*-9-desmethylretinal, 9-(2′,2′,6′-trimethylcyclohex-1′-enyl)-3-methylnona-4-cis-2,6,8-*trans*-retinal; *trans*-C-17 aldehyde, 7-(2′,2′,6′-trimethylcyclohex-1′-enyl)-5-methylsepta-2,4,6-trienal; *trans*-C-15 aldehyde, 5-(2′,2′,6′-trimethylcyclohex-1′-enyl)-3′ethylpenta-2,4-dienal.

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t-RDH could influence the rate of regeneration of rhodopsin and lead to bleaching adaptation (Jin et al., 1993); third, *all-trans*-retinal promotes the formation of multiply-phosphorylated forms of photolyzed rhodopsin (Ohguro et al., 1994); and finally, retinal may be involved in the formation of fluorescent components of lipofuscin, an aging pigment that accumulates in RPE (Eldred & Lasky, 1993).

In this study, we generated small amounts of *all-trans*-retinal by bleaching rhodopsin in ROS membrane preparations and measured the kinetics of reduction by *t*-RDH employing physiological ratios of NADP and NADPH (Matschinsky, 1968). We determined that *all-trans*-retinol could be oxidized to *all-trans*-retinal and give rise to pseudophotoproducts capable of activating G_t and of being phosphorylated by RK. In addition, we found that certain retinal analogues could also form pseudophotoproducts, and we discuss the influences of the chromophore structure on the formation of these photoproducts. Our data suggest that at high levels of illumination, the generation of *all-trans*-retinal may exceed its reduction by *t*-RDH *in vivo*. Accumulation of retinal could affect the activation and/or quenching of phototransduction and could contribute to light-dependent retinal degeneration.

MATERIALS AND METHODS

Materials. *all-trans*-Retinal, *all-trans*-retinol, 13- and 9-*cis*-retinal, and NADP were obtained from Sigma Chemical Co. (St. Louis, MO). [15-^{3}H]-*all-trans*-Retinol was obtained from DuPont, New England Nuclear (Boston, MA), and [15-^{3}H]-*all-trans*-retinal was prepared as described (Saari et al., 1993). 11-*cis*-Retinal was a generous gift of the National Eye Institute. The specific activities of [^{3}H]retinoids were adjusted to approximately 40 000 dpm/nmol, and impure retinoids were purified by HPLC (Saari et al., 1982). Retinoid analogues (Figure 1) were synthesized and characterized as described: desmethyl, *trans* C-15, and *trans* C-17 retinoid analogues (von Isler et al., 1956; Kropf et al., 1973); anhydro vitamin A (retro-retinol) (Shantz et al., 1943); retinyl bromoacetate (Gawinowicz & Goodman, 1982); and *all-trans*-retinoyl fluoride (Barua & Olson, 1985).

Synthesis of [^{3}H]NADPH. [^{3}H]NADPH was synthesized by chemical reduction of NADP with [^{3}H]$NaBH_4$ (Chaykin et al., 1966). The initial purification was performed on a column of Nucleosil NB 10 (Macherey-Nagel, Dueren, Germany) (Saari et al., 1993). The collected [^{3}H]NADPH fraction was separated a second time on the same column, this time equilibrated with 10 mM potassium phosphate buffer, pH 7.0 (buffer A), against a changing gradient of 10 mM potassium phosphate buffer, pH 7, containing 1 M NaCl (buffer B): (1) 0−20% buffer B over 5min; (2) 20−40% buffer B during the next 25 min, and (3) 40−50% buffer B for 10 min at a flow rate of 2 mL/min. [^{3}H]NADPH eluted at approximately 22.5 min. The purified, radiolabeled dinucleotide had spectral properties and chromatographic characteristics that were indistinguishable from unlabeled NADPH.

Isolation of Bovine Rod Outer Segments. ROS membranes were isolated from dark-adapted, freshly dissected bovine retinas (100 per preparation) in ice-cold solutions using a discontinuous sucrose gradient (Papermaster, 1982). Preparations referred to as *ROS membranes* were homogenized in 50 mM HEPES, pH 7.8, 60 mM KCl, and 20 mM NaCl, at a final concentration of 100 μM rhodopsin. Preparations washed and reconstituted with 11-*cis*-retinal (*washed ROS membranes*) were homogenized in 35 mL of water containing 20 μg/mL leupeptin at 0−5 °C and centrifuged at 47000*g* for 30 min. The pellet was resuspended in 35 mL of 10 mM Hepes buffer, pH 7.5, and centrifuged a second time (30 min at 47000*g*), and the washed pellet was resuspended in Hepes buffer (see above) at a final concentration of 100 μM rhodopsin. 11-*cis*-Retinal (in ethanol) was added to give a final concentration of 20 μM (final ethanol concentration, 0.2%). The mixture was incubated overnight at 4 °C, and excess retinoids were removed by extraction with 0.3 mL of petroleum ether per mL of ROS membrane suspension. The organic phase was discarded, the washed ROS membranes were collected by centrifugation for 60 min at 49000*g*, and the pellet was washed three times with 10 mM HEPES buffer, pH 7.5, containing 100 mM NaCl. Opsin-enriched membranes (referred to as opsin-enriched ROS membranes) were prepared by homogenizing ROS membranes in 35 mL of water containing 20 μg/mL leupeptin at 0−5 °C. The homogenate was centrifuged at 47000*g* for 30 min, and the pellet was resuspended in 35 mL of 10 mM HEPES buffer, pH 7.5, 500 mM NaCl, 0.5 mM EDTA, and centrifuged a second time (30 min at 47000*g*). The washed pellet was resuspended in 10 mM HEPES buffer, 20 mM NH_2OH (final concentration, 100 μM rhodopsin), placed on ice under a 150-W lamp, and illuminated for 30 min. *all-trans*-Retinal oximes and other retinoids were extracted with petroleum ether, and the opsin-enriched ROS membrane preparation was washed with HEPES buffer as described above. All ROS preparations were stored at −70 °C.

