The *Drosophila ninaG* Oxidoreductase Acts in Visual Pigment Chromophore Production*

Received for publication, October 28, 2004, and in revised form, January 3, 2005
Published, JBC Papers in Press, January 7, 2005, DOI 10.1074/jbc.M412236200

Shanta Sarfare‡‡, Syed Tariq Ahmad‡‡, Michelle V. Joyce†, Bill Boggess‡‡, and Joseph E. O’Tousa‡‡‡
From the ‡‡Departments of Biological Sciences and †Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556

The *Drosophila ninaG* mutant is characterized by low levels of Rh1 rhodopsin, because of the inability to transport this rhodopsin from the endoplasmic reticulum to the rhabdomere. *ninaG* mutants do not affect the biogenesis of the minor opsins Rh4 and Rh6. A genetic analysis placed the *ninaG* gene within the 86E4–86E6 chromosomal region. A sequence analysis of the 15 open reading frames within this region from the *ninaGP330* mutant allele identified a stop codon in the *CG6728* gene. Germ-line transformation of the CG6728 genomic region rescued the *ninaG* mutant phenotypes, confirming that *CG6728* corresponds to the *ninaG* gene. The *NinaG* protein belongs to the glucose-methanol-choline oxidoreductase family of flavin adenine dinucleotide-binding enzymes catalyzing hydroxylation and oxidation of a variety of small organic molecules. High performance liquid chromatography analysis of retinoids was used to gain insight into the in vivo role of the *NinaG* oxidoreductase. The results show that when Rh1 is expressed as the major rhodopsin, *ninaG* flies fail to accumulate 3-hydroxyretinal. Further, in transgenic flies expressing Rh4 as the major rhodopsin, 3-hydroxyretinal is the major retinoid in *ninaG*+, but a different retinoid profile is observed in *ninaGP330*. These results indicate that the *ninaG* oxidoreductase acts in the biochemical pathway responsible for conversion of retinal to the rhodopsin chromophore, 3-hydroxyretinal.

Vitamin A provides the retinal chromophores that mediate the sensing of light quanta by visual pigments. Vitamin A is a fat-soluble vitamin that cannot be synthesized *de novo* by most animals. Rather vitamin A is obtained from the diet, either as preformed vitamin A such as retinol from animal sources, or as provitamin A carotenoids from plant sources. These dietary forms must be both transported to the retina and chemically modified to play a key role in visual transduction (1, 2).

*Drosophila nina* mutations have provided some insights into processing of vitamin A for use in vision. Eight *Drosophila nina* genes are known (3). These *nina* mutants, named for the “neither inactivation nor afterpotential” electrotetrogramin defect, all possess low rhodopsin levels responsible for this phenotype. Vitamin A-deprived flies also show the same electroretinogram defects and low rhodopsin levels (4, 5), establishing the importance of vitamin A availability in the production of rhodopsin. The *ninaB* and *ninaD* mutants are also known to lower rhodopsin levels by disrupting vitamin A availability. The *ninaD* gene encodes a scavenger receptor (6) required for efficient use of carotenoids, such as β-carotene, from the diet. The *ninaB* encodes an oxygenase enzyme (7) responsible for cleaving β-carotene to generate retinal. Both of these gene products act outside of the retina to carry out their roles in vitamin A metabolism (8).

Five different retinoids are known to serve as visual pigment chromophores within the animal kingdom. These are retinal, (3,4-didehydroretinal, (3R)-3-hydroxyretinal, (3S)-3-hydroxyretinal, and (3R)-4-hydroxyretinal. Although the 3R enantiomer of 3-hydroxyretinal is found among many insect orders, the 3S enantiomer is found only among the Cyclorrhapha suborder of Diptera (9) that includes *Drosophila* (10). The biochemical pathways responsible for production of this retinoid and the other modified chromophores from retinal or other vitamin A precursors have not been characterized.

Here we describe the molecular characterization and phenotypic analysis of *ninaG*. The *ninaG* gene encodes a member of the glucose-methanol-choline (GMC) family of oxidoreductases (11). The biochemical substrates of most of these enzymes are not known, but some of the enzymes are dehydrogenases and oxidases carrying out redox reactions on a wide variety of organic metabolites. We show that NinaG is required for the biogenesis of the *Drosophila* rhodopsin chromophore, (3S)-3-hydroxyretinal. This study provides the first molecular description of an enzyme active in modification of retinal to an alternative visual pigment chromophore.

