Cloning of the gene encoding honeybee long-wavelength rhodopsin: a new class of insect visual pigments

(Molecular evolution; vision; Hymenoptera; G protein-coupled receptor; *Apis mellifera*; phylogeny; wavelength regulation)

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SUMMARY

Rhodopsins (Rh), G-protein-coupled receptors with seven transmembrane (TM) helices, form the first step in visual transduction in most organisms. Although many long-wavelength (LW) vertebrate opsin sequences are known, less information is available for invertebrate LW sequences. By a combination of RT-PCR and cDNA library screening, we have cloned and sequenced the honeybee LW *Rh* gene. The deduced protein is composed of 378 amino acids (aa), appears to have seven TM regions, and contains many of the structures and key aa thought to be important for Rh function. Phylogenetic analysis of this sequence in relation to other invertebrate *Rh* reveals it to be a member of a new group of insect LW *Rh*.

INTRODUCTION

Rhodopsins (Rh) are G protein-coupled seven TM helical receptors that form the first step in sensory visual transduction in most vertebrate and invertebrate organisms known to be responsive to light (Hargrave and McDowell, 1992; Khorana, 1992; Nathans, 1992). Within the opsin binding pocket, a chromophore is covalently bound via a protonated Schiff's base linkage to the side chain of a Lys

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Abbreviations: aa, amino acid(s); *Am*, *Apis mellifera*; bp, base pair(s); cDNA, complementary DNA; G protein, GTP-binding protein; GCG, Genetics Computer Group (Madison, WI, USA); kb, kilobase(s) or 1000 bp; LW, long wavelength; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; Rh, visual pigment(s) or rhodopsin(s); *Rh*, gene encoding Rh; PAUP, phylogenetic analysis using parsimony; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcription; TM, transmembrane (domain); UV, ultraviolet.

residue in the seventh TM domain (Birge, 1990). The chromophore, usually 11-*cis* retinal (Nakanishi, 1991) or other closely related compounds such as 3-hydroxyretinal, isomerizes upon absorption of a photon. This change in configuration of chromophore induces a conformational change in the opsin protein itself, opening a binding site for the heterotrimeric G-protein, transducin (Hargrave et al., 1993). Activated rhodopsin (Rh*) catalyzes the replacement of the GDP bound to transducin by GTP, forming the first step of a biochemical cascade within the photoreceptor that ultimately leads to release of neurotransmitter, and generation of a neural signal (for reviews, see Shichi, 1983; Stieve, 1986; Stryer, 1986). The only photosensitive step in visual transduction is the absorption of a photon by the chromophore. Consequently, color vision in organisms is based on input from two or more independent spectral classes of photoreceptors (Helmholtz, 1896; Young, 1802), due in many instances (but not always) to opsins maximally sensitive to photons of differing energies.

Honeybees have long been model systems for the study

of vision in insects, especially color vision (for reviews, see Menzel, 1985; Menzel and Backhaus, 1989). That they can be efficiently trained to associate different spectral cues with certain food sources has shown bees to have true color vision, and not simply wavelength-specific behaviors. Because honeybee vision is so well characterized, we cloned the *Am* LW *Rh* as part of a comparative study of the interrelationship between *Rh* sequence and function.

