

学 位 論 文

Functional analysis of chicken pineal  
photoreceptive molecule pinopsin

(ニワトリ松果体光受容タンパク質ピノプシンの機能解析)

平成11年12月博士(理学)申請

生物化学専攻

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## 論文の内容の要旨

論文題目 Functional analysis of chicken pineal photoreceptive molecule pinopsin

(ニワトリ松果体光受容タンパク質ピノプシンの機能解析)

氏名 仲村 厚志

ニワトリ松果体は光受容能を持ち、受容された光シグナルは松果体細胞に内在する概日時計の位相を調節する。ニワトリ松果体の光受容タンパク質ピノプシンは、網膜以外の組織から初めてクローニングされたオプシンであり、アミノ酸配列を基に作成した分子系統樹において、網膜の光受容タンパク質(視物質)とは異なる新しいグループを形成する。ピノプシンのクローニング以降、さらにいくつかの光受容タンパク質が網膜外組織からクローニングされ、概日時計の光位相同調や日長識別など多様な光生理現象に関与すると想定されてきた。しかしながら、視物質の性状解析に関しての膨大な研究がある一方で、網膜外の光受容タンパク質の性質については、試料の調製が困難なことから、そのほとんどが謎に包まれている。そこで本研究では、まず培養細胞でのピノプシンの大量発現および精製方法を確立した。次に、精製したピノプシンを用いて、光吸収後の構造変化過程とGタンパク質との共役過程を、分光学的・生化学的手法を駆使して詳細に検討した。

ピノプシンは、松果体に極く微量しか存在せず(2ng/松果体)、機能解析を行うために必要な量のピノプシンを精製することは極めて困難である。そこで、アフィニティー精製のためのタグ(6残基のヒスチジン)をN末端に導入したピノプシンを設計し、培養細胞293Sを用いて強制発現させた。発現し

たピノブシン(アポ蛋白質)に11シス型レチナルを加えて光受容タンパク質を再構成したのち、界面活性剤Dodecyl maltosideを用いて可溶化した。この細胞抽出液を出発材料として、3段階のカラムクロマトグラフィー(DEAE-Sepharoseカラム、ニケツルキレートカラムおよびSP-Sepharoseカラム)を行うことにより、ピノブシンを高純度に精製することに成功した。また、ヒステジンタグを付加したニワトリロドプシン(桿体視物質)とニワトリGreen(緑色感受性錐体視物質)を同様に発現させて精製し、ピノブシンと共に以下の測定に用いた。

まず、低温吸収スペクトル法によりピノブシンの光退色過程を調べた。その結果、ピノブシン(吸収極大波長:468nm)は視物質と同様、光を吸収した後、バソ(527nm)、ルミ(461nm)、メタI(460nm)、メタII(385nm)、そしてメタIII(460nm)という一連の中間体を経て退色した。この退色中間体系列とその分光学的性質は視物質のそれらと良く一致することから、ピノブシンの光受容後の構造変化は視物質と類似していることが示唆された。

次に、ウシ網膜より精製したGタンパク質トランスデュシン(Gt)を用いてGTP $\gamma$ S結合実験を行ったところ、視物質ロドプシンと同様、ピノブシンは光依存的にGtを活性化することがわかった。視物質では、メタII中間体がGt活性化能を持つことが知られている。そこで、ピノブシン光退色中間体のGt活性化能の寿命を解析し、分光学的に同定したメタII中間体の寿命と比較したところ、両者は酷似していた(図1)。この結果から、ピノブシンにおいてもメタII中間体がGtを活性化する生理活性を持つと考えられた。

ロドプシンと錐体視物質のメタII中間体の性質は互いに大きく異なり、この違いが光受容細胞(桿体と錐体)の光応答特性の特徴を生み出す一因と考えられている。また、アミノ酸配列の比較から、ピノブシンはロドプシンよりも錐体視物質に類似した性質を持つと推定される。そこで、ピノブシン

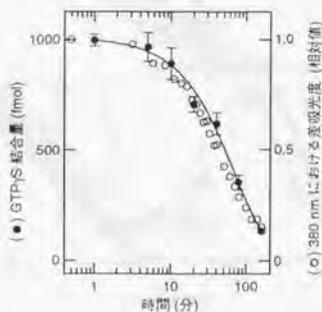


図1 ピノブシンのGt活性化能の時間変化とメタII中間体の崩壊過程の比較

ピノブシンに黄色光(>480 nm)を2°Cで30秒間照射し、その後、ピノブシンによるGt活性化能の時間変化とメタII中間体の崩壊過程を経時記録した。Gt活性化能は、GtへのGTP $\gamma$ S結合量を指標とした。一方、メタII中間体の崩壊過程は、380nmにおける吸光度の相対的減少によって示される。

のメタII中間体の性質(生成および崩壊の速度)をロドプシンおよびGreenと比較した(図2, 上段)。その結果、ビノプシンのメタII中間体の生成はロドプシンよりも20倍以上速く(-25°Cにおける時定数6.2分)、Greenとはほぼ同じ速度で生成した(つまり錐体タイプ)。一方、メタII中間体の崩壊過程は(図2, 下段)、予想に反してビノプシンはGreenより80倍以上遅く、すなわちロドプシンとはほぼ同じ速度(2°Cにおける時定数44分)でゆっくりと崩壊した(つまり桿体タイプ)。これらの結果より、ビノプシンのメタII中間体は桿体と錐体の両方の視物質の性質を兼ね備えていることが判明し、ビノプシンは新しいタイプの光受容タンパク質であることが明らかになった。ビノプシンのメタII中間体が予想以上に安定であることから、松果体における光情報伝達の過程では、網膜よりも時間分解能は低いものの光情報をより高度に増幅できる可能性が考えられる。このことは、脳における概日時計の光同調というビノプシンの生理機能に適した特性といえるかもしれない。また、ビノプシンとロドプシン・錐体視物質の系統関係を考え併せると、ビノプシンはメタII中間体の寿命を延ばす機能を、ロドプシンとは独立に獲得したことが示唆される。

そこで次に、ビノプシンのメタII中間体が錐体視物質よりも強く安定化される機構をアミノ酸レ

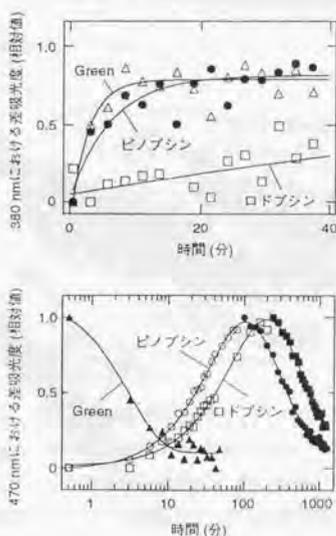


図2 ビノプシンのメタII中間体の生成および崩壊

上段: ビノプシンのメタII中間体の生成過程を、ロドプシンおよびGreenと比較した。67% (v/v) グリセリンを含むビノプシン試料を-25°Cまで冷却し、橙色光(>520 nm)を30秒間照射した。照射後の380 nmにおける吸光度の時間変化を示した。ロドプシンとGreenの場合は橙色光(>570 nm)を30秒間照射した。

下段: ビノプシンのメタII中間体の崩壊過程を、ロドプシンおよびGreenと比較した。ビノプシンに黄色光(>480 nm)を2°Cで30秒間照射した。照射後の470 nmにおける吸光度の時間変化を示した。ロドプシンとGreenの場合は橙色光(>520 nm)を30秒間照射した。ビノプシンとロドプシンでは、二相の吸光度変化が見られ、第一相はメタIIが崩壊しメタIIIが生成する過程で、第二相はメタIIIが崩壊する過程である。2°CではGreenのメタIIの崩壊は速く、メタIIIの崩壊のみが観察された。

ペルで明らかにするために、部位特異的な変異を導入した変異ピノブシンを用いた解析を行った。まず、候補となるアミノ酸残基を絞り込むために、既知の錐体視物質において保存されており、かつピノブシンにおいては異なるアミノ酸残基を検索した。この基準を満たす部位は、(i) ピノブシンの171番目のSer、(ii) 184番目のAsn、(iii) 2残基の欠失(190番目と191番目の間)、の3カ所であった。ピノブシンのこれらの部位を錐体視物質型のアミノ酸残基に置換した変異体を作成し、メタII中間体の崩壊過程を測定した(図3)。その結果、171番目および184番目それぞれの残基を置換した変異体P-S171RおよびP-N184Dでは、メタII中間体の寿命はそれぞれピノブシンの約70%および約80%となったが、両方の残基の2重変異体P-S171R/N184Dではピノブシンとほぼ同じ寿命を示した(図3、右図)。このことから、これら2残基(の組み合わせ)はピノブシンのメタII中間体の安定化にほとんど寄与していないと考えられる。一方、2残基を挿入した変異体P-188Gr193は11シス型レチナールと結合しなかったが、3つの部位を全て置換した変異体P-S171R/N184D/188Gr193では、メタII中間体の寿命がピノブシンの約30%まで減少した(図3、右図)。以上の結果より、190番目と191番目の間の2残基の欠失が、ピノブシンのメタII中間体の安定性に重要な役割をはたしていることが明らかになった。この2残基の欠失はピノブシンの細胞外側の第2ループにみられ、桿体視物質のロドプシンでは、この領域がタンパク質全体の正常なフォールディングに大きな影響を与えることが知られている。私の得た結果は、ピノブシンにおいてもこの領域がタンパク質の高次構造の形成、ならびにメタII中間体の安定性の制御に関わっていることを示している。