MATERIALS AND METHODS

*ninaG* Mutant Analysis—The ethylmethane sulfonate-induced *ninaGP330* strain was obtained from W. L. Pak, Purdue University (3). *ninaGP330* was identified following an ethylmethane sulfonate screen done in the O’Tousa laboratory and *ninaG* was identified within an existing laboratory stock. Deficiency chromosomes were obtained from the *Drosophila* Stock Center, Bloomington, Indiana and W. Engels, University of Wisconsin. *Drosophila* strains were maintained at room temperature and usually reared on standard cornmeal-molasses medium. Pseudopup and protein blots assessment of rhodopsin content was carried out as described in Hsu et al. (12).

Electroretinogram and electron microscopic analyses were performed as described previously (13), in both cases using 2-day-old flies maintained in 12-h light/12-h dark light cycle. Rhodopsin cellular location was visualized using transgenic flies carrying *pRh1:Rh1-GFP*, constructed using the Rh1-GFP coding sequence described by Pichaud and

---

* This work was supported by Grant EY06808 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ Current address: Dept. of Vision Sciences, University of Alabama at Birmingham, Birmingham, AL 35294-4390.

† To whom correspondence should be addressed. Tel.: 574-631-6093; Fax: 574-631-7413; E-mail: jotousa@nd.edu.

The abbreviations used are: GMC, glucose-methanol-choline; PDA, prolonged depolarizing afterpotential; HPLC, high performance liquid chromatography; MS, mass spectroscopy; GFP, green fluorescent protein.
Desplan (14) and the Rh1 promoter region. Ommatidia of newly closed Drosophila carrying this element in appropriate genetic backgrounds were prepared by modification of described protocols (15, 16). The dispersed ommatidia were immersed in a drop of chilled phosphate-buffered saline, pH 7.4, on a glass slide. To-Pro-3 iodide (Molecular Probes) was added to visualize nuclei, anti-fade reagent (Molecular Probes) was added in equal volume, and the GFP fluorescence was then imaged with Bio-Rad MRC 1024 Scanning Confocal microscope. Vitamin A-free medium was prepared as described by Nichols and Pak (17).

To generate flies deficient in vitamin A, a life cycle (from egg to adult) was completed on this medium. Replenishment with all-trans-retinal (Sigma) was added by placing adult flies on a supplemented medium for 3 days prepared by adding 10 μl of a 10 mM solution/ml of vitamin A-free medium.

For Rh1 RNA analysis, adult flies were flash-frozen in liquid nitrogen and vortexed to separate the heads from the bodies. Total RNA was isolated from different fly strains using the RNeasy mini kit (Qiagen). 15 μg of total RNA was subjected to 1.2% formaldehyde-agarose gel electrophoresis. 18 and 28 S ribosomal RNA standards were visualized on the same gel with ethidium bromide staining to evaluate RNA integrity and as loading controls. The gel was blotted onto Hybond-XL membrane (Amersham Biosciences) using the manufacturer’s instructions, probed with Rh1 cDNA, random-labeled with [32P]dCTP using the Megaprime DNA labeling system (Amersham Biosciences) and autoradiographed to visualize Rh1 RNA levels. FRT/FLP (flippase recombinase/flippase recombinase target) genetic mosaics were constructed and analyzed as described previously (8).

**ninaG Molecular Analysis**—For sequence analysis of the 15 genes in the ninaG region, 1 μl of genomic DNA, prepared using the single fly DNA prep protocol (18), was used in PCR with Platinum Taq DNA High Fidelity proofreading polymerase (Invitrogen) and unique oligonucleotide primers designed to amplify a 1-kb segment from the region. The PCR products were cloned into pCR4 TOPO cloning vector (Invitrogen). Plasmids containing the correct inserts were bidirectionally sequenced using T3 and T7 sequencing primers and Big Dye Terminator v.3 on autoclaved ABI Prism® 3700 automated sequencer (Applied Biosystems). For transgenic rescue of ninaG, a 3.8-kb KpnI-StuI fragment from BAC013PO8 containing 1.1 kb of the ninaG (CG8728) promoter region, the entire open reading frame, and 0.7 kb of the 3′-untranslated region was cloned into the P element vector pCaSpeR4 (19) and transformed into the ninaG<sup>P330</sup> mutant using standard procedures (20).