EXPERIMENTAL AND DISCUSSION

(a) Cloning and sequencing of *Am Rh* cDNA

Total RNA was isolated from *Am* heads frozen in liquid nitrogen using a standard guanidinium isothiocyanate – CsCl preparation (Sambrook et al., 1989). This RNA was used as a template for reverse transcription using Superscript enzyme (Life Technologies, Gaithersburg, MD). The first-strand cDNA was used directly for PCR using degenerate oligo primers derived from conserved
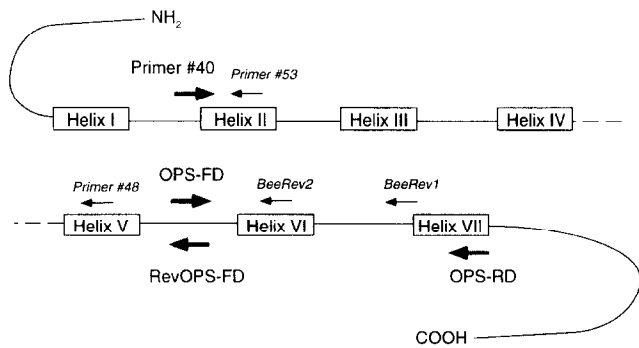
Fig. 1. Primers used for RT-PCR amplifications of *Am Rh*. Heavy arrows indicate degenerate primers, thin arrows specific primers. Primer sequences: OPSFD: 5'-GANCARGCNAARAARATGAAY, OPSRD: 5'-CCRTANACRATNGGRTTRTA, Rev-OPSFD: 5'-RTTCATYT-TYTTNGCYTGNTC, Primer #40: 5'-GSIICIAARKCIYTIC-GNACNCC; specific primers used to generate overlapping clones: Primer #53 (5' end at nt 353), Primer #48 (5' end at nt 767), BeeRev1 (5' end at nt 1007), BeeRev2 (5' end at nt 945). For degenerate primers, cycling parameters were 95°C, 45°C, 72°C for 30 s each step, 25 cycles. Annealing temperatures were higher (45–55°C) for amplification reactions involving specific primers.

regions of other insect *Rh* (Fig. 1). PCR products were ligated directly into the pCR II vector (Invitrogen, San Diego) without further purification. Transformants were screened for inserted fragments of the appropriate size PCR directly from the bacterial colonies using M13 primers flanking the cloning site of the pCR II vector. Vectors containing fragments of the appropriate size were then cycle-sequenced with fluorescently-labelled sequencing primers or dideoxynucleotides, and run on an Applied Biosystems automated sequencer (Model 373A, Foster City, CA).

We used a nested primer strategy to generate overlapping PCR clones for sequencing (Fig. 1). For each segment at least three or more independent PCR clones, in most cases from separate PCR reactions, were sequenced on both strands in order to minimize PCR artifacts and confirm the nucleotide sequence shown in Fig. 2. Two of the degenerate oligo primers, OPS-FD and OPS-RD amplified a short segment (248 bp), from which specific primers for 3' and 5' RACE were constructed. In addition to our RT-PCR strategy, we also constructed a plasmid cDNA library from *Am* heads in pSPORT (Life Technologies, Gaithersburg, MD). To obtain the extreme 5' end of the *Am Rh*, we used a DNA prep from our cDNA library in a PCR with a reverse primer specific to our *Rh* sequence, and a forward primer containing pSPORT sequences flanking the cloning site. A further clone containing the complete coding sequence was also obtained from a cDNA library constructed in λgt22A. A Northern blot using the 248-bp fragment as a probe detected a transcript of about 1.6 kb (Fig. 3).