Protein Preparation. Rhodopsin, in the native disk membrane, was prepared by removing the soluble and membrane-associated proteins at low ionic strength (Kühn, 1981). Bleached membranes used for G_t assays were prepared from rhodopsin by thorough bleaching with white light for 30 min on ice in 10 mM BTP buffer, pH 7.5, containing 130 mM NaCl, in the presence of 10 mM NH_2OH to facilitate conversion of retinals to their oximes (Wald & Brown, 1953). NH_2OH was removed by two passes over a G25 Sephadex desalting column. Spectral analysis of the solubilized preparation indicated the presence of small amounts of residual retinyl oxime. G_t was isolated from washed ROS membranes by elution with GTP (Heck & Hofmann, 1993). Traces of opsin contaminating G_t were removed by a concanavalin A-Sepharose column. RK was purified as described (Buczyłko et al., 1991). Rhodopsin and opsin concentrations were calculated from their absorbances at 500 nm (E = 40 000 M^{-1} cm^{-1}), and 280 nm (E = 64 000 M^{-1} cm^{-1}), respectively (Applebury et al., 1974). G_t and RK concentrations were determined by the method of Bradford (1976), using bovine serum albumin as the standard.

Activity of Rhodopsin Kinase. RK activity was measured using urea-washed ROS membranes as the substrate (Palczewski et al., 1988; Buczyłko et al., 1991). The reaction mixture (140 μL) contained 20 μM urea-washed ROS, 5 mM $MgCl_2$, and 100 μM [γ-^{32}P]ATP (100−500 cpm/pmol; DuPont New England Nuclear, Boston, MA) in 20 mM BTP buffer, pH 7.5. Phosphorylation was initiated by illumination and addition of radioactive ATP. The reaction was stopped by mixing the sample with 10% trichloroacetic acid (Palczewski et al., 1988).

Intrinsic G_t Fluorescence. Activation of the G_α subunit induces a conformational change that is reflected by an increase in tryptophan fluorescence (Higashijima et al., 1987). A spectrofluorometer (Spex Fluorolog-2) was used to measure the fluorescence enhancement at 340 nm. The excitation wavelength was 300 nm. Samples containing G_t (400 nM) and rhodopsin or opsin (40 nM) were prepared at 20 °C in 50 mM BTP buffer, pH 6 or pH 8, containing 130 mM NaCl and 5 mM $MgCl_2$. The membranes were sonicated (Branson Sonifier S 125, 80 W, 10 s) to obtain a more uniform size distribution. G_t was activated by adding 20 μM GTPγS. Rhodopsin was photoactivated for 5 s immediately prior to the measurement; within this time, the active form of rhodopsin is Meta II (see, for example, Hofmann, 1993). The relative rate constant k was derived from the function: $A(t) = A_{max}(1 - \exp(-kt))$, where A_{max} is the maximal G_t fluorescence enhancement, $A(t)$ is the amplitude, and k is the relative rate of G_t activation in s^{-1}.

t-RDH Assay by Phase Partition. *t*-RDH activity was assayed by monitoring the production of either [15-^{3}H]-*all-trans*-retinol (reduction) or [^{3}H]NADPH (oxidation) (Saari et al., 1993). The reduction reaction mixture contained 10 mM HEPES, pH 7.5, 14.2 or 20.0 μM [^{3}H]NADPH (specific activity, 83 000 dpm/nmol), 0 or 56.8 μM NADP, and ROS membranes (final concentration, 0.5−1.0 mg/mL rhodopsin), in a final volume of 60 μL per determination. Measurements performed at controlled concentrations of free calcium utilized an EGTA/$CaCl_2$ buffering system (Schoenmakers et al., 1992). Reactions were initiated by the addition of ROS membranes under red illumination and were either incubated in the dark (control) or bleached at different levels (between 0.1% and 30%) with a single flash of light from an electronic flash (Auto 322 Thyrister, SunPak, Newark, NJ) and then incubated at 37 °C in the dark for the time indicated in the text. Alternatively, *all-trans*-retinal in ethanol was added at the concentrations indicated in the text (final concentration of ethanol < 1%). Reactions were quenched with 400 μL of methanol, 100 μL of 10 mM NH_2-OH, pH 7.0, and 100 μL of 0.1 M NaCl and extracted with 500 μL petroleum ether. Radioactivity was measured in 250 μL of the organic phase.

The assay for the reverse reaction (oxidation of retinol) was similar to that described for 11-*cis*-retinol dehydrogenase of RPE (Saari et al., 1993). Reaction mixtures included [15-^{3}H]-*all-trans*-retinol at concentrations indicated in the text, 200 μM NADP, 60 μM bovine serum albumin, and 1 mM DTT in 50 mM Tris buffer, pH 7.5. Reactions were started with addition of 10 μL of ROS membranes per 1-mL volume (final rhodopsin concentration, approximately 40 μg/mL) and were carried out at 37 °C. At specified times (for reaction progress studies) or after 10 min, 200 μL of the reaction was mixed with 400 μL of cold methanol and 50 μL of freshly neutralized NH_2OH (final concentration 50 mM). After 5 min at 37 °C, 150 μL of 1 M NaCl was added followed by 400 μL of CH_2Cl_2. After mixing and centrifuging to separate the phases, the lower phase was removed and the upper phase was extracted two more times with 400 μL of CH_2Cl_2. Radioactivity in 400 μL of the upper phase was determined by liquid scintillation.

t-RDH Assay-HPLC. The activity of *t*-RDH in washed ROS membranes was performed using the substrate and reaction conditions described above (*t*-RDH assay by phase partition). Retinoids were extracted as described (Saari et al., 1982), with the exception that *all-trans*-retinal was first converted to *O*-ethyloxime, which improves its separation from retinols by HPLC (Van Kuijk et al., 1985). Reactions were mixed with 1 volume of cold ethanol and 0.1 volume of *O*-ethylhydroxylamine (final concentration 40 mM). After overnight incubation at 4 °C, a second volume of cold ethanol and five volumes of petroleum ether (bp range, 35−60 °C) were added and mixed with a Pasteur pipette. Two additional volumes of H_2O were added, and the phases were separated by centrifugation. The upper phase was removed, dried in a stream of argon, taken up in acetonitrile, and separated by isocratic HPLC with a 0.45 cm × 25 cm column of C_{18} Vydac TP silica (pore size, 5 μm) (The Sep/a/ra/tions Group, Hesperia, CA), using 81% acetonitrile, 10 mM ammonium acetate, pH 5.5, at 2 mL/min, and monitoring at 350 nm. Specific radioactivities of HPLC-purified fractions of radiolabeled retinoids were determined by measuring the amount of radioactivity in the fraction and relating it to the spectrally determined retinoid concentration. All reactions involving retinoids were carried out under red illumination. Retinoid concentrations were calculated using extinction coefficients reported by Hubbard et al. (1971) and Elving et al. (1973).