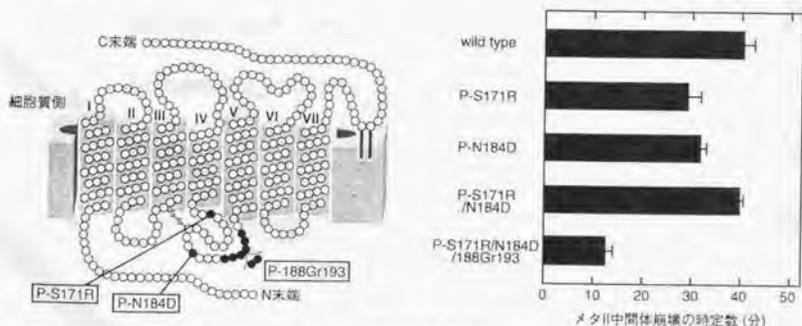


図3 部位特異的な変異ピノブシンのメタII中間体の崩壊速度

左図: 部位特異的な変異を導入した位置を示した。右図: 変異ピノブシンのメタII中間体の崩壊速度を比較した。分光学的測定は図2の下段の実験と同様に行い、それぞれの変異体のメタII中間体の崩壊速度を求めた。なお、作成したピノブシン変異体のうちP-188Gr193は、11シス型レチナールを加えても光受容能が検出されなかった。

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1. Abbreviations

DM: dodecyl- $\beta$ -D-maltoside

DTT: dithiothreitol

GMP: guanosine 5'-monophosphate

GTP $\gamma$ S: guanosine 5'-O-(3-thiotriphosphate)

G-protein: guanine nucleotide-binding protein

HEPES: *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

$\lambda_{\max}$ : absorption maximum in the visible region

MAPK: mitogen-activated protein kinase

NAT: serotonin *N*-acetyltransferase

PC: L- $\alpha$ -phosphatidylcholine from egg yolk

PCR: polymerase chain reaction

SCN: suprachiasmatic nucleus

## 2. General Introduction

Many vertebrates retain photoreceptor cells in several extra-retinal tissues such as the pineal gland, deep brain and skin (von Frisch, 1911; Oliver and Baylè, 1976; Hartwig and van Veen, 1979; Foster *et al.*, 1985; Yoshikawa and Oishi, 1998). Among these extra-retinal photoreceptive tissues, the pineal gland of many species in nonmammalian vertebrates has been shown to play an important role in photo-entrainment of circadian rhythms.

Chicken pineal gland has been widely used to investigate the circadian clock system for several advantages. When the chicken pineal gland is isolated and cultured in constant darkness, the change in activity of serotonin *N*-acetyltransferase (NAT), an important enzyme in a rhythmic production of melatonin, continued to oscillate (Binkley *et al.*, 1978; Deguchi, 1979a; Kasal *et al.*, 1979), indicating that a circadian oscillator is located within the chicken pineal gland. In addition, a light pulse given in the subjective night not only shifts the phase of the oscillator (Binkley *et al.*, 1981) but also suppresses acutely the NAT activity (Deguchi, 1979b). On the basis of these findings, it was concluded that the chicken pineal gland has an endogenous photoreceptive molecule which transmits the light signal to the oscillator for resetting the phase.

In 1981, Deguchi measured the action spectrum of the inhibitory effect of light on NAT activity, and suggested that a rhodopsin-like photosensitive molecule is involved (Deguchi, 1981). Immunocytochemical studies have shown that the avian pineal gland contains retinal photoreceptor cell-specific proteins. These include rhodopsin-like pigment (Foster *et al.*, 1989; Araki *et al.*, 1992; Masuda *et al.*, 1994) and transducin-like proteins (van Veen *et al.*,

1986). Furthermore, electrophysiological study detected cyclic GMP-dependent cation-channel activity in the chicken pinealocytes (Dryer and Henderson, 1991). In spite of several lines of evidence suggesting the similarity between retinal and pineal photon-signal transducing proteins, the molecular identity of the pineal photosensitive pigment had been an open question. In 1994, a cDNA encoding a novel photoreceptive molecule was cloned from a chicken pineal cDNA library, and it was named pinopsin after pineal opsin (Okano *et al.*, 1994).

For understanding the photon-signaling in extra-retinal tissues, it is important to characterize the photoreceptive molecule in those tissues because, in the retina, the difference in cellular responses to light may be associated to different properties between rod and cone visual pigments (Shichida and Imai, 1998). Since the identification of chicken pinopsin, several opsin genes for non-visual purposes have been cloned from retinal and extra-retinal tissues (Soni and Foster, 1997; Sun *et al.*, 1997; Blackshaw and Snyder, 1997; Provencio *et al.*, 1998; Wada *et al.*, 1998; Yoshikawa *et al.*, 1998; Blackshaw and Snyder, 1999; Mano *et al.*, 1999). As compared to the abundance of visual pigments in the retina, the relative contents of the extra-retinal pigments in the tissues are very low. For this reason, an over-expression system in cultured cells is required for extensive study of the molecular properties of the extra-retinal pigments. So far, only pinopsin and VA-opsin have been successfully reconstituted with 11-*cis*-retinal and their  $\lambda_{\max}$  had been determined (Okano *et al.*, 1994; Kawamura and Yokoyama, 1998; Soni *et al.*, 1998; Kojima *et al.*, in press), but the amounts of these pigments were not enough to study their photochemical and biochemical properties. In the present study, I have established the over-expression

system of pinopsin, which enabled examination of its detailed properties not only by photochemical techniques but also by biochemical analyses.

### References

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### 3. Chimeric nature of pinopsin between rod and cone visual pigments

#### 3.1 Introduction

A blue-sensitive pigment pinopsin was previously cloned from a chicken pineal cDNA library (Okano *et al.*, 1994), and it was classified into a novel subtype of vertebrate rhodopsin family. Interestingly, the phylogenetic analysis (Figure 1) showed that pinopsin has diverged from an ancestor of cone pigments before the divergence of rhodopsins from an ancestor of cone pigments (Okano *et al.*, 1994), suggesting that pinopsin would be a cone-type pigment. If this is the case, the formation and decay of the meta II intermediate of pinopsin would resemble those of cone pigments, because there is a notable difference in properties of meta II intermediate between rod and cone pigments (Shichida *et al.*, 1994; Okada *et al.*, 1994; Imai *et al.*, 1995; Imai *et al.*, 1997a; Imai *et al.*, 1997b). Among a series of photobleaching intermediates of visual pigments, meta II intermediate is identified as a physiologically active intermediate activating a retinal G-protein transducin (Figure 2, Fukada and Yoshizawa, 1981; Emeis and Hofmann, 1981; Bennett *et al.*, 1982; Okada *et al.*, 1994). In this respect, the difference in meta II lifetime has been implicated in cellular responses characteristic of rod and cone cells (Shichida and Imai, 1998). Therefore, I have focused my interests on the thermal behavior of meta II intermediate of pinopsin for better understanding of pineal cell physiology.

In the present study, the meta II formation kinetics of pinopsin is shown to be comparable to that of a cone pigment, but its decay profile is much

similar to that of rhodopsin. These unique properties of pinopsin are the first example for chimeric nature between rod and cone type pigments in vertebrates.



① A family of rhodopsin genes encodes a rod opsin  
② A family of rhodopsin genes encodes a cone opsin  
③ A family of rhodopsin genes encodes a rod opsin  
④ A family of rhodopsin genes encodes a rod opsin

The rhodopsin gene family is a large family of genes that encode the opsin part of the visual pigment. The rhodopsin gene family is divided into two main groups: rod opsins and cone opsins. Rod opsins are found in the rod cells of the retina and are responsible for vision in low light conditions. Cone opsins are found in the cone cells of the retina and are responsible for vision in bright light conditions. The rhodopsin gene family is highly conserved across vertebrates, indicating its importance in vision.