(b) Comparative sequence analysis of *Am Rh*

The putative *Am Rh* cDNA that we isolated encodes a 378-aa protein. It has about 62% identity to *Drosophila melanogaster* Rh1 and Rh2 at the aa level, and only about 42% identity at the aa level to *Drosophila* UV opsins, Rh3 and Rh4. The *Am Rh* shows the greatest identity to the mantid *Rh*, about 65% at the nt level (76% identity for aa). We reconstructed a phylogeny from known invertebrate Rh sequences from GenBank, including our novel
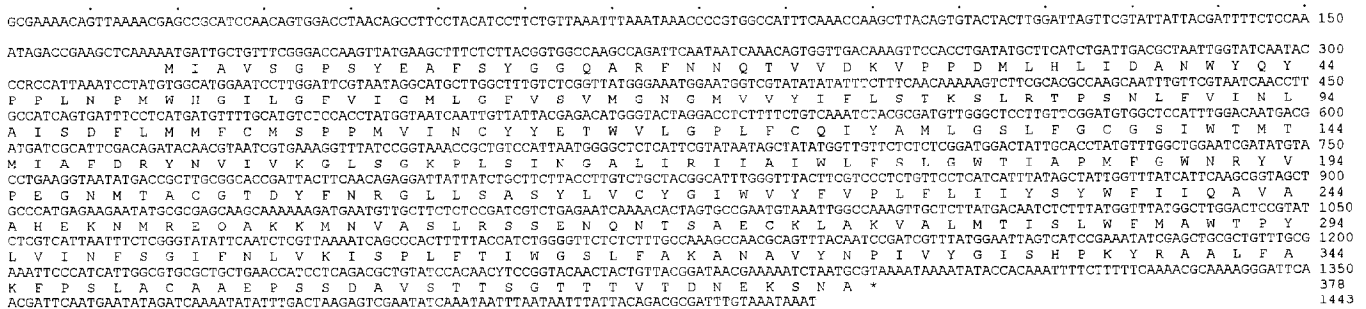
Fig. 2. cDNA sequence for *Am* LW *Rh*. Two polymorphic sites derived from independent cDNA clones are R^{303} and Y^{1257}.

1   2

kb
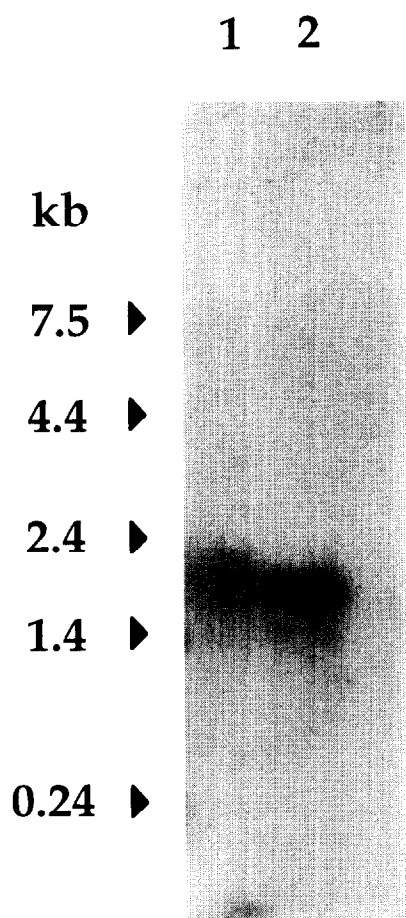
7.5 ▶

4.4 ▶

2.4 ▶

1.4 ▶

0.24 ▶

Fig. 3. Total RNA (lane 1) was isolated from *Am* heads purified using a phenol-guanidinium isothiocyanate preparation (Trizol, Life Technologies, Gaithersburg, MD). Poly(A)⁺mRNA (lane 2) was selected using oligo(dT) attached to magnetic beads (Promega, Madison, WI). These were run several hours at 100 V on a 2.2 M formaldehyde 1% agarose gel, and hybridized at 42°C overnight with the [^{32}P]dCTP random hexamer-labelled probe, a 258-bp fragment of *Am Rh*. Size standards (0.24–9.5 kb RNA Ladder, Life Technologies, Gaithersburg, MD) in kb are indicated in the left margin.

Am Rh. The phylogeny shown in Fig. 4 was reconstructed using maximum parsimony on opsin aa data, with boot-strap values from 100 replications indicated above the nodes. The results of our phylogenetic analysis support the grouping of the *Am* Rh with mantid Rh, a LW Rh, and not with any of the fly Rh. These two Rh form a distinct clade from all other fly Rh, indicating a separate, new class of Rh absent in higher flies.