The K_m for *all-trans*-retinal was determined by varying the photolyzed rhodopsin concentration from 0.2 to 20 μM at an NADPH concentration of 60 μM. The reaction time was 3.5 min. The K_m for NADPH was determined by varying the NADPH concentration from 2 to 25 μM at an *all-trans*-retinal concentration 40 μM. The reaction time was 10 min. The K_ms were calculated by Lineweaver−Burk analysis as described by Segel (1975). The K_m for *all-trans*-retinol or NADP was determined in a similar manner using exogenous substrates. Initial rates were determined from linear portions of reaction progress curves.

RESULTS

Kinetic Analysis of t-RDH. The sensitivity of a recently described phase partition assay of *t*-RDH activity allowed examination of the kinetic properties of *t*-RDH with low levels of exogenous *all-trans*-retinal or endogenous *all-trans*-retinal generated by bleaching rhodopsin (Saari et al., 1993). This method is based on the use of [^{3}H]NADPH (second substrate, *all-trans*-retinal) or [^{3}H]-*all-trans*-retinol (second substrate, NADP) to provide transfer of tritium during the reaction to retinal or NADP, respectively. The validity of the results obtained with this assay was assessed by comparison with those from conventional HPLC analysis in which retinol and retinal *O*-ethyloximes were separated with a C18 HPLC column and quantified. Figure 2 illustrates the HPLC profiles obtained after extraction of retinoids from the enzymatic oxidation of *all-trans*-retinol. The upper panel (in which ROS membranes and *all-trans*-retinol were incubated for 10 min at 37 °C without NADP) illustrates the endogenous levels of retinals found in ROS membranes preparations. The middle and lower panels illustrate the effect of incubating two concentrations of *all-trans*-retinol with ROS membranes and NADP. It is evident from these analyses that increasing amounts of the reaction product, *all-trans*-retinal, are detected with increasing concentrations of substrate in the reaction.

The K_m for the reduction of exogenous *all-trans*-retinal was 5 ± 2 μM, and the V_{max} was found to be 5 ± 0.6 nmol/

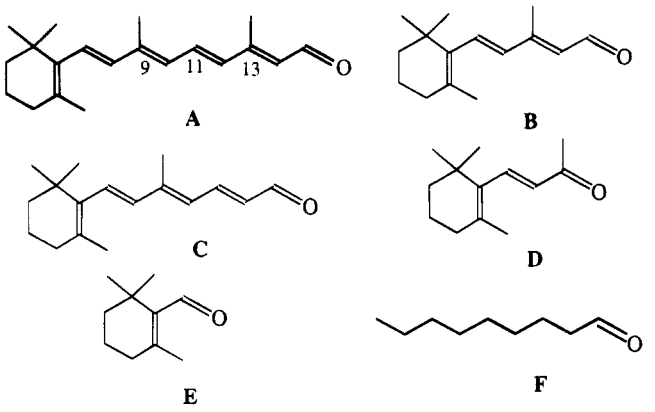

FIGURE 1: Structures of retinal analogues: A, *all-trans*-retinal; B, C-15 aldehyde; C, C-17 aldehyde; D, β-ionone; E, citroellal; F, *n*-nonylaldehyde.

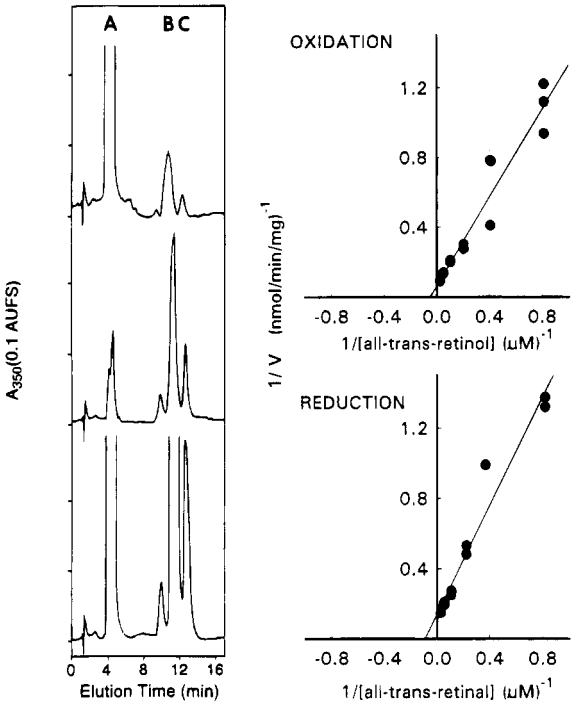

FIGURE 2: (Left) HPLC analysis of the enzymatic oxidation of *all-trans*-retinol. The upper panel illustrates the HPLC trace obtained with retinoids extracted from a control in which 39 μM *all-trans*-retinol was incubated for 10 min at 37 °C with 40 μg/mL of ROS membranes in the absence of NADP. The level of endogenous retinal in the ROS membrane preparation is shown by the syn- and anti-retinal *O*-ethyloximes (peaks B and C, respectively). Peak A is unreacted retinol. The middle and lower panels illustrate retinoids extracted from reaction mixtures of ROS, 200 μM NADP, and 2.5 and 39 μM *all-trans*-retinol, respectively. Increasing amounts of retinal oximes are generated as the substrate concentration is increased. The identity of the component eluting just before peak B is unknown. (Right) double-reciprocal plots displaying the kinetic characteristics of retinol dehydrogenase with exogenous substrates. Washed ROS membranes in pH 7.5 buffer were incubated with either 200 μM NADP plus different concentrations of *all-trans*-retinol (oxidation) or NADPH plus different concentrations of *all-trans*-retinal (reduction) at 37 °C. Retinoid production was monitored by HPLC analysis of aliquots removed at timed intervals. The results of duplicate or triplicate determinations are shown.

min/mg rhodopsin with the phase partition assay (Figure 3 and Table 1). Similar results were obtained using HPLC analysis (K_m = 12 μM and V_{max} = 8 nmol/min/mg rhodopsin; Figure 2). Similar values for these kinetic constants were found for the oxidation of *all-trans*-retinol: K_m = 10.2 ± 1.2 μM (phase partition assay, Table 1), 20 μM (HPLC, Figure 2), and V_{max} = 11.2 ± 2.1 nmol/min/mg rhodopsin (phase partition assay; Figure 3), 15 nmol/min/mg rhodopsin (HPLC). Similar kinetic results were obtained when ROS, rather than washed ROS, were used as a source of enzyme.