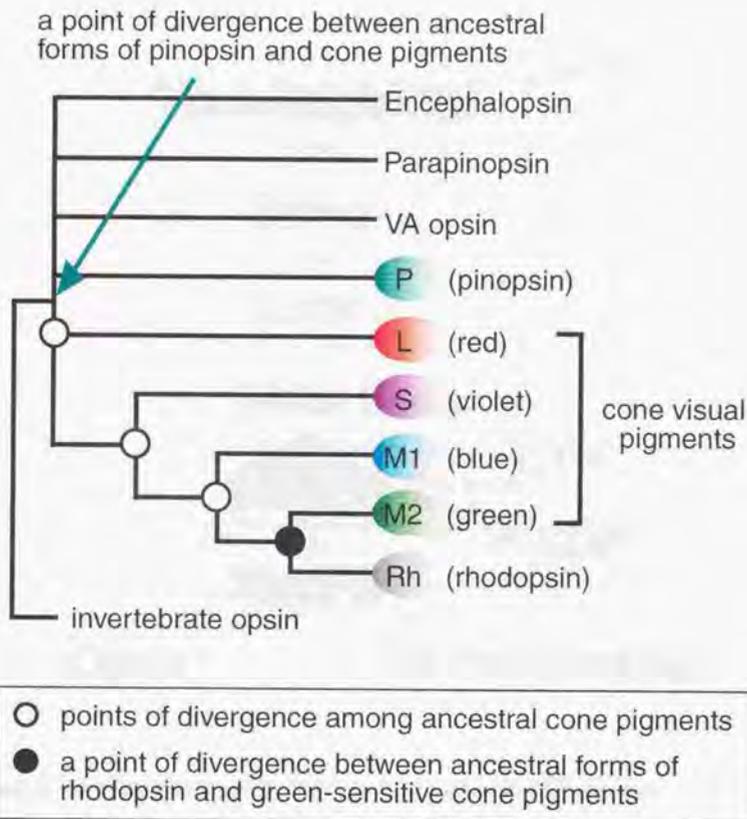


Figure 1. Phylogenetic relationship between vertebrate opsins. Pinopsin and other non-visual-type vertebrate opsins have diverged from an ancestor of cone pigments before the divergence of rhodopsins from an ancestor of cone pigments (Okano *et al.*, 1994; Soni and Foster, 1997; Blackshaw and Snyder, 1997; Blackshaw and Snyder, 1999). Red, violet, blue or green represents red-, violet-, blue- or green-sensitive cone visual pigment, respectively.

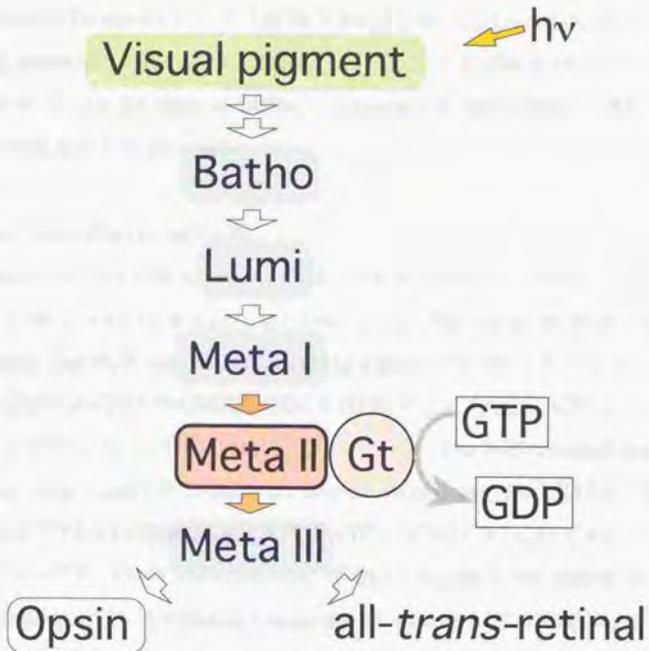


Figure 2. Schematic drawing of the photobleaching process of visual pigments.

### 3.2 Experimental procedures

#### *Buffers.*

Buffer P-0 contains 50 mM HEPES-NaOH, 50 kallikrein inhibitor units/mL aprotinin, and 4 µg/mL leupeptin (pH 6.6). Buffer P-10, P-90, P-130, and P-140 are the same as Buffer P-0 except for containing 10, 90, 130, and 140 mM NaCl, respectively.

#### *Expression Constructs and Cells.*

Pinopsin cDNA (Okano *et al.*, 1994) was modified as shown in Figure 3. To introduce six histidine residues and a Factor Xa site at the N-terminus of pinopsin, first PCR was performed using a pair of primers; 5'-CATCATC ACCATCACCACATCGAGGGGCGCATGTCCTCCAACAGCTCC-3' and 5'-CCCGAATTCACACGGGGTGTGCTGG C-3'. The PCR product was subjected to the second PCR using another set of primers; 5'-CGGTATCGTC GATAAGCTTAAACCGCAGCCATGCATCATCACCATCACCAC-3' and 5'-CCCGAATTCACACGGGGTGTGCTGGC-3' to add 11 bp stretch of chicken rhodopsin 5'- untranslated sequence in upstream of initiation methionine. The resulting amplified product was digested with *Hind*III and *Eco*RI, and subcloned into pBluescript II KS+ (Stratagene), and fully sequenced on both strands to confirm the absence of PCR errors. Similarly, the cDNAs of chicken rhodopsin and green (Okano *et al.*, 1992), a green-sensitive cone visual pigment, were modified so as to have the same N-terminal tag as described above. To construct opsin expression vectors, each of the modified cDNA was subcloned into the *Hind*III-*Eco*RI site of mammalian expression vector pUSRα (Kayada *et al.*, 1995), which is a derivative of pUC-SRα (Shimamoto *et al.*, 1993).

A suspension-adapted variant 293S (Nathans *et al.*, 1989) of the human embryonic kidney cell line (ATCC CRL 1573) was used as the recipient for transient transfection. The cells were grown in 10% fetal bovine serum, 50:50 DMEM/F12 with low glucose (Gibco) at 37 °C in a 5% CO<sub>2</sub> atmosphere. In a typical experiment, 10 µg/plate of the opsin expression vector and 0.5 µg/plate of pRSV-TAg (an SV40 T-antigen expression vector) were coprecipitated onto two hundred 10-cm diameter plates of 293S cells by the calcium phosphate method (Gorman *et al.*, 1990). Forty hours after transfection, the cells were collected with Buffer P-10, and stored at -80 °C until use.

#### *Reconstitution.*

11-*cis*-Retinal was purified as previously reported (Maeda *et al.*, 1978) and stored at -80 °C until use. The following procedures were performed in the dark or under dim-red light (>660 nm) at 4 °C unless otherwise specified. To generate photopigments, the cells collected as described above were suspended with 25 mL of Buffer P-10, and were incubated with an excess amount of 11-*cis*-retinal (about 500 nmol in 250 µL ethanol) for 1 h at room temperature, except for chicken green-expressing cells which were incubated with 11-*cis*-retinal for 4 h at 4 °C. The regenerated photopigment in the suspension was solubilized by mixing with an equal volume (25 mL) of Buffer P-10 containing 2% (w/v) dodecyl-β-D-maltoside (DM; Dojindo Laboratories). The mixture was incubated for 1 h at 4 °C and centrifuged to isolate solubilized proteins (termed "cell extract").

*Purification of Pigments.*

Pinopsin was purified from the cell extract in the dark or under dim-red light (>660 nm). To increase relative content of pinopsin, the cell extract was passed through a DEAE-Sepharose column (10 x 38 mm; Amersham Pharmacia Biotech) at a flow rate of 36 mL/h. The flow-through fraction containing most of solubilized pinopsin was incubated with 2 mL resin of Probond nickel-charged agarose (Invitrogen) for about 12 h at 4 °C. The resin was packed into a column (10 mm diameter), and washed at a flow rate of 36 mL/h with the following buffers successively; 150 mL of Buffer P-140 containing 0.02% DM (w/v) and 50 mL of Buffer P-140 containing both 0.02% DM (w/v) and 20 mM imidazole. Then, pinopsin was eluted from the column with 3 mL of Buffer P-140 containing both 0.02% DM (w/v) and 200 mM imidazole (pH 6.6). The eluate was dialyzed against Buffer P-10 containing 0.02% DM (w/v), and centrifuged at 125000g for 30 min at 4°C to remove insoluble materials. The clear supernatant containing pinopsin was applied to a SP-Sepharose column (7 x 13 mm; Amersham Pharmacia Biotech) at a flow rate of 18 mL/h. The column was washed with 10 mL of Buffer P-10 containing 0.02% DM (w/v), and then pinopsin was eluted with Buffer P-130 containing 0.02% DM (w/v). The eluate was dialyzed against Buffer P-140 containing 0.02% DM (w/v), and used for spectroscopic and biochemical experiments. For low-temperature spectroscopy, the pinopsin solution was concentrated by using Microcon 10 (Amicon), dialyzed, and mixed with 2-fold or 3-fold volume of glycerol.

Similarly, histidine-tagged chicken green was expressed and purified from the cell extract. In the final step of the SP-Sepharose column chromatography, green was eluted with Buffer P-90 containing 0.02% DM (w/v).

Histidine-tagged chicken rhodopsin was expressed and purified from the cell extract with some modifications: To increase relative content of solubilized rhodopsin, the cell extract was passed through a CM-Sepharose column (Amersham Pharmacia Biotech) instead of the DEAE-Sepharose column. The flow-through fraction containing rhodopsin was similarly purified by the nickel-charged agarose column, and the eluate was dialyzed against buffer P-10 containing 0.02% DM (w/v). After centrifugation, the clear supernatant was dialyzed against Buffer P-140 containing 0.02% DM (w/v), and used for spectroscopic and biochemical experiments.

#### *Spectrophotometry.*

The absorption spectra of purified pigments were recorded in a previously reported system (Imai *et al.*, 1995) with a Shimadzu Model MPS-2000 spectrophotometer interfaced with an NEC PC-9801 computer. Oxford model CF-1204 cryostat was used for low-temperature spectroscopy. The sample temperature was controlled within  $\pm 0.1$  °C by a temperature controller (ITC-4, Oxford) attached to the cryostat. The sample was irradiated with light from a 1 kW tungsten halogen lamp (Rikagaku Seiki). The wavelengths of the irradiation light were selected with a glass cutoff filter (VY-50, VO-54, 59, VR-63; Toshiba), or an interference filter (436 nm; Nihonshinku).