Honeybees are known to have three types of photoreceptors with different absorption sensitivities that are characteristic of many hymenopteran insects (Peitsch et al., 1992): green, blue, and UV (544 nm, 436 nm, 344 nm). Studies of LW (green) photoreceptors in *Am* lacking screening pigments indicate an Rh with maximal sensitivity at about 526 nm in these cells (Gribakin, 1988). The putative *Am Rh* cDNA sequence shows the greatest identity to the green mantid *Rh* which is thought to have wavelength sensitivities in the green (Towner and
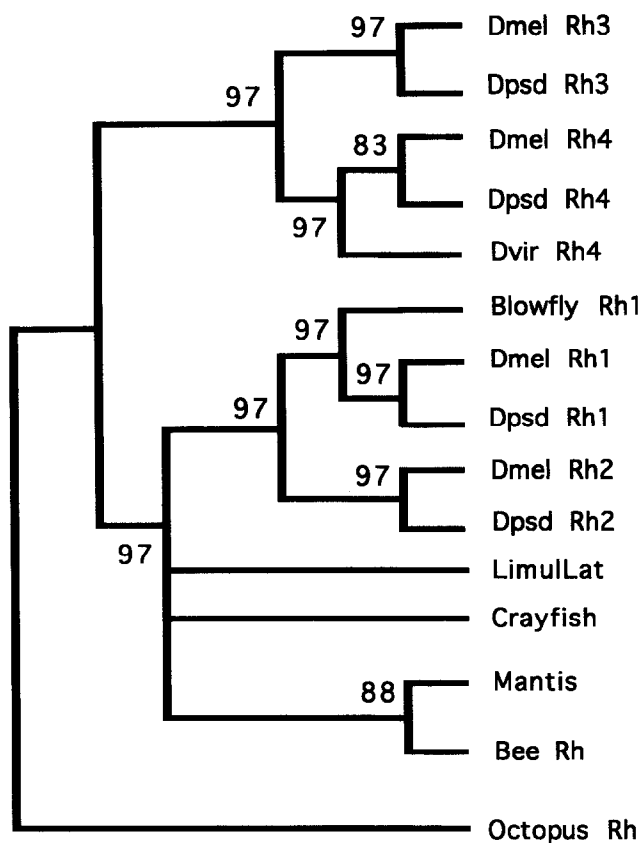
Fig. 4. A phylogenetic tree of invertebrate Rh aa sequences, reconstructed using maximum parsimony as implemented by PAUP (Swofford, 1991). This tree is a strict consensus of the two shortest trees found in 100 replications of a heuristic search with random addition and tree bisection-reconnection branch-swapping. The stepmatrix used was BLOSUM-100 (Henikoff and Henikoff, 1992). The one node not well-supported in bootstrap analysis, in which crayfish and limulus lateral eye Rh emerge basal to the mantis and *Am* Rh, was collapsed in the figure. Numbers above nodes indicate percentage bootstrap values out of 100 replications with random addition. Sequences were aligned using ClustAlV (Higgins et al., 1992), with further refinement made by eye. Most of the alignment, except for small portions of the N- and C-termini, were used for phylogenetic reconstruction. Trees were rooted using the octopus Rh sequence as an outgroup. Taxa as indicated on tree, listed with GenBank accession Nos.: *Drosophila melanogaster* DmelanRh1 (K02315); DmelanRh2 (M12896); DmelanRh3 (M17718); DmelanRh4 (M17719); *Calliphora erythrocephala* BlowflyRh (M58334); *Drosophila pseudoobscura* DpseudoRh1 (X65877); DpseudoRh2 (X65878); DpseudoRh3 (X65879); DpeudoRh4 (X65880); *Drosophila virilis* DvirilRh4 (S66727); *Limulus polyphemus* LimulLat (L03791); *Procambarus clarkii* Crayfish (S53494); *Sphodromantis spp.* Mantis (X71665); *Paroctopus defleini* Octopus (X07797); *Apis mellifera* BeeLW (U26026).

Gärtner, 1994). In addition, the results of our phylogenetic analysis support the grouping of the *Am* LW *Rh* with mantid *Rh*. Thus we infer that it is most likely the LW (P526) Rh.