**Retinal Extraction and HPLC⁄MS Analysis**—Methodology for retinal extraction was developed from the published protocol of Suzuki et al. (21). 700–800 Drosophila were flash frozen in liquid nitrogen, and the heads were separated from bodies by vigorous shaking, homogenized in 100 μl of 6 M formaldehyde, 0.1 M phosphate buffer (pH 7.5), and incubated at 30 °C for 2 min. 0.5 ml of dichloromethane was then added, the homogenate was vortexed, and further incubated at 30 °C for 10 min. 1 ml of n-hexane was added, mixed, and spun at 3,000 rpm for 5 min. The upper organic layer was collected, the aqueous phase was reincubated at 30 °C for 2 min. 0.5 ml of dichloromethane was then added, and 1 ml of the initial HPLC mobile phase, and then filtered through a 0.22 μm PTFE filter (Nalgene) prior to HPLC analysis. A Waters 2690 HPLC system equipped with a 996 photodiode array detector and interfaced with a Micromass Quatro LC triple quadrupole mass spectrometer (Waters) was used for liquid chromatography/UV-visible/MS experiments. Absorbance spectra were monitored from 200 to 800 nm, and mass spectra were acquired over the mass range 150–800 a at a scan rate of 2 s. A 10-μl sample was injected onto a 4.6 × 20-mm, 3-μm particle size Atlantis dC18 guard column and a 4.6 × 150-mm, 3-μm particle size Atlantis dC18 analytical column (Waters). The mobile phase gradient consisted of 80:20 (v/v) acetonitrile/water for 3 min, a 12-min linear gradient to 100% acetonitrile, and 5 min of 100% acetonitrile delivered at a flow rate of 1 ml/min. The HPLC eluent was split 50:50 prior to injection into the electrospray ion source of the mass spectrometer, and formic acid (0.1%) was added to all mobile phase solvents as a means of increasing ionization efficiency for MS.

**RESULTS**

The ninaG Mutant Phenotype—A collection of vision-defective mutants called nina, for “neither inactivation nor afterpotential” were identified on the basis of their electroretinogram phenotype (22). In wild type, a bright blue stimulus induces a depolarized state that persists after termination of the stimulus (Fig. 1A, top trace). This is referred to as the prolonged depolarizing afterpotential (PDA). The lack of the PDA response in nina mutants, such as shown for ninaG<sup>P330</sup> in Fig. 1A, bottom trace, is because of low levels of Rh1 rhodopsin within the R1-6 photoreceptors (3, 23). The ninaG mutant possesses very low levels of Rh1 rhodopsin when assayed in protein blots (Fig. 1B, upper panel). The requirement for ninaG in production of other rhodopsins was tested by protein blots against the R7 opsin Rh4 and the R8 opsin Rh6. Fig. 1B, middle panels, show that Rh4 and Rh6 opsin production does not require ninaG activity. Similar results were seen for two other ninaG alleles. Thus, ninaG function is only required for effi-
cient biogenesis of a subset of *Drosophila* visual pigments. To investigate whether maturation of other transmembrane proteins is affected in *ninaG* mutants, Trp and RdgB protein levels were examined. Trp is a calcium channel that co-localizes with rhodopsin in the rhabdomeres (24). RdgB is a membrane protein that is localized to the photoreceptor subrhabdomeric cisternae (25). Neither Trp nor RdgB protein levels are affected in *ninaG* mutants (Fig. 1B, lower panels).

Northern analysis was used to determine whether *ninaG* was required for expression of Rh1 mRNA. Similar levels of *ninaE* transcript of 1.5 kb representing the Rh1 opsin mRNA was detectable by Northern analysis in wild type, *ninaG*P330, *ninaG*1, and *ninaG*2 heads (Fig. 1C). Heads from the eye (eyes absent) mutant, lacking the compound eyes, were used as the negative control. These results show that *ninaG* mutants do not affect Rh1 mRNA levels, establishing that *ninaG* acts in translational or posttranslational control of rhodopsin production.