#### *Transducin Activation Assay.*

Transducin was purified from dark-adapted bovine retinas as described elsewhere (Fukada *et al.*, 1994). Transducin activation assays were performed as previously reported (Terakita *et al.*, 1998) with some modifications. Briefly, transducin was mixed with the pigment solution in 50

mM HEPES (pH 6.6), 5 mM MgCl<sub>2</sub>, 140 mM NaCl, 1 mM DTT, 0.01% (w/v) DM, and 3 μM [<sup>35</sup>S]GTPγS (1500-3000 cpm/pmol) in the dark. After 30 s preincubation, the reaction was started by exposing the mixture containing either pinopsin or rhodopsin to light (>480 nm or >520 nm, respectively) for 30 s at 2 °C, followed by incubation in the dark for 30 s at this temperature. Under the irradiation conditions, the pigment in the mixture was completely bleached. The reaction was terminated with a stop solution [20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 25 mM MgCl<sub>2</sub>, and 5 μM GTPγS], and the amount of [<sup>35</sup>S]GTPγS bound to transducin α-subunit was measured as previously reported (Fukada *et al.*, 1994).





### 3.3 Results

#### *Purification of Histidine-tagged Pinopsin.*

Pinopsin present in the chicken pineal gland is so small in quantity (Okano *et al.*, 1997; Takanaka *et al.*, 1998) that an over-expression system in cultured cells is required to obtain a sufficient amount of pinopsin. For affinity purification, the recombinant pinopsin was designed to carry additional six histidine residues at the N-terminus (Figure 3). I avoided tagging at the C-terminus which, in the case of rhodopsin, is exposed to the molecular surface interacting with transducin. The N-terminal tagging was expected to minimize the effect on the signaling properties (see Discussion). The histidine-tagged pinopsin expressed in 293S cells was reconstituted with 11-*cis*-retinal, and solubilized with a detergent, dodecyl- $\beta$ -D-maltoside (DM). The amount of regenerated pinopsin was spectrophotometrically estimated to be about 4  $\mu\text{g}/10^7$  cells. Usually 400  $\mu\text{g}$  of recombinant pinopsin was obtained from cells cultured in two hundred 10-cm plates. Then, pinopsin was purified by three steps of column chromatography (see Experimental procedures and Figure 4), with a recovery of about 25%. The absorption spectrum of the purified histidine-tagged pinopsin (Figure 5) showed  $\lambda_{\text{max}}$  at 468 nm which is very close to  $\lambda_{\text{max}}$  of non-tagged pinopsin (about 470 nm; Okano *et al.*, 1994). Similarly, I prepared modified chicken rhodopsin and green each having a histidine tag at the N-terminus. The recombinant rhodopsin and green showed  $\lambda_{\text{max}}$  at 504 nm and 507 nm, respectively, which were also close to the reported values (Okano *et al.*, 1989) of native chicken rhodopsin (503 nm) and green (508 nm). Thus, I confirmed that the histidine tag attached to N-terminus gives little effect on the conformation of

these pigments, and I used these samples for characterization of pinopsin in comparison with rhodopsin and green.

#### *Photoreaction of Pinopsin at Liquid Nitrogen Temperature.*

Primary photoreactions of pinopsin were investigated by low-temperature spectroscopy. When pinopsin was cooled to  $-196^{\circ}\text{C}$ , its absorption spectrum was sharpened with a slight red-shift of  $\lambda_{\text{max}}$  to 475 nm (Figure 6, curve 1). Irradiation with blue light (436 nm) at this temperature caused a red-shift of the spectrum indicating the formation of a batho-product (bathopinopsin). Prolonged irradiation finally produced a photosteady-state mixture containing mainly bathopinopsin (Figure 6, curve 2). Irradiation of the mixture with deep-red light ( $>610$  nm) gave a spectrum (curve 3) identical with curve 1. Subsequent irradiation with orange light ( $>560$  nm) resulted in formation of a blue-shifted iso-product (curve 4; isopinopsin). This product was irradiated with the blue light giving a spectrum (curve 5) identical with curve 2. These photoreactions indicate that the original pigment (pinopsin), batho-product and iso-product were perfectly interconvertible by light at  $-196^{\circ}\text{C}$ , just like retinal visual pigments (Yoshizawa and Wald, 1963; Yoshizawa and Wald, 1967; Imamoto *et al.*, 1989; Imai *et al.*, 1995; Kojima *et al.*, 1995; Imai *et al.*, 1997b).

#### *Intermediates in the Photobleaching Process of Pinopsin.*

In the case of retinal visual pigments, warming of the batho intermediate results in sequential formation of lumi, meta I, and enzymatically active meta II intermediates (Matthews *et al.*, 1963; Yoshizawa and Wald, 1967; Shichida *et al.*, 1994; Kojima *et al.*, 1995; Imai *et al.*, 1997b). To identify the intermediates in the photobleaching process of pinopsin, the photosteady-

state mixture containing mainly bathopinopsin (Figure 6, curve 5) was warmed in a stepwise manner (Figure 7). Bathopinopsin was stable up to  $-190^{\circ}\text{C}$  (Figure 7, curve 2), and the following two intermediates, lumi and meta I, were stable up to  $-70^{\circ}\text{C}$  (curve 11) and  $-50^{\circ}\text{C}$  (curve 13), respectively. As was observed for the bleaching process of chicken blue-sensitive cone pigment (Imai *et al.*, 1997b), I detected a small change in half band width without remarkable shift in  $\lambda_{\text{max}}$  during the transition from lumi to meta I intermediates (curves 11-13). Above  $-40^{\circ}\text{C}$ , meta I intermediate was converted to the next meta II intermediate, and its  $\lambda_{\text{max}}$  ( $\sim 380\text{ nm}$ ) was quite similar to those of meta II of retinal visual pigments (Matthews *et al.*, 1963; Shichida *et al.*, 1993; Shichida *et al.*, 1994; Imai *et al.*, 1994; Kojima *et al.*, 1995; Imai *et al.*, 1995; Imai *et al.*, 1997b). The absorption maxima of the four intermediates were calculated by using a conventional method (Yoshizawa and Shichida, 1982; Imai *et al.*, 1995): 527 nm (bathopinopsin), 461 nm (lumipinopsin), 460 nm (metapinopsin I), and 385 nm (metapinopsin II). To sum up, the bleaching process of pinopsin is similar to those observed for visual pigments with respect to (i) the number, (ii) the transition temperature of the intermediates, and (iii) the transition profile of the absorption maxima (see also Figure 11).

#### *The Formation Process of Metapinopsin II.*

Previous studies revealed that the meta II formation of cone visual pigments is faster than that of rhodopsin (Shichida *et al.*, 1993; Shichida *et al.*, 1994; Imai *et al.*, 1995). To investigate whether pinopsin is a cone-type pigment from this point of view, the time constant for the formation of meta II intermediate of pinopsin was compared with those of rhodopsin and green (a green-sensitive cone visual pigment). Green was used as a representative

of cone pigments, because its photochemical reaction has been investigated most extensively in comparative studies with rhodopsin (Shichida *et al.*, 1994; Imai *et al.*, 1995). The thermal reaction of photoactivated pinopsin was recorded by time-resolved spectroscopy at  $-25^{\circ}\text{C}$  (Figure 8). After the irradiation, absorbance at 380 nm increased while that at 460 nm decreased (Figure 8A), representing the conversion of metapinopsin I to metapinopsin II. The kinetic profile at 380 nm (Figure 8B, closed circles) was simulated by a single-exponential curve, from which the time constant of the metapinopsin II formation was estimated to be 6.2 min. This rate was comparable to that of the meta II formation of green (time constant, 2.7 min; Figure 8B), and much faster than that of rhodopsin (time constant, 130 min; Figure 8B). Thus, I concluded that the formation of meta II intermediate of pinopsin is as fast as those of cone pigments.

#### *Transducin Activation by Pinopsin.*

Similarity in photobleaching process between pinopsin and the visual pigment, together with the presence of transducin-like G-protein in the chicken pineal gland (Okano *et al.*, 1997), raised the possibility that pinopsin can activate transducin. As shown in Figure 9, pinopsin activated bovine retinal transducin in a light-dependent manner, and the activation efficiency of pinopsin was comparable to that of rhodopsin. To see whether metapinopsin II is responsible for the activation, I measured the decay time course of the activation ability at  $2^{\circ}\text{C}$  (closed circles; Figure 10A), and compared it with the spectral change at the same temperature (open circles, Figure 10A). In the thermal reaction of photoactivated pinopsin at  $2^{\circ}\text{C}$  (Figure 10B), the absorbance at  $\sim 380$  nm decreased with concomitant increase in absorbance at  $\sim 450$  nm, representing the conversion process of

metapinopsin II to metapinopsin III. This decay process of metapinopsin II (reproduced in Figure 10A, open circles) was nearly identical to the decay of the active state, though the calculated time constant for the latter ( $69 \pm 11$  min) was slightly larger than that for the former (44 min). Such a small difference could be due to the existence of the equilibrium between metapinopsin II and metapinopsin III (Kibelbek *et al.*, 1991), and thus I assigned metapinopsin II as an important intermediate activating transducin.