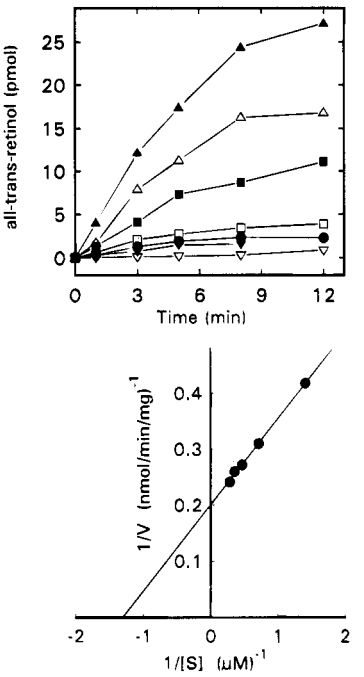

FIGURE 3: Reduction of *all-trans*-retinal generated by bleaching rhodopsin. Upper panel: washed ROS, at a final concentration of 10 μM rhodopsin, were resuspended in 50 mM HEPES buffer, pH 7.5, containing 20 μM [^{3}H]NADPH in a final volume of 30 μL. *all-trans*-retinal was generated by a flash from a photographic flash lamp, and the amount of *all-trans*-retinol produced was quantified by the phase partition assay as described in Materials and Methods. The figure indicates the amount of *all-trans*-retinol formed after bleaching different amounts of rhodopsin: 10% (▲); 5% (△); 2.5% (■); 1.25% (□); 0.62% (●); 0.31% (▼); 0.15% (▽). Lower panel: Double-reciprocal plot of initial reaction rates determined over 3.5 min after bleaching different amounts of rhodopsin. The concentration of bleaced rhodopsin was determined as described in the Materials and Methods.

Table 1: Kinetic Parameters for *t*-RDH[a]

substrates	K_m (μM)	V_{max} (nmol/min/mg rhodopsin)
generated by bleaching rhodopsin		
all-trans-retinal	1.1 ± 0.8	7.1 ± 3.5
added:		
13-*cis*-retinal	10 ± 0.5	5 ± 0.2
all-trans-retinal	5 ± 2	5 ± 0.6
all-trans-retinol	10.2 ± 1.2	11.2 ± 2.1
all-trans-9-desmethylretinal	8 ± 2.8	3 ± 0.3
all-trans-13-desmethylretinal	13 ± 1.4	4 ± 0.2
all-trans-9,13-desmethylretinal	25 ± 1.0	4 ± 0.3
NADP	21.5 ± 2.5	
NADPH	13 ± 1.2	
NAD	0	
NADH	0	

[a] The activity of *t*-RDH was measured in washed ROS membranes by the phase partition assay. K_m and V_{max} were determined from the double-reciprocal plot shown in Figure 2 (Segel, 1975).

The results of experiments in which the activity of *t*-RDH was measured after bleaching washed ROS membranes preparations with different intensities of white light indicated

Table 2: Relative Rates of Reduction of Selected Aldehydes by *t*-RDH[a]

substrates	activity[b] (%)
all-trans-retinal	100 ± 4
13-*cis*-retinal	100 ± 2
all-trans-9-desmethylretinal	51 ± 4
all-trans-13-desmethylretinal	81 ± 7
all-trans-9,13-desmethylretinal	46 ± 5
9-*cis*-retinal	8 ± 2
11-*cis*-retinal	6 ± 4
citronellal	0.8 ± 0.4
β-ionone	1.5 ± 0.5
n-nonylaldehyde	2.5 ± 0.7
trans C-17 aldehyde	3.0 ± 0.5
trans C-15 aldehyde	1.5 ± 0.5

[a] The activity of *t*-RDH was measured by the phase partition assay. Substrate concentrations were 20 μM, and the values are from 3–6 determinations. [b] Activities are expressed as the percent of that obtained with exogenous *all-trans*-retinal as substrate.

adherence to classical Michaelis–Menten kinetics. A double-reciprocal plot of the initial rate of the reaction versus the amount of bleached rhodopsin was linear. The K_m of the enzyme for *all-trans*-retinal generated by bleaching rhodopsin (1.1 μM, Table 1) was lower than the K_m determined when reactions were supplemented with exogenous substrate, whereas the V_{max} of reduction of endogenous substrate was similar to that observed for the reduction of exogenous *all-trans*-retinal (Table 1). In comparison to the reaction velocities of several other enzymes of phototransduction [reviewed by Pugh and Lamb (1993)], the reaction velocity of *t*-RDH is slow.

Several other substances known to be present in ROS membranes were examined for their effect on the dehydrogenase. 11-*cis*-Retinal, which must be supplied to rod photoreceptors to regenerate visual pigment, was without effect on the initial rate or extent of reduction. Taurine, present in outer segments in relatively high concentrations (30–80 mM; Voaden et al., 1977), retarded the rate of reduction by 50% (data not shown). Ornithine, a diamine whose concentration is elevated in gyrate atrophy (Takki & Simell, 1974), and octylamine also reduced the rate of reduction (40%). The mechanism(s) for the observed inhibitions were not investigated in this study but could involve the formation of a Schiff base between the amines and *all-trans*-retinal.

Since the concentration of free calcium changes during phototransduction, we investigated the effect of calcium on *t*-RDH activity. Both oxidation and reduction were insensitive to changes in free calcium concentration in the physiological range (5 nM to 1 μM), when ROS membranes, washed ROS membranes, or opsin-enriched ROS membranes were used.

Substrate and Inhibitor Specificity of t-RDH. The activity of *t*-RDH is dependent on NADP or NADPH, suggesting that the 2′-phosphate of the dinucleotide is critical in recognition and high affinity binding to the catalytic site of the enzyme (K_m = 21.2 ± 2.5 μM and 13 ± 1.2 μM for NADP and NADPH, respectively, Table 1).

13-*cis*-Retinal is a good substrate for *t*-RDH with K_m (10 ± 0.5 μM) and V_{max} (5 ± 0.2 nmol/min/mg rhodopsin) values similar to those of *all-trans*-retinal (Table 1). The 13-desmethyl analogue of *all-trans*-retinal was also a good substrate for *t*-RDH (Table 2) with K_ms in the low micromolar range (Table 2). Removal of the 9-methyl group reduced the activity by half but the resulting product was still active. 9- and 11-*cis*-retinals were not substrates for the enzyme (Table 2). The hydrophobic aldehyde, *n*-nonylaldehyde, which is not constrained by double bonds and lacks the cyclohexyl ring was also not a substrate. Likewise, retinal analogs with short polyene tails (i.e., citronellal, β-ionone, and *trans* C-15 and *trans* C-17 aldehydes) did not serve as substrates for *t*-RDH (Table 2). These results indicate that the binding site of *t*-RDH both recognizes the cyclohexenyl ring and requires the extended polyene chain with bonds in the trans conformation up to the terminal 13 position.