**Defective Rh1 Transport in *ninaG* Mutants**—To investigate the effect of the *ninaG* mutation on the localization of Rh1 rhodopsin in the photoreceptor cells, we created flies containing rhodopsin-GFP (Rh1-GFP) transgene driven by the *Drosophila* Rh1 promoter. The location of Rh1-GFP was visualized in whole mounts of isolated ommatidia, the unit cluster of the R1-8 photoreceptor cells. In the *ninaG*G1 background, the Rh1-GFP localizes predominantly to the rhabdomeres of R1-6 cells (Fig. 2A, labeled *R*). In *ninaG* mutants, Rh1-GFP localized exclusively to the cytoplasmic compartments (Fig. 2B, labeled *C*), failing to decorate the rhabdomeres (*R*). The redistribution of Rh1-GFP seen in *ninaG* mutants is similar to that observed in vitamin A-deprived flies (data not shown). At the ultrastructural level, *ninaG*P330 (Fig. 2D) shows a reduction in the size of the R1-6 rhabdomeres (Fig. 2, *C* and *D*), one rhabdomere is labeled *R* when compared with wild type (Fig. 2C). We also observed proliferation of the endoplasmic reticulum in the cell bodies (Fig. 2D, ER), and similar results were observed in *ninaG*2 (data not shown). The *ninaG*P330 mutant shows these defects at all ages examined, with no other significant changes, including retinal degeneration, observed up to 20 days of age (data not shown).

**The Retina Is the Site of *ninaG* Activity**—To test whether NinaG activity is required within photoreceptors, we created genetic mosaics in which only the eye tissue was mutant for *ninaG*. Fig. 3 shows the rhodopsin content in *ninaG* mosaics and an appropriate set of control genotypes. In the *ninaG* mosaic (Fig. 3, far right), the retinal tissue is mutant for *ninaG*, but the rest of the animal is *ninaG*G1. These mosaic flies show low levels of rhodopsin, showing that NinaG activity is required within photoreceptors or other cell types of the retina for normal rhodopsin expression.

**Identification of the *ninaG* Gene**—We determined the cytological position of *ninaG* as a first step toward identifying and characterizing the *ninaG* gene. Flies heterozygous for *ninaG* and each of a set of deficiencies spanning much of the right arm of the third chromosome were examined for rhodopsin content using the presence/absence of the deep dark pseudopupil (26). The presence of this structure in white-eyed flies is indicative of low rhodopsin levels. Df(3R)M-Kx1*ninaG* and Df(3R)/T.322/ninaG and Df(3R)/T.61/ninaG flies showed no dark deep pseudopupil suggestive of low rhodopsin levels. Reduced rhodopsin levels in these three genotypes were confirmed by Western analysis (data not shown). This analysis placed the map location of *ninaG* within region 86E3–87A9 on the right arm of the third chromosome. To further refine this map position, P-element-induced deletions within the 86E region (27) were tested for complementation with *ninaG* mutants. Df(3R)theoR1, Df(3R)/pros235, and Df(3R)/pros640, but not Df(3R)/77, failed to complement the low rhodopsin phenotype of *ninaG* (Fig. 1B,
**ninaG Oxidoreductase Acts in Retinal Metabolism**

**ninaG activity is required within retinal tissue.** Genetic mosaics were constructed in which the eye tissue was mutant for ninaG<sup>P330</sup> but the rest of the animal is ninaG<sup>+</sup>. The far right lane of this protein blot shows that these mosaics have low levels of Rh1. The other lanes serve as controls, with the wild type and Rh1 null mutant (ninaE<sup>Y17</sup>) lanes establishing antibody specificity, and ninaG<sup>P330</sup> homozygotes and FRT-ninaG<sup>P330</sup>/ninaG<sup>P330</sup> show that ninaG<sup>P330</sup> is present on the FRT chromosome.

These results placed ninaG into a small region bracketed proximally by the pros gene and distally by the tho gene. P-element alleles of pros (P[PZ]pros<sup>10419</sup>) and tho (P[PZ] tho<sup>+</sup>) complemented ninaG. Therefore, ninaG is likely to lie in the region between pros and tho. This is a genomic region of 79,639 bp containing 15 annotated genes.

To identify the gene responsible for the ninaG mutant, we sequenced the coding regions of the 15 identified genes within the 86E5–86E8-E11 cytological region from ninaG<sup>P330</sup>. 36 different PCRs generated products of ~1-kb length to examine all genes. The coding sequences within these PCR products were compared with the Drosophila genome sequence (28, 29). The gene designated CG6728 showed a notable alteration in the ninaG<sup>P330</sup> mutant allele, a C to T change at nucleotide 241. This resulted in a nonsense codon, altering a CAG (Gln) codon to a TAG (STOP) codon. No other mutations resulting in nonsense mutations were identified, although three other genes contained single nucleotide changes resulting in amino acid substitutions.