The deduced aa sequence for *Am* LW Rh has several motifs and residues known to be critical for Rh function (Fig. 5). It has the expected seven hydrophobic domains as indicated on a standard hydropathy plot (GCG
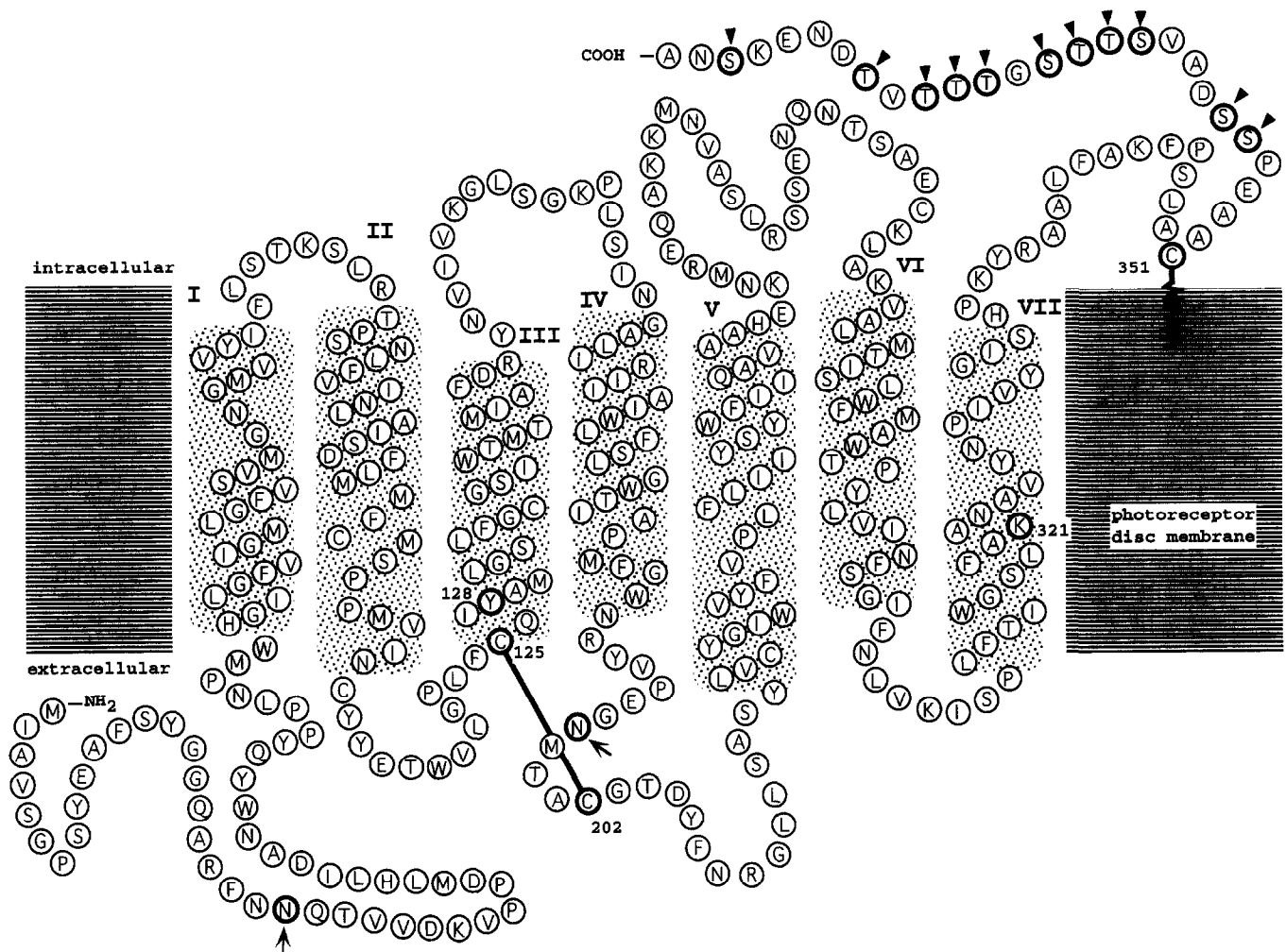
Fig. 5. Model for *Am* Rh, adapted from Mirzadegan et al., 1992. Lys^{321} is indicated as the probable site of retinal attachment, Tyr^{128} as the site of the counterion, and the two Cys residues, Cys^{125} and Cys^{202}, as forming a disulfide bond. Cys^{351} is indicated as being a possible site of palmitoylation. Arrows indicate potential sites for *N*-linked glycosylation, and arrowheads sites for phosphorylation. Roman numerals I–VII indicate TM domains.