Our conclusions regarding the binding specificity of *t*-RDH for particular retinoids were confirmed by studies carried out with potential inhibitors, including retinoic acid, 13-*cis*-retinoic acid, retro-retinol, *n*-nonylaldehyde, *all-trans*-retinoyl fluoride, and retinyl bromoacetate (data not shown). Only *all-trans*-retinoic acid was a relatively good competitive inhibitor of the enzyme, with K_i = 44 μM, in agreement with previously published results (Nicotra & Livrea, 1982). Although 13-*cis*-retinal is a good substrate for *t*-RDH, 13-*cis*-retinoic acid is only a modest inhibitor of the enzyme (K_i > 80 μM).

Reversibility of the Reaction Catalyzed by t-RDH. Evidence for reversibility of the dehydrogenase reaction in ROS membrane homogenates was obtained by carrying out the reaction under conditions that favored recycling of retinol and retinal. Washed ROS, [C-15-^{3}H]-*all-trans*-retinol, NADP, and NADPH were incubated, and the specific activity of the radiolabeled *all-trans*-retinol was determined at timed intervals over 4 h. It is well established that a specific prochiral proton is removed from an alcoholic substrate in a dehydrogenase reaction (Loewus et al., 1953). If the [^{3}H]NADPH produced from oxidation of [C-15-^{3}H]-*all-trans*-retinol is diluted into a large pool of NADPH, the hydride ion added during reduction will not be radioactive and the number of tritium atoms of the original substrate should decrease from 2 to 1 per mole as a result of continual recycling of product and reactant. We calculated that the actual dilution of [^{3}H]NADPH with unlabeled NADPH in our experiments should lead to a 40% decrease in specific radioactivity. In agreement with this prediction, our results (Figure 4) showed that the specific radioactivity of [^{3}H]-*all-trans*-retinol gradually decreased to approximately 63% of the original value.

Relevance of the Reversibility of t-RDH to Phototransduction. An interesting aspect of *all-trans*-retinal metabolism is its effect on phototransduction processes. As indicated earlier, *t*-RDH in ROS membranes is insensitive to changes in free calcium in the physiological range. However when ATP and GTP are added to ROS membranes (which retain arrestin), the initial rate and maximal velocity of the reduction reaction are inhibited (Figure 5). Since the enzyme is not inhibited by addition of nucleotides when washed ROS membranes are used as the source of *t*-RDH, the results suggest that inhibition is the result of phosphorylation of photolyzed rhodopsin and the binding of the regulatory protein arrestin, rather than direct inhibition of the dehydrogenase. The photolyzed, phosphorylated rhodopsin–arrestin complex is stable for minutes (Hofmann et al., 1992). Hydrolysis of the *all-trans*-retinal–opsin Schiff base may be retarded in this complex.

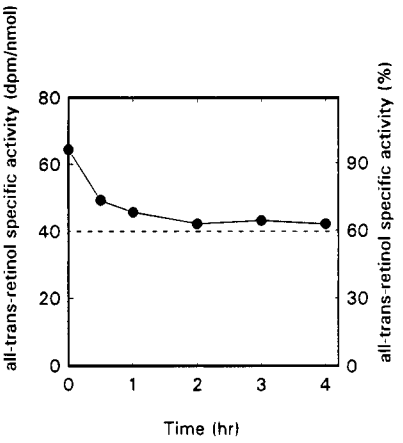

FIGURE 4: Changes in the specific radioactivity of *all-trans*-retinol as a result of enzymatic cycling. [15-^{3}H]-*all-trans*-retinol (27 μM) was incubated with 26 μg/mL washed ROS membranes in pH 7.2 buffer, 100 μM NADPH, and 100 μM NADP at 37 °C. At the times indicated, samples of the reaction mixture were withdrawn and retinoids extracted and analyzed by HPLC, and the specific radioactivity of *all-trans*-retinol was determined as described in Materials and Methods. The results shown are the averages of two determinations. The dotted line indicates the predicted specific radioactivity of retinol in the reaction mix after continued recycling of reactants and products (see text for details).

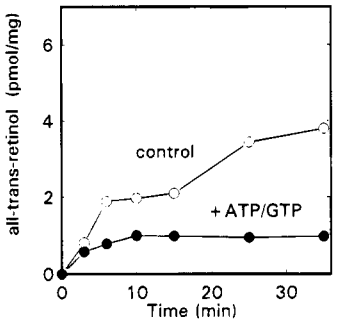

FIGURE 5: Effect of ATP/GTP on the rate of *all-trans*-retinol formation. Whole ROS, at a final concentration of 12 μM, were resuspended in 50 mM HEPES buffer, pH 7.5, containing 10 μM [^{3}H]NADPH in a final volume of 30 μL. *all-trans*-Retinal was generated by a flash from a photographic flash lamp that bleached 0.62% rhodopsin. Final concentrations of ATP and GTP in the reaction mix were 2.9 mM and 0.1 mM, respectively. The free calcium concentration was maintained at 10 nM by using an EGTA/calcium buffer system as described in Materials and Methods.

We also investigated whether *all-trans*-retinal, generated by reversal of the dehydrogenase reaction, affects components of the phototransduction cascade. Bleached membranes containing *t*-RDH were incubated with *all-trans*-retinal and NADP (to substantiate the previous observation that opsin forms a complex with exogenous *all-trans*-retinal, Hofmann et al., 1992) or *all-trans*-retinol and NADP (to promote retinol oxidation). All reactions contained RK and were initiated with the addition of [^{32}P]ATP. As reported, exogenous *all-trans*-retinal forms a complex with opsin that is phosphorylated by RK (Figure 6, curve a). Phosphorylation of opsin is enhanced in reactions supplemented with *all-trans*-retinol and NADP (Figure 6, curve b). The stimulation of phosphorylation is clearly linked to the *t*-RDH reaction since substitution of NADPH for NADP in the oxidation reaction did not stimulate phosphorylation. This conclusion is supported by the observed lack of phosphorylation in reactions carried out in the presence of NH_2OH, which is known to form retinal oximes and prevent formation