As a result of the sequence analysis, CG6728 emerged as the best candidate for the ninaG gene. Fig. 4A shows a diagram of the gene structure, identifying the site of the mutation found in ninaG<sup>P330</sup>. We used a 3.8-kb genomic KpnI-Stul fragment that contained this open reading frame and 1.1 kb of upstream sequences to construct transgenic flies. Fig. 4B shows that this CG6728 genomic fragment rescues the low rhodopsin phenotype of ninaG<sup>P330</sup> mutants. Further, electrophoretic analysis showed that the CG6728 transgene restores the PDA response in ninaG mutants (Fig. 4C). These results establish that CG6728 is the ninaG gene.

**NinaG Is a Member of GMC Oxidoreductase Enzyme Family**—Comparison of the NinaG protein sequence with GenBank<sup>TM</sup> protein databases indicated that NinaG is a member of the GMC oxidoreductase family. This is a diverse family of flavin adenine dinucleotide flavoproteins. There are 15 identified Drosophila genes in this family. Only one, glucose dehydrogenase (30), codes for a characterized enzyme. Fig. 5A shows the neighbor-joining gene tree (31) of the Drosophila GMC oxidoreductase family members and characterized GMC oxidoreductase enzymes from other organisms. The analysis shows high divergence within this family in general and the NinaG protein in particular. Such results preclude any inference of substrate specificity or enzymatic action from this type of analysis.

Sequence analysis showed that the amino-terminal region of NinaG contains a signal recognition sequence (Fig. 5B). This amino-terminal domain is predicted to contain a signal peptide in NinaG protein with cleavage between residues 26 and 27 (32). The other members of the family lack any predicted signal sequence. Other features of the NinaG protein structure are as expected for the GMC oxidoreductase family, including the ADP-binding domain described as the βαβ fold (33) within the amino-terminal region.

**NinaG Acts in Chromophore Production**—The identification of NinaG as a member of the GMC oxidoreductase family suggests that the NinaG is an enzyme responsible for a redox reaction on small organic molecules. Given that the Rh1 accumulation in the endoplasmic reticulum observed in ninaG mutants is similar to other genotypes, and vitamin A-free diets deficient in rhodopsin chromophore availability, we hypothesized that the NinaG enzyme acts in the production of 3-hydroxyretinal, the Rh1 chromophore.

To investigate this possibility, HPLC was used to profile the retinal extracts from ninaG<sup>+</sup> and ninaG mutant heads. A similar number of heads were used for preparation of these extracts, and the results reported here were reproduced in independently prepared samples. Fig. 6 displays representative chromatograms of these extracts at two wavelengths, 324 and 374 nm. These chromatograms have been scaled to the same y axis to allow direct comparison of the samples. Significant differences were observed among three peaks eluting at 3.5, 3.8, and 4.1 min, whereas the remaining chromatographic profiles of all samples were similar. To prove that all observed components are because of retinoid species, wild type flies were reared on a vitamin A-free diet. Extracts from such flies showed no chromatographic peaks in the region of interest at the wavelengths listed above. Furthermore, feeding retinal back to these adult flies restored the chromatographic profile observed for wild type flies reared on normal medium (data not shown).
Fig. 5. Protein relationships and domains of NinaG. A, neighbor-joining sequence tree analysis of GMC oxidoreductases. All Drosophila gene products encoding GMC oxidoreductases as predicted by annotation of the Drosophila genome are shown. The NinaG protein is a distant relative of other family members. Also shown are characterized enzymes in the family from other eukaryotic species. These are methanol oxidase (from Pichia, yeast), eddysone oxidase (Spodoptera, moth), choline dehydrogenase (human and mouse), cellobiose dehydrogenase (Trametes, fungus), and glucose oxidase (Aspergillus, fungus). Branch lengths are given in parentheses as uncorrected p distances analyses, and bootstrap supports (10,000 repetitions) above 70% are shown in bold. For all proteins, the amino and carboxyl-terminal sequences were discarded before computer analysis to give the best alignment to amino acids 45–591 of NinaG. B, predicted NinaG protein domains. Sequence comparison analysis predicts a cleavable signal peptide (32) and the ADP binding site of flavin adenine dinucleotide (32). The length of the NinaG protein matches the amino-terminal (PP00732, GMC_oxred_N) and carboxyl-terminal (PF05199, GMC_oxred_C) conserved domains (43) found in all members of the GMC oxidoreductase family.