*Decay Processes of Metapinopsin II, Metarhodopsin II and Meta-green II.*

The lifetime of metapinopsin II was compared with those of green and rhodopsin. As described above, the decay of metapinopsin II at 2 °C was a relatively slow process with a time constant of 44 min estimated from the kinetic profile at 470 nm (Figure 10C, open circles). This decay time constant was comparable to that of metarhodopsin II (91 min; Figure 10C, open squares). Under the same conditions, the decay of meta II intermediate of green was not detected, and instead the decay process of meta III was observed (Figure 10C, closed triangles), indicating that the decay of meta-green II was completed within 0.5 min. Thus, the decay time constant for metapinopsin II (44 min) is rather similar to that of metarhodopsin II (91 min), but much larger (at least 80 times) than that of meta-green II.

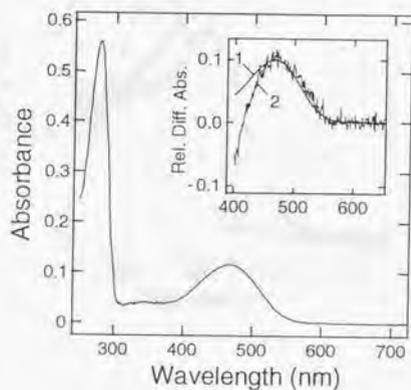


Figure 5. Absorption spectra of purified pinopsin. The purified histidine-tagged pinopsin had  $\lambda_{\text{max}}$  at 468 nm, and a typical ratio of  $A_{280}/A_{468}$  was 4.8. *Inset:* The absorption spectrum of purified histidine-tagged pinopsin (smooth line; curve 1) is compared with the difference spectrum of non-tagged pinopsin before and after complete photobleaching (curve 2, reproduced from Okano *et al.*, 1994).

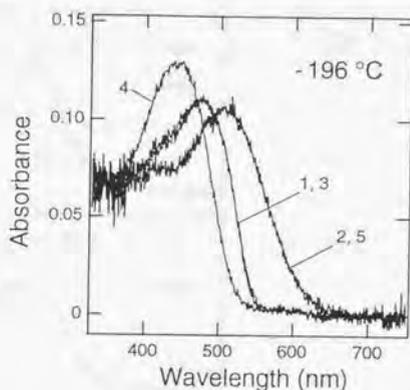


Figure 6. Photochemical reactions of pinopsin at liquid nitrogen temperature. Purified chicken pinopsin in 75% (v/v) glycerol mixture was cooled to  $-196^{\circ}\text{C}$  (curve 1) and irradiated with blue light (436 nm) for 1280 s to form a photosteady-state mixture (curve 2). It was then irradiated with deep-red light ( $>610$  nm) for 240 s (curve 3), followed by irradiation with orange light ( $>560$  nm) for 1920 s (curve 4). This sample was irradiated again with the blue light (436 nm) for 640 s (curve 5). In every case, a more prolonged irradiation gave no spectral change, indicating formation of the photosteady-state mixture.

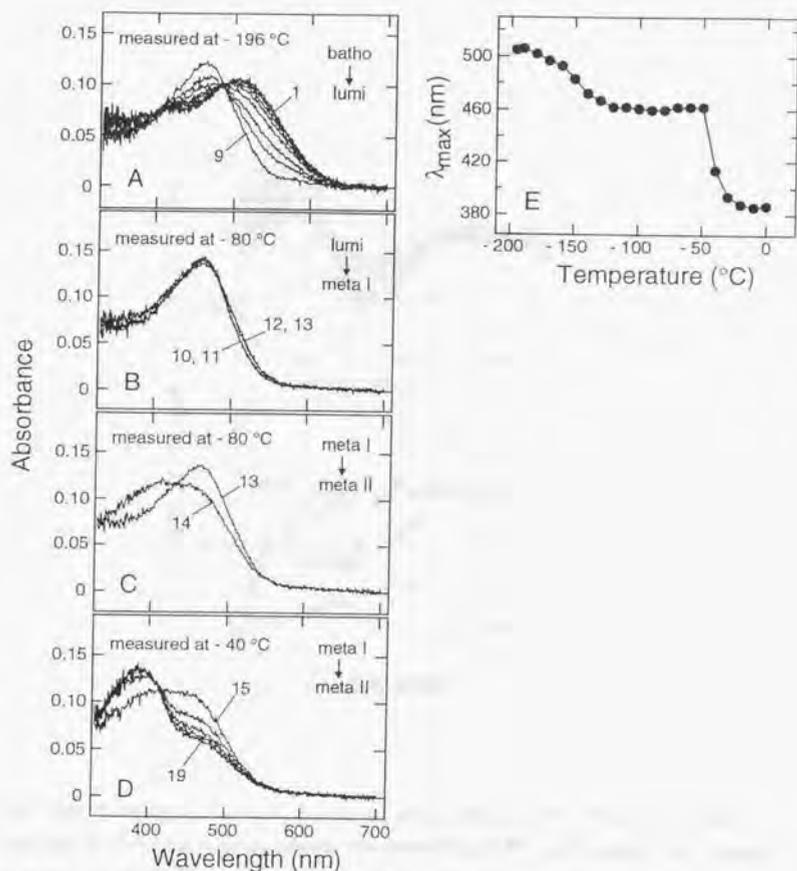


Figure 7. Photobleaching process of pinopsin. The photosteady-state mixture containing mainly batho-intermediate formed at  $-196^{\circ}\text{C}$  (curve 1) was warmed in a stepwise manner. *Panel A:* Absorption spectra were recorded at  $-196^{\circ}\text{C}$  after warming to  $-190, -180, -170, -160, -150, -140, -130,$  and  $-120^{\circ}\text{C}$  (curves 2-9). *Panel B:* Absorption spectra were recorded at  $-80^{\circ}\text{C}$  after warming to  $-80, -70, -60,$  and  $-50^{\circ}\text{C}$  (curves 10-13). *Panel C:* Absorption spectra were recorded at  $-80^{\circ}\text{C}$  after warming to  $-40^{\circ}\text{C}$  (curve 14). *Panel D:* Absorption spectra were recorded at  $-40^{\circ}\text{C}$  after warming to  $-40, -30, -20, -10,$  and  $0^{\circ}\text{C}$  (curves 15-19). *Panel E:* Absolute  $\lambda_{\max}$  of the recorded spectra were plotted against the temperatures to which the sample was warmed.

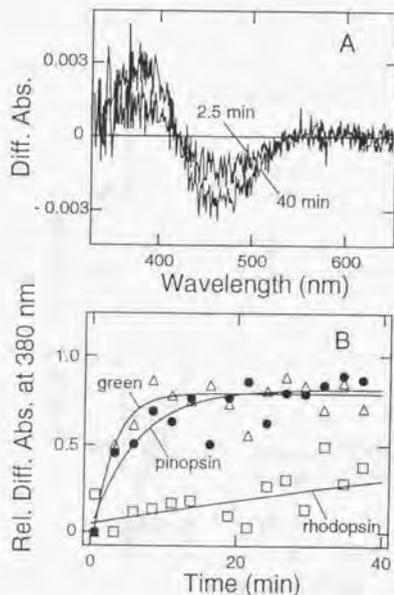


Figure 8. Formation of meta II intermediate of pinopsin at  $-25^{\circ}\text{C}$ . *Panel A*: Purified pinopsin in 67% (v/v) glycerol mixture was cooled to  $-25^{\circ}\text{C}$ , and irradiated with orange light ( $>520\text{ nm}$ ) for 30 s. After the irradiation, the sample was incubated in the dark at the same temperature. The curves were the difference spectra between the spectrum recorded immediately after irradiation and those recorded 2.5 min and 40 min after the irradiation. *Panel B*: Kinetic profiles of the meta II formation of pinopsin (closed circles) was compared with those of rhodopsin (open squares) and green (open triangles). Relative absorbance changes at 380 nm after the irradiation were plotted against incubation time after the irradiation. The irradiation of rhodopsin and green was performed with orange light ( $>570\text{ nm}$ ) for 30 s. Solid curves represent single-exponential curves fitted with time constants of 6.2 min (pinopsin), 2.7 min (green), and 130 min (rhodopsin), respectively.

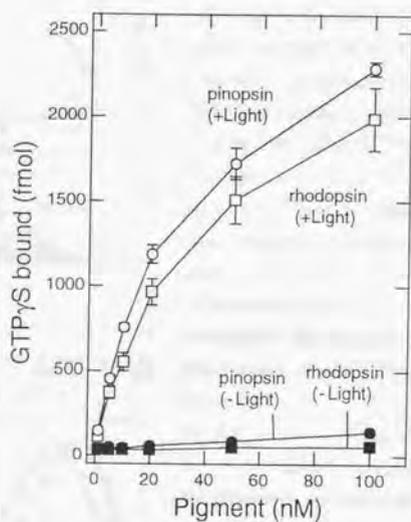


Figure 9. Light-dependent activation of transducin by pinopsin and rhodopsin. Pinopsin or rhodopsin at various concentrations (final concentrations are indicated on the abscissa) was mixed with 500 nM of bovine transducin and 3  $\mu$ M [ $^{35}$ S]GTP $\gamma$ S in 0.01% DM (final concentrations). Reactions were proceeded under irradiation (pinopsin, >480 nm; rhodopsin, >520 nm) for 30 s and subsequently in the dark for 30 s at 2  $^{\circ}$ C, and then the amount of GTP $\gamma$ S bound to transducin  $\alpha$ -subunit was measured. Average values and standard deviations from three independent experiments are presented.