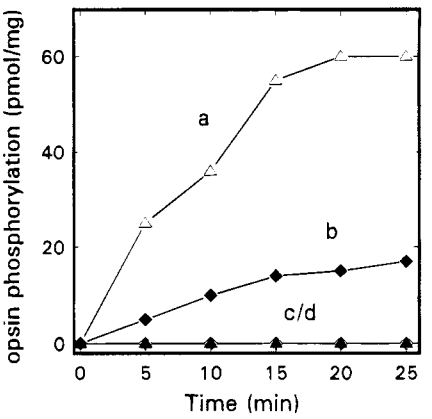

FIGURE 6: Phosphorylation of the opsin−*all-trans*-retinal complex generated by oxidation of *all-trans*-retinol. The phosphorylation reaction was carried out under dim red illumination in the presence of 50 μM *all-trans*-retinol or 50 μM *all-trans*-retinal, 250 μM NADP, 0.2 μM purified RK, 20 μM opsin in 50 mM 1,3-bis[tris(hydroxymethyl)methylamine]propane buffer, pH 6.5. Control experiments contained 20 mM NH_2OH. The reaction was triggered by addition of [γ-^{32}P]ATP at a final concentration of 200 μM. The amount of phosphorylated opsin produced is plotted as a function of time for reactions containing *all-trans*-retinal (a), *all-trans*-retinol (b), *all-trans*-retinol plus NH_2OH (c), or *all-trans*-retinal plus NH_2OH (d).

of the complex between opsin and *all-trans*-retinal (Figure 6, curves c and d).

G_t-Activation by Opsin Complexed with all-trans-Retinal and β-Ionone. The intrinsic fluorescent method employed in our studies measures $G_{t\alpha}$ since it is based on the increase in tryptophan fluorescence of G_t that follows exchange of GDP for GTP [see Hofmann (1993) and citations therein for comparison with other assays and for kinetic evaluations]. The kinetic analysis uses the following equation

$$dG^*/dt = k[R^*][G_0 - G^*]$$

where $[G_0]$ is the total concentration of G_t, and G^* and R^* are activated forms of G_t and rhodopsins, respectively. It is assumed the $[R^*]$ does not change during the experiment. At pH 6, opsin complexed with *all-trans*-retinal activates G_t almost as efficiently as Meta II (Figure 7, Table 3), and photoactivated rhodopsin and opsin complexed with *all-trans*-retinal display similar relative activation rates of $k = 0.051 - 0.055\ s^{-1}$. The maximal level of activation achieved with Meta II is approximately 10% lower than that produced with the opsin/*all-trans*-retinal complex, presumably because the assay is too slow to recognize the G_t fraction that is activated by a preformed complex with Meta II. At pH 8, the relative activation rate of opsin complexed with *all-trans*-retinal is substantially less than that of Meta II ($k = 0.02\ s^{-1}$ vs $k = 0.052$, respectively), which indicates that a pH-dependent product is reversibly formed between opsin and *all-trans*-retinal. Interestingly, we also observed that β-ionone promotes opsin's activity toward G_t implying that formation of the Schiff base is not necessary to induce the active conformation of the protein.

Bleached membranes in the presence of residual retinal oxime have a basal activity of $k = 0.018\ s^{-1}$ and $k = 0.013\ s^{-1}$ at pH 6 and pH 8, respectively (Table 3). This activity is probably due to opsin·*all-trans* retinal complexes that are formed as a result of the reversible disassociation of retinal oximes. Incubation of opsin with 10 mM NH_2OH for 30

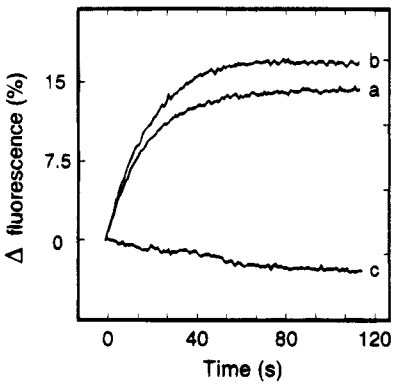

FIGURE 7: G_t activation by photoactivated rhodopsin, opsin—*all-trans*-retinal, and opsin. G_t (400 nM) was resuspended in 50 mM 1,3-bis[tris(hydroxymethyl)methylamine]propane buffer, pH 6, containing either 40 nM of photoactivated rhodopsin (a), opsin (40 nM) with *all-trans*-retinal (400 nM) (b), or 40 nM opsin (c). The reactions were recorded at 20 °C, and measurements were initiated with 20 μM GTPγS. Reaction b was preincubated for 10 min. Reaction c was preincubated with 1 mM NH_2OH for 30 min to produce retinal oxime. The relative rates (K) of G_t activation were 0.051 (a), 0.055 (b), and 0 (c) s^{-1} (see Table 4).

Table 3: G_t Activation by *all-trans*-Retinal and β-Ionone Compared to Bleached Rhodopsin[a]

substrates[b]	rate of activation[c] (k, s^{-1}) pH 6	pH 8
rhodopsin + light[d]	0.051 ± 0.008	0.052 ± 0.008
rhodopsin + light[d] + 1 mM NH_2OH	0.056 ± 0.008	0.046 ± 0.007
bleached membranes	0.018 ± 0.001	0.013 ± 0.001
bleached membranes + 0.1 mM NH_2OH	0.011 ± 0.001	0.006 ± 0.0005
bleached membranes + 1 mM NH_2OH	0[e]	0.001 ± 0.0001
bleached membranes + 10 mM NH_2OH	0[e]	
bleached membranes + *all-trans*-retinal	0.055 ± 0.008	0.020 ± 0.002
bleached membranes + *all-trans*-retinal + 1 mM NH_2OH	0[e]	0.0014 ± 0.0001
bleached membranes + β-ionone	0.031 ± 0.003	0.014 ± 0.001

[a] The activity of G_t was assayed as described in Materials and Methods. [b] Concentrations of rhodopsin and opsin were 40 nM and retinoids 400 nM. Retinoids were allowed to incubate with opsin for 10 min at the pH indicated (T = 20 °C); NH_2OH was incubated with opsin for 30 min prior to measurement (T = 20 °C, pH as indicated). [c] The uncertainty of the rate is mainly due to the mixing artifact in the first 1−2 s of the measurement. For the fast rates, the uncertainty is therefore higher (approximately 15%) than for the slow rates (<8%). [d] Samples were illuminated for 5 s (see Materials and Methods). [e] The rate k = 0.0002−0.0008 is the lower limit of the method.

min causes a shift to the inactive state (both pHs), whereas incubation of opsin with a 10-fold excess of *all-trans*-retinal increases the activation to k = 0.055 s^{-1} (pH 6) and k = 0.02 s^{-1} (pH 8). NH_2OH (up to 1 mM) does not affect G_t activation by Meta II generated by photolyzed rhodopsin; the photoproduct remains stable during the assay, consistent with previously reported spectrophotometric data (Hofmann et al., 1983). The data suggest that NH_2OH competes to remove the *all-trans*-retinal from the binding site.