Fig. 6A shows that wild type flies possess a major retinoid species with a retention time of 4.1 min, with a secondary species indicated by the peak at 3.8 min. The ninaG flies extract yielded significantly smaller peaks at 4.1 and 3.8 min, indicating that they possess lower levels of both of these species. The \( \lambda_{\text{max}} \) of both the 4.1- and 3.8-min peaks was 374 nm, and the electrospray mass spectrum (data not shown) of each peak showed an ion at \( m/z \) 301.5 for \([M+H]^+\) of 3-hydroxyretinal. These peaks were assigned as 11-cis-3-hydroxyretinal (4.1 min) and all-trans-3-hydroxyretinal (3.8 min), in accordance with earlier work showing these are the predominant retinoids extracted from Drosophila heads (10). Extracts from wild type flies raised in the dark and provided with all-trans-retinal as the only retinoid source yielded a large increase in the 3.8-min peak at the expense of the 4.1-min peak (data not shown). This result is in agreement with the isomer assignments of the chromatographic peaks, because flies require light to convert 3-hydroxyretinal from the all-trans isomer (34).

Fig. 6B shows the chromatograms at 324 nm for wild type and ninaG extracts. The predominant retinoid elutes at 3.5 min and has a \( \lambda_{\text{max}} \) of 324 nm. Interestingly, the electrospray mass spectrum of the corresponding chromatographic peak showed an ion at \( m/z \) 285.5 (data not shown), which is the expected \( m/z \) value for \([M+H]^+\) of retinal. The HPLC/UV-visible/MS results of the all-trans-retinal standard yielded a chromatographic peak at 13.2 min with a \( \lambda_{\text{max}} \) of 374 nm and \( m/z \) 285.5 (data not shown). Although the presence of an ion at \( m/z \) 285.5 suggests that the 3.5-min peak is an isomer of retinal, the major change in \( \lambda_{\text{max}} \) (324 versus 374 nm) and retention time (3.5 versus 13.2 min) relative to the all-trans-retinal standard does not support this assignment.

If the majority of the 3-hydroxyretinal extracted from wild type is complexed to Rh1 rhodopsin, the absence of 3-hydroxyretinal in the ninaG mutant background might be a secondary effect of ninaG action reducing Rh1 rhodopsin protein production. To discount this possibility, the profile of retinoids in flies expressing Rh4 in place of Rh1 in ninaG\(^+\) (black) and ninaG mutant (outlined) samples are shown. Note the large increase in the unidentified retinoid eluting at 3.5 min, \( \lambda_{\text{max}} = 324 \) nm, in the ninaG mutant.

ninaG Oxidoreductase Acts in Retinal Metabolism

Fig. 6. HPLC profiles of retinoids extracted from wild type and ninaG heads. Absorbance profiles at 374 nm (A) and 324 nm (B) of wild type (black) and ninaG (outlined) samples are shown. Note the reduction in the retinoid eluting at 4.1 min, identified as 11-cis-3-hydroxyretinal and the increase in the unidentified retinoid at 3.5 min, \( \lambda_{\text{max}} = 324 \) in the ninaG mutant. Absorbance profiles at 374 nm (C) and 324 nm (D) of flies expressing Rh4 in place of Rh1 in ninaG\(^+\) (black) and ninaG mutant (outlined) samples are shown. Note the large increase in the unidentified retinoid eluting at 3.5 min, \( \lambda_{\text{max}} = 324 \) nm, in the ninaG mutant. 

![Figure 6](http://www.jbc.org/)
for chromophore production also appears to act only on β-carotene (7, 40). These results, then, are more consistent with the view that the following steps are required for use of zeaxanthin and related xanthophylls, 1) xanthophylls in the diet are reduced to β-carotene, 2) β-carotene is then cleaved to generate retinal, and, finally, 3) the retinal ring is oxidized to produce the hydroxylated and didehydrogenated retinoid structures used in some animals.

To determine whether the NinaG oxidoreductase acts in chromophore biogenesis, we characterized the retinoid content in the ninaG mutant using high performance liquid chromatography. There is a significant reduction in 3-hydroxyretinal in ninaG mutants, suggesting that ninaG mutants are defective in chromophore biogenesis. Even more compelling is the observation that the Rh4 rhodopsin uses a different chromophore in the ninaG+ and ninaG backgrounds. In ninaG+ Rh4 rhodopsin contains, as expected, 3-hydroxyretinal. In the ninaG mutant, Rh4 contains a distinct chromophore, distinguished from 3-hydroxyretinal by retention time, absorption maximum, and mass spectrum. These results are best explained by a defect in the production of 3-hydroxyretinal in the ninaG mutant. The results further suggest that Rh1 is selective in the use of 3-hydroxyretinal as a chromophore, whereas Rh4 will tolerate this alternative chromophore. The chemical structure of this alternative chromophore is currently under investigation but likely represents an intermediate, or product of alternative pathway, accumulating in the absence of NinaG activity.