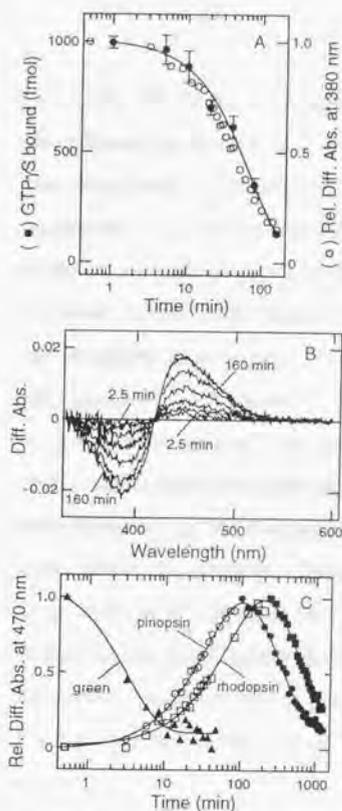


Figure 10. Thermal decay of meta II intermediate of pinopsin at 2 °C. *Panel A:* The mixture of pinopsin and [ $^{35}$ S]GTP $\gamma$ S was irradiated at 2 °C with yellow light (>480 nm) for 30 s. After the incubation for indicated time (abscissa) in the dark at 2 °C, 15  $\mu$ L aliquots were withdrawn and mixed with 5  $\mu$ L transducin solution (final concentrations: 15 nM pinopsin, 500 nM transducin, 3  $\mu$ M [ $^{35}$ S]GTP $\gamma$ S) for measurement of the GTP $\gamma$ S binding activity. Average values (closed circles) with standard deviations from three independent experiments were fitted with a single-exponential curve with a time constant of  $69 \pm 11$  min. The thermal decay of metapinopsin II at 2 °C (open circles) was assessed by the decrease in absorbance at 380 nm (see below). *Panel B:* Pinopsin was irradiated with yellow light (>480 nm) at 2 °C for 30 s, and the sample was subsequently incubated in the dark at the same temperature. The curves were the difference spectra between the spectra recorded immediately after the irradiation and those recorded 2.5, 5, 10, 20, 40, 80, and 160 min after the irradiation. *Panel C:* The thermal conversion of meta intermediates of pinopsin (circles), rhodopsin (squares), or green (triangles)

was assessed by relative absorbance changes at 470 nm plotted against incubation time after irradiation. Rhodopsin or green was irradiated with orange light (>520 nm) at 2 °C for 30 s. The absorbance changes of pinopsin and rhodopsin were simulated by a combination of two sequential single-exponential curves. The first phase of each curve represents the formation of meta III from meta II, i.e., meta II decay (open symbols), and the second phase represents the decay of meta III (closed symbols). The calculated time constants for the first phase of pinopsin and rhodopsin are 44 and 91 min, respectively, and those for the second phase are 320 and 590 min, respectively. As for green, only the decay of meta III (closed triangle) was observed (time constant, 3.9 min) due to a rapid conversion of meta II to meta III intermediates at this temperature.

### 3.4 Discussion

Using recombinant pinopsin, I investigated for the first time the photobleaching process of an extra-retinal pigment, pinopsin, in detail by low-temperature spectroscopy. When a retinal visual pigment such as rhodopsin is exposed to blue light at  $-196^{\circ}\text{C}$ , batho intermediate is observed as the primary photo-product with a twisted all-*trans*-chromophore (Shichida and Imai, 1998). This intermediate is converted to iso-product with 9-*cis* chromophore upon exposure to red light at  $-196^{\circ}\text{C}$ . That is, light induces only geometric isomerization of the chromophore at  $-196^{\circ}\text{C}$ , leaving the protein moiety relatively unaltered (Shichida and Imai, 1998). In the present study, quite similar photoreversibilities were observed among pinopsin, bathopinopsin and isopinopsin (Figure 6), suggesting strongly that 11-*cis* retinylidene chromophore of pinopsin is isomerized to all-*trans* form by light. This seems to be supported by the occurrence of 11-*cis*- and all-*trans*-retinal in the chicken pineal gland (Sun *et al.*, 1991; Masuda *et al.*, 1994), though the chromophore analysis of native pinopsin has not been performed due to the low content in a single pineal gland [approx. 2 ng (Takanaka *et al.*, 1998)].

In the photobleaching process of pinopsin, I detected several intermediates, each of which spectrophotometrically corresponds to that of a retinal visual pigment. As shown in Figure 11,  $\lambda_{\text{max}}$ 's of meta I, meta II, and meta III of pinopsin respectively converge to those of visual pigments, while batho and lumi intermediates of these pigments have their unique  $\lambda_{\text{max}}$ 's. This suggests that the chromophore/opsin interactions and opsin conformations of meta I, II, and III of pinopsin are respectively similar to the corresponding intermediates of visual pigments. Judging from the  $\lambda_{\text{max}}$

profile, I speculate that the conformational change of pinopsin after the photon absorption resembles those of rod and cone visual pigments, among which chicken blue seems most similar to pinopsin in overall structural change (Figure 11).

The present result demonstrated that chicken pinopsin activates transducin in a light-dependent manner. Recently, transducin activation by pinopsin has been reported by Max *et al.* (1998), but their data are somewhat different from the present results in two aspects. First, the lifetime of active state of pinopsin is about twice longer than that of rhodopsin purified from bovine retinas (Max *et al.*, 1998), but the present data (Figure 10C) show that the lifetime of metapinopsin II is about half of that of metarhodopsin II derived from histidine-tagged recombinant rhodopsin. Second, the initial rate of transducin activation by pinopsin is 2-3 times lower than that by retinal rhodopsin in their report (Max *et al.*, 1998), while I observed that pinopsin can activate transducin with an initial rate comparable to that of recombinant rhodopsin upon exposure to light (Figure 9). Such disagreements might come from a difference in preparation of pinopsin (and rhodopsin). Max *et al.* (1998) used recombinant pinopsin with 1D4 epitope tag added to the C-terminus. Transducin activation by pinopsin may be perturbed due to the tagging to the C-terminal tail, which is located at the molecular surface interacting with G-protein. To avoid such a possible perturbation, I introduced a histidine tag to the N-terminus located at the extracellular surface. In addition, I prepared recombinant rhodopsin having the same histidine-tag at the N-terminus to minimize or cancel out a possible artificial effect of the tagging on the evaluation of the functional difference between pinopsin and rhodopsin. My preparation would help detailed characterization of unidentified functional importance of pinopsin C-terminal tail, of which

the amino acid sequence is noticeably diverged from those of rod and cone visual pigments (Okano *et al.*, 1994).

As schematically illustrated in Figure 12, the present results demonstrated that the decay of metapinopsin II is relatively slow (rod-type), while its formation is relatively rapid (cone-type). The rod-type decay process of metapinopsin II was unexpected, because pinopsin was predicted to be a cone-type pigment on the basis of the following three reasons: First, like cone pigments, pinopsin has an isoelectric point much more basic than rhodopsins (Figure 13; modified from Okano and Fukada, 1998), and the difference in isoelectric point between cone pigments and rhodopsins is one of the important factors regulating the thermal behaviors of meta II intermediate (Imai *et al.*, 1995). Second, a phylogenetic tree of vertebrate pigments (Figure 1) indicated that pinopsin has diverged from an ancestor of cone pigments before the divergence of rhodopsins from an ancestor of cone pigments (Okano *et al.*, 1994). Third, chicken pinopsin as well as most of the cone visual pigments has a neutral amino acid residue at position 122 (a number in chicken rhodopsin), while all the sequenced rhodopsins have a glutamate at this position (Figure 14). This single amino acid residue is a major determinant of the meta II decay rate of rod and cone pigments (Imai *et al.*, 1997). In spite of these cone-type features of pinopsin, I found a long-lived metapinopsin II, suggesting an unidentified regulatory mechanism of the meta II lifetime unique to pinopsin. Possible residues responsible for this phenomenon are investigated in the next section of this thesis.

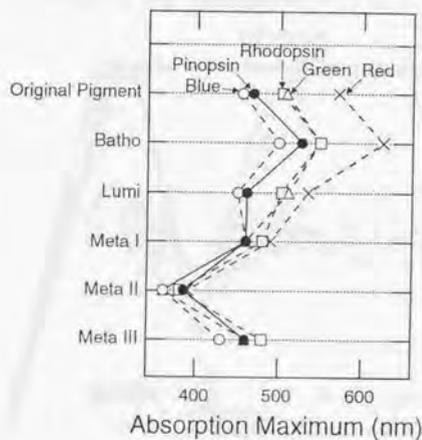


Figure 11. The shift of absorption maxima of the intermediates appearing in the photobleaching process of pinopsin and chicken visual pigments. Plotted are the absorption maxima of the intermediates of pinopsin (closed circles), rhodopsin (open squares), green (open triangles), blue (open circles), and red (crosses), among which those of rhodopsin, red, green, and blue are reproduced from previous reports (Fukada *et al.*, 1990; Kandori *et al.*, 1990; Shichida *et al.*, 1993; Imai *et al.*, 1994; Imai *et al.*, 1995).