Although it is difficult to extrapolate these data to *in vivo* conditions, important conclusions can be drawn nonetheless. On the basis of our assay, it is apparent that opsin has very low if any activating ability, whereas opsin·*all-trans*-retinal is effective in G_t activation at low, nonphysiological pH and has reduced activity at higher pH. These data are consistent with a model in which Meta II is significantly different than the opsin—*all-trans*-retinal complex, in agreement with similar observations made by Fukada and Yoshizawa (1981) made using the PDE-assay.

Table 4: Rate of Opsin Phosphorylation by RK in the Presence of Different Retinoids[a]

substrates	activity[b] (nmol/min/mg)
without retinoids	4 ± 1
all-trans-retinal	56 ± 1
all-trans-retinol	10 ± 3
13-*cis*-retinal	45 ± 2
all-trans-9-desmethylretinal	14 ± 1
all-trans-13-desmethylretinal	72 ± 10
all-trans-9,13-desmethylretinal	15 ± 1
11-*cis*-9-desmethylretinal	10 ± 0
trans C-17 aldehyde	55 ± 2
trans C-15 aldehyde	30 ± 1
β-ionone	19 ± 2
all-trans-retinoic acid	6 ± 1
11-*cis*-retinal	0

[a] The activity of RK was assayed as described in Materials and Methods. The concentration of opsin was 20 μM and retinoids 50 μM, and the values are obtained from five to eight determinations. [b] The activity is expressed per mg of RK.

Specificity of a Pseudophotoproduct for RK. The reaction between opsin and *all-trans*-retinal is strongly pH-dependent (data not shown). The rate of opsin phosphorylation in the presence of *all-trans*-retinal is approximately seven times faster at pH 6 than it is at pH 8.3. This pH dependence was observed with all retinoids tested and is consistent with the pH dependence of G_t activation by opsin in the presence of *all-trans*-retinal. *all-trans*-Retinal can be replaced by a series of derivatives and isomers (Table 4). 13-*cis*-Retinal and *all-trans*-13-desmethylretinal are also efficient in the activation of opsin. Interestingly, β-ionone, *trans*-C-15 aldehyde, and *trans*-C-17 aldehyde, which can occupy the binding site of opsin without pigment regeneration (Matsumoto & Yoshizawa, 1975; Crouch et al., 1982; Jin et al., 1993), activate phosphorylation of opsin as well. Conversely, the 11-*cis* isomer and retinoic acid do not generate conformations that are phosphorylated by RK.

These data provide the first indication that pseudophotoproducts exist both with (Hofmann et al., 1992) and without Schiff base formation. The molecular nature of the different products is currently under investigation.

DISCUSSION

Retinol Dehydrogenase—An Enzyme of Two Pathways. *t*-RDH is at the intersection of two pathways initiated by bleaching rhodopsin. Reduction of *all-trans*-retinal by *t*-RDH is the final step in quenching activated photoproducts (Hofmann et al., 1992) and the first step in the enzymatic pathway leading to regeneration of the 11-*cis* configuration of the retinal required for visual pigment regeneration (Rando, 1991; Saari, 1994). Published accounts of the activity of this enzyme *in vitro* indicate a surprisingly slow activity by comparison with the enzymes of the phototransduction cascade and the rate of *all-trans*-retinal reduction under ambient light (Kaplan, 1984). We were intrigued by this apparent disparity which would appear to allow *all-trans*-retinal levels and, consequently, phosphorylated opsin·*all-trans*-retinal·arrestin complex(s) to build up during illumination within the physiological range. We were also intrigued by the apparent similarity of the rate of retinal formation *in vitro* and the rate of rod visual pigment regeneration in psychophysical experiments (Rushton, 1981; Alpern et al., 1970).

Kinetic and Thermodynamic Parameters of the Reaction Catalyzed by t-RDH. This important enzyme has been the subject of numerous investigations; however, there is little agreement among previous reported kinetic constants. The reaction has been carried out with exogenous substrate or substrate generated by large fractional bleaches of rhodopsin under various conditions and pHs outside the physiological range. Reported K_m values range from 4 to 85 μM for *all-trans*-retinal and from 9 μM to 6.6 mM for *all-trans*-retinol (Blaner & Churchich, 1980; Ishiguro et al., 1991). Much of this variability may be attributed to the use of insensitive assays such as the thiobarbituric acid method (Ishiguro et al., 1991) and the insolubility and chemical reactivity of *all-trans*-retinal. The latter property leads to nonspecific association of retinal with amino groups to form a variety of complexes.

Recent development of a phase partition assay with enhanced sensitivity (Saari et al., 1993) prompted us to reexamine the enzyme's kinetic parameters with endogenous substrate generated by bleaching small amounts of visual pigments, its substrate specificity, and the reversibility of the reaction. In addition, the ability of retinal analogues to generate activated photoproducts was measured. Generation of substrate with small fractional bleaches has the advantages that the molar ratio of *all-trans*-retinal to opsin never exceeded 1 and *all-trans*-retinal was generated within the disk membrane, where presumably it is in proximity to the membrane-associated dehydrogenase. *t*-RDH activity could be detected with the partition assay after bleaching as little as 0.15% of the visual pigment. The sensitivity of this assay depends on the specific radioactivity of [^{3}H]NADPH, and no attempt was made in this study to determine the minimum detectable bleach.

The rate of reduction is relatively slow, requiring minutes for completion, whereas phototransduction is completed within seconds, including the rapid activation and slower quenching reactions (Pugh & Lamb, 1993; Pepperberg et al., 1988). Phosphorylation of opsin however, is relatively slow (Biernbaum et al., 1991). Perhaps the slow reduction allows more complete phosphorylation of photolyzed opsin by rhodopsin kinase and prevents the accumulation of potentially activatable opsin.