In vivo, the Rh1 visual pigment is characterized by a dual peak of maximum spectral sensitivity, a pronounced UV light sensitivity in addition to the blue sensitivity expected for the purified Rh1 protein. This UV light sensitivity is because of the presence of a retinoid pigment that is distinct from the visual pigment chromophore. This UV-sensitizing pigment is able to absorb in the UV light and transfer this energy to the Rh1 chromophore (41). The organisms in the suborder Cyclorrhapha containing (3S)-3-hydroxyretinal are the same organisms capable of the secondary UV spectral sensitivity (9). Thus, the use of (3S)-3-hydroxyretinal may be critical to biogenesis of Rh1 but not to the biogenesis of other visual pigments.

The results presented here show that the NinaG protein acts within the biochemical pathways responsible for the formation of (3S)-3-hydroxyretinal. The biochemical steps involved in this process are not understood. The involvement of a P-450 cytochrome in creation of (3R)-3-hydroxyretinal was previously demonstrated in Drosophila head extracts (42), but an isomerization reaction converting (3R)-3-hydroxyretinal to (3S)-3-hydroxyretinal has not been described. The NinaG protein could act in this process or, alternatively, within a distinct pathway responsible for (3S)-3-hydroxyretinal production. Either scenario predicts that the residual 3-hydroxyretinal present in the ninaG mutant is the 3R enantiomer, providing an explanation for the inability of this 3-hydroxyretinal to serve as the Rh1 chromophore.

In Drosophila, cleavage of β-carotene to retinal occurs outside the retina (8); therefore, retinal or other product must be transported to the retina for use as the visual pigment. The cell-specific requirement of ninaG documented here suggests the final steps of 3-hydroxyretinal biogenesis occur within the retina. The identification of a signal recognition sequence in the NinaG protein further suggests that the protein is targeted to the endoplasmic reticulum. If true, the oxidative environment of the endoplasmic reticulum could be essential to the NinaG oxidoreductase activity. Another reason for potential endoplasmic reticulum localization of NinaG is that newly synthesized Rh1 opsin protein needs to be linked via a Schiff base...
to the chromophore. This event needs to occur within the ER for further maturation of the visual pigment. Also, as discussed above, the Rh1 rhodopsin also associates with a second chromophore, the UV-sensitizing pigment. Thus, it is plausible that efficient biogenesis of visual pigments requires coordinate synthesis of the chromophore and sensitizing pigment.

Acknowledgments—We thank the following people for contributing reagents and strains used in this work: William Pak, the ninaG<sup>250</sup> strain; Bill Engels, chromosome deficiencies in the ninaG region; Karen Hibbard, the pRh1:Rhl1-GFP strain; Steve Britt, the pRh1:Rhl4 and pRh1:Rhl6 strain; and Armin Huber, anti-Rh4 and anti-Rh6 antibodies. We thank Neil Lobo for help with the DNA sequence effort, Sheila Adams and Kathleen Mitchell for assistance with genetic and histological procedures, and Lei Li, Karen Hibbard, and Jing Yang for reviewing earlier versions of this manuscript. Finally, we are indebted to the Center for Environmental Science and Technology at the University of Notre Dame for allowing generous access to the Waters 2690 HPLC and Micromass Quattro LC mass spectrometer.