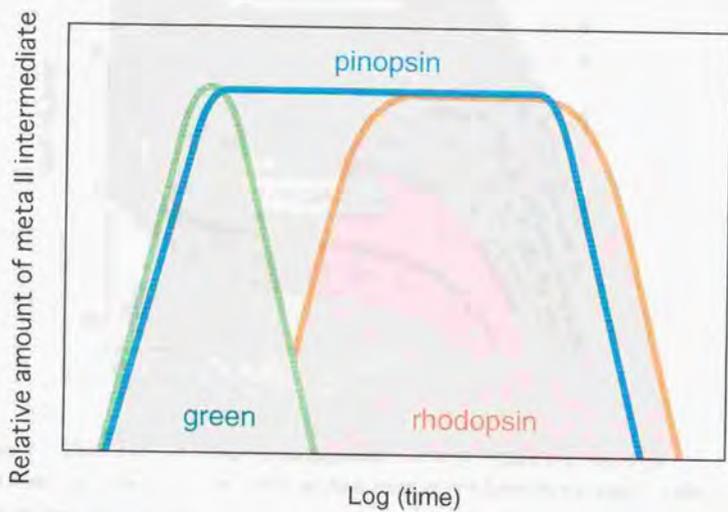


Figure 12. Schematic drawing of formation and decay of meta II intermediate of pinopsin, green and rhodopsin.

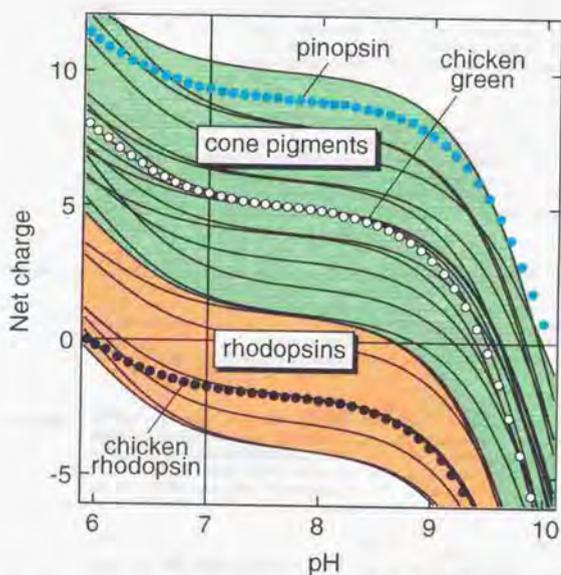


Figure 13. Calculated net charge of the pigments. The net charges at given pH of vertebrate pigments were calculated from their amino acid sequences according to the formula described previously (Okano *et al.*, 1992). The curves for cone pigments were clustered in the green zone. In order of the degree of positive charge at pH 7 in the green zone (from +10.37 to +1.65), each curve represents, human blue, chicken pinopsin, marmoset red, human red, chicken blue, chicken blue, gecko green, gecko blue, chicken red, chicken green, cavfish red, chicken violet, goldfish green 1, goldfish red, goldfish green 2, cavfish green 2 and cavfish green 1. Similarly, the curves for rhodopsins were clustered in the orange zone. In order of the degree of positive charge at pH 7 in the orange zone (from +1.55 to -3.44), each curve indicates, frog (*Rana pipiens*) rhodopsin, frog (*Xenopus laevis*) rhodopsin, lamprey rhodopsin, sheep rhodopsin, mouse rhodopsin, human rhodopsin, dog rhodopsin, hamster rhodopsin, chicken rhodopsin, sandgoby rhodopsin, goldfish rhodopsin and bovine rhodopsin, respectively. (modified from Okano and Fukada, 1998)

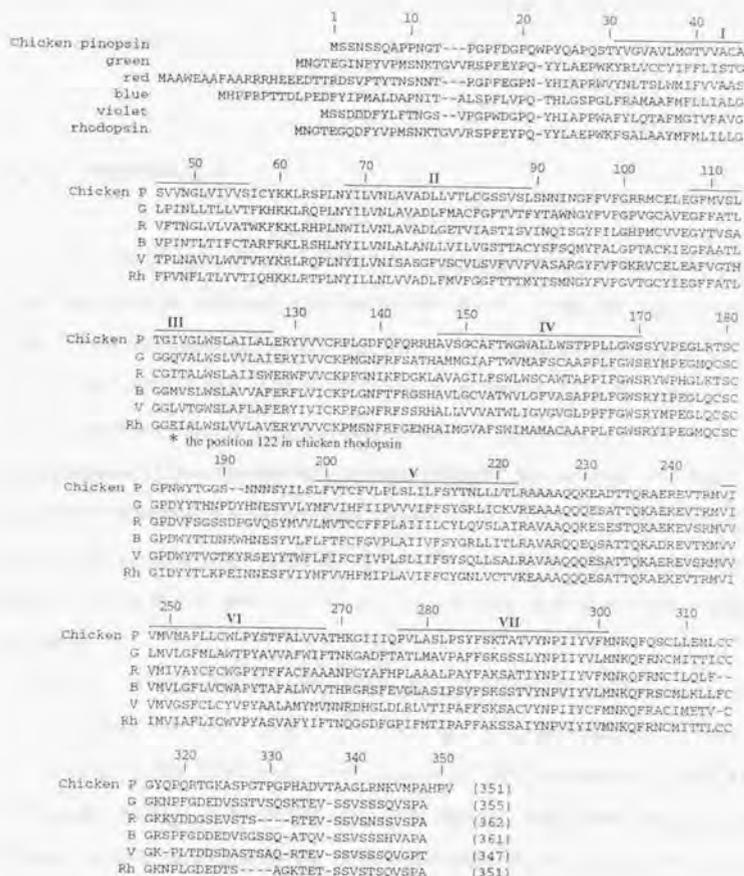


Figure 14. Amino acid sequence alignment of chicken pinopsin and chicken visual pigments. Gaps (-) are inserted for the optimal alignment of the sequences. Putative transmembrane domains (I-VII) are indicated by horizontal lines. Asterisk indicates amino acid residues at the position 122 in chicken rhodopsin which mainly determine the meta II decay rate of rod and cone pigments.

#### 4. Regulatory mechanism of stability of pinopsin meta II intermediate

##### 4.1 Introduction

In the previous section, I demonstrated that the decay of metapinopsin II is relatively slow (rod-type) and that its formation is relatively rapid (cone-type). This indicates that pinopsin is a new type pigment with chimeric nature between rod and cone visual pigments in terms of the thermal behaviors of the meta II intermediate. The rod-type decay process of metapinopsin II was unexpected, because pinopsin has several cone-type features as described in the previous section (3.4). It is now most likely that the meta II lifetime of pinopsin is regulated by an unidentified mechanism different from that determining the meta II life time of rhodopsin and cone pigments.

Relatively unstable property of the meta II intermediate seems to be common to all the cone pigments investigated so far (Shichida *et al.*, 1994; Okada *et al.*, 1994; Imai *et al.*, 1995; Imai *et al.*, 1997a; Imai *et al.*, 1997b). Then I searched for characteristic sequence features which are common to all the known cone pigments but are diverged in pinopsin to identify the amino acid residue(s) responsible for the difference in the meta II stability between pinopsin and cone visual pigments. This comparison revealed that two amino acid residues, Ser171 and Asn184 in pinopsin (Figure 15B), are replaced in all the known cone pigments by Arg and Asp, respectively, and that two amino acid residues are inserted into cone pigments at a position between 190 and 191 in pinopsin (Figure 15B). Importantly, all the three positions are

located in the second extracellular loop (Figure 15A), and these sequence features of cone pigments are also found in almost all the vertebrate rhodopsins. In the present study, I designed site-directed mutants of pinopsin mutated at these positions, and investigated the decay processes of their meta II intermediates. The results suggest that the lack of two amino acid residues between Ser190 and Asn191 in pinopsin participates in the stabilization of metapinopsin II.

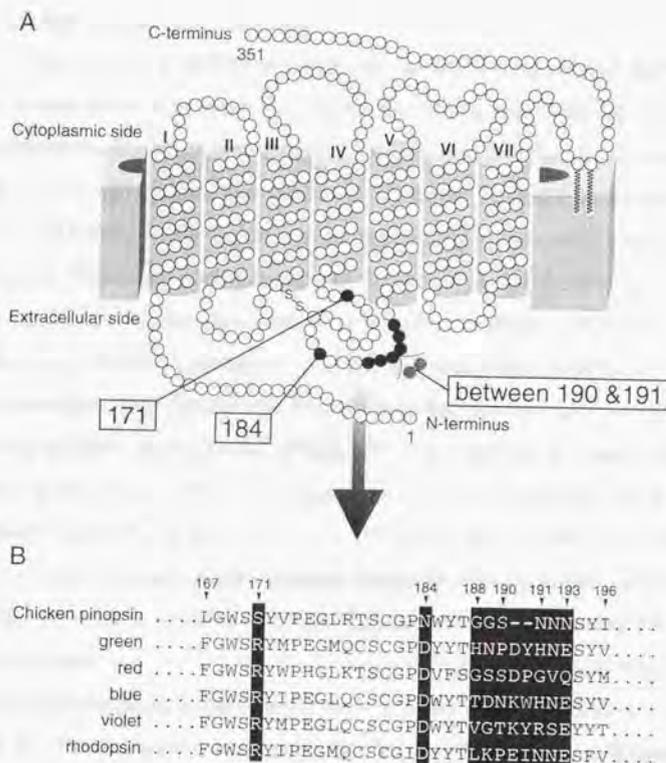


Figure 15. Selection of amino acid residues of pinopsin for site-directed mutagenesis.