The equilibrium constant K = [retinal][NADPH][[H$^+$]/[retinol][NADP] was found to be 3.3×10^{-9} M in detergent solution (Bliss, 1951; Blaner & Churchich, 1980), approximately 330 times larger than the value determined for reduction of acetaldehyde (Racker, 1950; Bliss, 1951). At pH 7.5, the equilibrium constant for the dehydrogenation of retinal (K' = [retinal][NADPH]/[retinol][NADP]) is approximately 0.1 (Bliss, 1951), and the reduction of retinal is freely reversible. However, it is important to note that this value may differ with retinoids solubilized in ROS membranes. The Haldane relationship (Segel, 1975) between kinetic parameters for an ordered bisubstrate reaction in both directions (Nicotra & Livrea, 1982) is

$$[(1/K' = [\text{products}]_{eq}/[\text{substrates}]_{eq} = (V_{max(forward)}/V_{max(reverse)})^2/K_{i(retinol)}K_{m(NADP)}/K_{i(retinal)}K_{m(NADPH)}]$$

Assuming $K_{i(retinol)} = K_{m(retinol)}$ and $K_{i(retinal)} = K_{m(retinal)}$, we found K' to be 0.18 to 0.28 when retinal was generated by weak bleaches or 1.0 to 1.9 when retinal was added. The agreement is reasonable considering that bleaching generates *all-trans*-retinal within the disk membranes, in proximity to *t*-RDH, whereas much of the exogenous retinal may be lost in complexes with amino groups or adsorbed to surfaces.

Specificity of t-RDH. *t*-RDH exhibits relatively strict retinoid substrate specificity. *all-trans*-Retinal and 13-*cis*-retinal are good substrates whereas the 9-*cis* or 11-*cis* isomers are poor substitutes. The carbonyl oxygen of *all-trans*-retinal is only displaced by 3−5 Å in 13-*cis*-retinal (estimated from space-filling models), suggesting that these two isomers are sterically similar. Since 11-*cis*-retinal is continuously supplied to the outer segment for visual pigment regeneration, the exclusion of this isomer as a substrate would appear to be a necessity. The 9- and 13-methyl groups contribute to substrate recognition but are not required for binding to the enzyme. The apparent specificity pattern could be explained by postulating a substrate binding pocket that includes the β-ionone ring located at specific distance from the aldehyde group, the *all-trans*-side chain up to C-12, with less steric constraints for C-13, -14, and -15. This hypothesis is strengthened by the observation that neither β-ionone, citronellal, nor the shorter analogs, *trans* C-15 and *trans* C-17 aldehydes, are substrates or inhibitors for the enzyme.

Interestingly, the second substrate, NADPH (or NADP), exhibits absolute specificity, suggesting that 2′-phosphorylation of the dinucleotide is necessary for high affinity binding. As noted previously, this specificity couples NADPH production by the pentose phosphate pathway to its utilization in the outer segment (Futterman et al., 1970). Recent studies have demonstrated the presence of enzymes of the pentose phosphate pathway in ROS membranes (Hsu, 1993), which are presumably involved in the production and maintenance of an NADPH/NADP ratio of 1:4 (Matschinsky, 1968).

all-trans-Retinal·Opsin Photoproduct. Removal of *all-trans*-retinal from ROS membranes is critical for the regeneration of photolyzed rhodopsin. As we have shown in this study, *all-trans*-retinal formed an active complex with opsin that is capable of G_t activation. The nature of this complex is under investigation, but interestingly the activation of opsin could be also detected in the presence of β-ionone, suggesting that the transition from quiescent to active receptor capable of catalyzing the exchange of nucleotides on G_t results not only from interruption of the salt bridge between Lys 229 and Glu 113 (Robinson et al., 1992), but also from conformational changes resulting from interactions controlled by the cyclohexyl ring during photoisomerization of 11-*cis*-retinal to *all-trans*-retinal.

A similar conclusion can be drawn from studies of the stimulation of opsin phosphorylation by different analogs of *all-trans*-retinal [present studies, but also Hofmann et al. (1992)]. Interestingly, the 9-methyl group of the retinal appears to have a major effect on the level of phosphorylation, which may be an explanation for previous finding of Corson et al. (1994) that the quantal response of 9-desmethylrhodopsin to a light flash decays 5−7 times more slowly than the native pigment. Our data indicate that if *all-trans*-retinal were to accumulate in ROS, it very likely could form an active complex with opsin that would be capable of G_t stimulation. Such a complex is also a substrate for RK, and phosphorylated forms of this complex will bind arrestin (Hofmann et al., 1992).

t-RDH in Phototransduction. Bleaching rhodopsin produces an activated photopigment, Meta II, that consists of *all-trans*-retinal covalently linked to opsin as a deprotonated Schiff base. Meta II interacts with G_t, leading to propagation of the signal initiated by the absorption of a photon. Since the half-life of Meta II is relatively long, continued activation of transducin is prevented by phosphorylation, binding of arrestin, eventual hydrolysis of the Schiff base, and reduction of free retinal by *t*-RDH.

Using the kinetic parameters determined in this study, one can now construct a reasonable scenario for *t*-RDH function at various illumination intensities. At bleach rates that do not saturate rods, *t*-RDH is unlikely to play an important role since the concentration of *all-trans*-retinal would be well below the K_m of the enzyme and the total enzymatic activity is low. As the bleach rate rises above rod saturation, the increase in *all-trans*-retinal concentration would eventually allow its reduction to *all-trans*-retinol by *t*-RDH, thereby freeing opsin for complex formation with 11-*cis*-retinal and regeneration of the visual pigment. Increasing the intensity of illumination further could produce substrate levels that exceed the rate of reduction of *all-trans*-retinal by *t*-RDH, resulting in its accumulation, as noted by Zimmerman (1974) during bleaching of rat visual pigments. The complex(s) of phosphorylated opsin•*all-trans*-retinal•arrestin could withhold opsin from the regeneration process, reducing the quantum catch, and could relay a desensitizing signal to the phototransduction cascade. The sensitivity of the visual system is known to respond to bleaching with a decrease in sensitivity that is greater than predicted by the reduced quantum catch, a phenomenon known as bleaching adaptation. Perhaps the function of desensitization is to decrease energy-consuming processes that occur at levels of illumination intensity above rod saturation.

The results elucidate the role of *t*-RDH in rod photoreceptor physiology and suggest that impairment of its activity could have an impact on the quenching of activated photoproducts that could result in retinal degeneration (Law & Rando, 1989; Eldred & Lasky, 1993).

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