REFERENCES

1. Blomhoff, R., Green, M. H., Berg, T., and Norum, K. R. (1990) Science 250, 399–404.
2. Yeum, K. J., and Russell, R. M. (2002) Annu. Rev. Nutr. 22, 483–504.
3. Pak, W. L. (1995) Investig. Ophthalmol. Vis. Sci. 36, 2340–2357.
4. Harris, W. A., Ready, D. F., Lipson, E. D., Hudsptch, A. J., and Stark, W. S. (1977) Nature 266, 646–650.
5. Ozaki, K., Nagatani, H., Ozaki, M., and Tokunaga, F. (1993) Neuron 10, 1113–1119.
6. Kiefer, C., Sumsen, E., Wernet, M. F., and Von Lintig, J. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 10581–10586.
7. von Lintig, J., Dreher, A., Kiefer, C., Wernet, M. F., and Vott, K. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 1130–1135.
8. Gu, G., Van Vactor, Mitchell, K. A., and O'Tousa, J. E. (2004) J. Biol. Chem. 279, 18608–18613.
9. Seki, T., and Vott, T. F. (1998) Comp. Biochem. Physiol. B 119, 53–64.
10. Seki, T., Isono, K., Ito, M., and Katsuta, Y. (1994) Eur. J. Biochem. 226, 691–696.
11. Lamark, T., Kaasen, I., Eshoo, M. W., Falkenberg, P., McDougall, J., and Gloor, G. B. (1986) in Nichols, R., and Pak, W. L. (1985) J. Biol. Chem. 260, 839–850.
12. Pichaud, F., and Desplan, C. (2001) J. Neurosci. 21, 7525–7535.
13. Pichaud, F., and Desplan, C. (2001) J. Neurosci. 21, 7536–7545.
14. Kirschfeld, K., and Franceschini, N. (1977) Genetics 81, 953–964.
15. Kirschfeld, K., and Franceschini, N. (1977) Nature 269, 386–390.
16. Cavener, D. R. (1992) J. Biol. Chem. 267, 19787–19793.
17. Kolodziej, B., and Desplan, C. (1997) J. Cell. Biol. 138, 1057–1069.
18. Kiefer, C., Sumsen, E., Wernet, M. F., and Von Lintig, J. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 10581–10586.
19. von Lintig, J., Dreher, A., Kiefer, C., Wernet, M. F., and Vott, K. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 1130–1135.
20. Seki, T., Isono, K., Ito, M., and Katsuta, Y. (1994) Eur. J. Biochem. 226, 691–696.
21. Lamark, T., Kaasen, I., Eshoo, M. W., Falkenberg, P., McDougall, J., and Gloor, G. B. (1986) in Nichols, R., and Pak, W. L. (1985) J. Biol. Chem. 260, 839–850.
22. Pichaud, F., and Desplan, C. (2001) J. Neurosci. 21, 7525–7535.
23. Pichaud, F., and Desplan, C. (2001) J. Neurosci. 21, 7536–7545.
24. Kirschfeld, K., and Franceschini, N. (1977) Genetics 81, 953–964.
25. Kirschfeld, K., and Franceschini, N. (1977) Nature 269, 386–390.
26. Cavener, D. R. (1992) J. Biol. Chem. 267, 19787–19793.
27. Kolodziej, B., and Desplan, C. (1997) J. Cell. Biol. 138, 1057–1069.
28. Kiefer, C., Sumsen, E., Wernet, M. F., and Von Lintig, J. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 10581–10586.
29. von Lintig, J., Dreher, A., Kiefer, C., Wernet, M. F., and Vott, K. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 1130–1135.
30. Seki, T., and Vott, T. F. (1998) Comp. Biochem. Physiol. B 119, 53–64.
31. Seki, T., Isono, K., Ito, M., and Katsuta, Y. (1994) Eur. J. Biochem. 226, 691–696.
32. Pichaud, F., and Desplan, C. (2001) J. Neurosci. 21, 7525–7535.
33. Pichaud, F., and Desplan, C. (2001) J. Neurosci. 21, 7536–7545.
34. Kirschfeld, K., and Franceschini, N. (1977) Genetics 81, 953–964.
35. Feiler, R., Bjornson, R., Kirschfeld, K., Mismer, D., Rubin, G. M., Smith, D. P., Pasquinelli, A. E., and Paro, R. (2000) Cell 101, 399–408.
36. Colley, N. J., Baker, E. K., Stamnes, M. A., and Zuker, C. S. (1991) J. Biol. Chem. 266, 4295–4300.
37. Cavener, D. R. (1992) J. Biol. Chem. 267, 19787–19793.
38. Kolodziej, B., and Desplan, C. (1997) J. Cell. Biol. 138, 1057–1069.
39. Kirschfeld, K., and Franceschini, N. (1977) Genetics 81, 953–964.
40. Kirschfeld, K., and Franceschini, N. (1977) Nature 269, 386–390.
41. Kirschfeld, K., and Franceschini, N. (1977) Nature 269, 386–390.
The *Drosophila ninAG* Oxidoreductase Acts in Visual Pigment Chromophore Production

Shanta Sarfare, Syed Tariq Ahmad, Michelle V. Joyce, Bill Boggess and Joseph E. O'Tousa

*J. Biol. Chem. 2005, 280:11895-11901.*
do: 10.1074/jbc.M412236200 originally published online January 7, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M412236200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 16 of which can be accessed free at [http://www.jbc.org/content/280/12/11895.full.html#ref-list-1](http://www.jbc.org/content/280/12/11895.full.html#ref-list-1)