Wisconsin software, not shown). The Lys residue known to be the site of retinal attachment in bovine Rh (Bownds, 1967) is present in a homologous position in the seventh TM domain (Lys^{321}). The position known to be the counterion to the protonated Schiff's base in bovine Rh (Nathans, 1990; Sakmar et al., 1989; Zhukovsky and Oprian, 1989), is a Tyr (at position 128) in *Am* Rh as in all other invertebrate opsins except for the UV opsins, which may have an unprotonated Schiff's base chromophore. The cytoplasmic tail of *Am* Rh contains many Ser and Thr, potential sites for phosphorylation by Rh kinase (McDowell et al., 1993; Ohguro et al., 1993), a regulatory feature common to many seven TM G-protein-coupled receptors (Hollenberg, 1991). *N*-linked glycosylation is thought to be important for proper folding and transport of Rh (Kaushal et al., 1994). Two *N*-linked glycosylation signals (N X S/T) are present in *Am* Rh, one at the N terminus (Asn^{22}), and one in extracellular loop 4–5 (Asn^{198}). The N-terminal site has been shown to be glycosylated in *Calliphora* Rh (Huber et al., 1990). *Am* Rh also

contains Cys^{125} and Cys^{202} required to form the disulfide bond, a critical component of Rh structure thought to be involved in stabilizing the metarhodopsin II active state and its activation of transducin (Davidson et al., 1994). Bovine Rh has two Cys in its C terminus that are palmitoylated, forming a fourth intracellular loop (Ovchinnikov et al., 1988; Papac et al., 1992). *Am* Rh has only one of these cysteines, Cys^{351} in its C terminus.

In contrast to *Drosophila*, *Am* Rh are known to be A1 visual pigments, i.e., they contain 11-*cis* retinal instead of the 3-hydroxyretinal characteristic of many insects including most flies (Gleadall et al., 1989; Smith and Goldsmith, 1990). It has been postulated that Ser residues at two positions in TM domain V may form hydrogen bonds with the extra hydroxyl group present to stabilize the 3-hydroxyretinal chromophore (Oprian, 1992). Visual pigments that use 11-*cis* retinal, such as in the vertebrates, do not have these serine residues. Consistent with this hypothesis, *Am* LW Rh does not have Ser residues at either of these two positions (Gly^{221} and Val^{224}).

Comparative analysis of Rh sequences has proven useful in identifying residues involved in determining Rh absorption spectra (Chang ct al., 1995). However, similar comparative analysis in invertebrates has been hampered by the lack of diversity in Rh sequences from different species, as well as in Rh possessed of varied wavelength absorption sensitivities. Our cloning studies of *Am* LW *Rh* is a necessary step toward a more complete comparative analysis of Rh function.

(c) Conclusions

(*1*) A putative *Am* LW *Rh* cDNA sequence was obtained using RT-PCR, as well as by screening of a cDNA library.

(*2*) Phylogenetic analysis indicates that this *Am Rh* sequence is a member of a new class of LW insect opsins.

(*3*) Polar residues in helix V postulated to stabilize the extra hydroxyl group in A3 pigments are lacking in *Am* LW *Rh*. This is consistent with evidence that honeybees have A1, not A3 visual pigments.

ACKNOWLEDGMENTS

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