*Panel A:* The residues of pinopsin mutated in this study are indicated by closed circles.

*Panel B:* The amino acid sequence of the loop domain between helices IV and V of pinopsin is aligned with those of visual pigments, and the mutated residues of pinopsin are compared with the others (white characters on black background).

## 4.2 Experimental procedures

### *Preparation of Mutant Pigments.*

The cDNA of chicken pinopsin was modified so as to have additional six histidine residues at the N-terminus for affinity purification as described in the previous section (see section 3.2). Site-directed mutations were introduced into the modified constructs by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's manual. The entire coding regions of the modified cDNA's were sequenced by the dideoxy termination method. Each of the mutated cDNA in pBluescript II KS+ (Stratagene) was excised with *Hind*III and *Eco*RI and subcloned into the *Hind*III and *Eco*RI site of the mammalian expression vector pUSR $\alpha$  (Kayada *et al.*, 1995), which is a derivative of pUC-SR $\alpha$  (Shimamoto *et al.*, 1993). Each pinopsin mutant was expressed by transient transfection of 293S cells as described (Gorman *et al.*, 1990). Reconstitution, extraction, and purification of mutant pigments were performed as described in the previous section (section 3.2). Briefly, the cells expressing the recombinant opsin were harvested, and incubated with 11-*cis*-retinal to regenerate the photopigment in the dark or under dim-red light (>660 nm), and the proteins were solubilized with 1% (w/v) dodecyl- $\beta$ -D-maltoside (DM; Dojindo Laboratories). The mutant pigments were purified by three steps of column chromatography by using DEAE-Sepharose (Amersham Pharmacia Biotech), Probond nickel-charged agarose (Invitrogen), and SP-Sepharose (Amersham Pharmacia Biotech).

*Spectrophotometry.*

The absorption spectra of purified pigments were recorded in a system reported previously (Kojima *et al.*, 1995). The sample was irradiated with light from a 1 kW tungsten halogen lamp (Rikagaku Seiki). The wavelengths of the irradiation light were selected by using a glass cutoff filter (VY-50; Toshiba).

### 4.3 Results

First, I prepared two kinds of single amino acid substitution mutants, P-S171R and P-N184D termed P-m1 and P-m2, respectively. In the study on a mutational insertion of the two amino acid residues in the second extracellular loop, a remarkable divergence in sequence found in this region (188-193) made it difficult to determine the position where two residues are to be inserted (see Figure 15B). For example, the two amino acid insertions are depicted at the position between 190 and 191 of pinopsin in one model (Okano *et al.*, 1994), but the insertions are at the position between 187 and 188, or between 193 and 194 in the others (Blackshaw and Snyder, 1997; Blackshaw and Snyder, 1999). Therefore, instead of a simple insertional mutagenesis, I prepared a mutant P-188Gr193 termed P-m3, in which 6 amino acid stretch (188-193 in chicken pinopsin) was replaced by corresponding 8 amino acid residues of chicken green (194-201). I also prepared two kinds of their combined mutants P-m1/m2 and P-m1/m2/m3. All the mutants except for P-m3 bound 11-*cis*-retinal and formed photopigments. The  $\lambda_{\max}$ 's of P-m1, P-m2, P-m1/m2, and P-m1/m2/m3 were almost identical with that of wild-type pinopsin (Table 1), suggesting that these mutations give no significant effect on the conformation of pinopsin (especially that near the chromophore).

The lifetimes of meta II intermediate of the pinopsin mutants were compared with that of wild-type pinopsin (Figure 16). In the thermal reaction of photoactivated wild-type pinopsin at 2 °C (Figure 16A and F), the absorbance at ~380 nm decreased with concomitant increase in absorbance at ~450 nm, representing the conversion process of metapinopsin II to metapinopsin III (see also section 3.3). At this temperature, the conversion

processes of meta II to meta III intermediate were observed in all the pinopsin mutants examined (Figure 16B, C, D, E, G, H, I and J). The time constants for the meta II decay processes of P-m1 (29 min) and P-m2 (32 min) were slightly smaller than that of wild-type pinopsin (41 min, Figure 16G, H and Figure 17). These effects on the meta II decay were, however, canceled in the double mutant P-m1/m2, whose time constant (40 min, Figure 16I and Figure 17) was almost the same as that of wild-type pinopsin. Thus, the set of Ser171 and Asn184 seems to contribute little to stabilization of metapinopsin II. The Arg and Asp residues at the corresponding positions of visual pigments might cooperatively stabilize the meta II of visual pigments, possibly through an electrostatic interaction between them. In contrast, the meta II decay of the triple mutant P-m1/m2/m3 is much faster (13 min; Figure 16J and Figure 17) than that of the double mutant P-m1/m2. These results indicate that the deletion of two amino acid residues between Ser190 and Asn191 of pinopsin plays a critical role in stabilization of metapinopsin II.

Table 1:  $\lambda_{\max}$  of pinopsin mutants

Mutant	$\lambda_{\max}$ (nm)
Wild type	468
P-S171R (m1)	468
P-N184D (m2)	468
P-188Gr193 (m3)	— *
P-S171R/N184D (m1/m2)	469
P-S171R/N184D/188Gr193 (m1/m2/m3)	465

\* no pigment formation with 11-*cis*-retinal

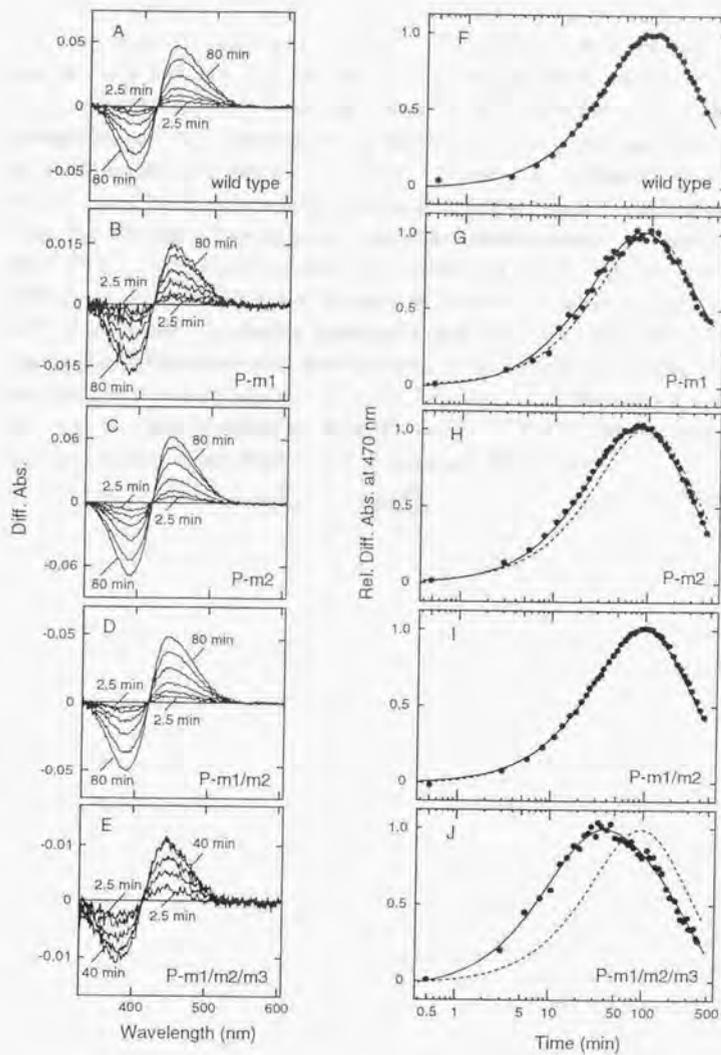


Figure 16. Thermal decay processes of meta II intermediates of pinopsin mutants at 2 °C.

Figure 16. Thermal decay processes of meta II intermediates of pinopsin mutants at 2 °C.

*Panel A-E:* Wild-type pinopsin and its mutants were irradiated with yellow light (>480 nm) for 30 s at 2 °C, and the samples were subsequently incubated in the dark at the same temperature. The curves were the difference spectra between the spectrum recorded immediately after the irradiation and those recorded 2.5, 5, 10, 20, 40, and 80 min after the irradiation (Panel A, wild type; Panel B, P-m1; Panel C, P-m2; Panel D, P-m1/m2), or those recorded 2.5, 5, 10, 20, and 40 min after the irradiation (Panel E, P-m1/m2/m3).

*Panel F-J:* The thermal conversion processes of meta intermediates of wild-type pinopsin (Panel F), P-m1 (Panel G), P-m2 (Panel H), P-m1/m2 (Panel I) and P-m1/m2/m3 (Panel J) were shown by relative absorbance changes at 470 nm plotted against incubation time after the irradiation. The absorbance changes of pigments were simulated by a combination of two sequential single-exponential curves. The first phase of the transitions represents the formation of meta III from meta II (i.e., the decay of meta II), and the second phase represents the decay of meta III. The profile of the wild-type pinopsin (Panel F) is reproduced in each of Panels G-J (broken line).

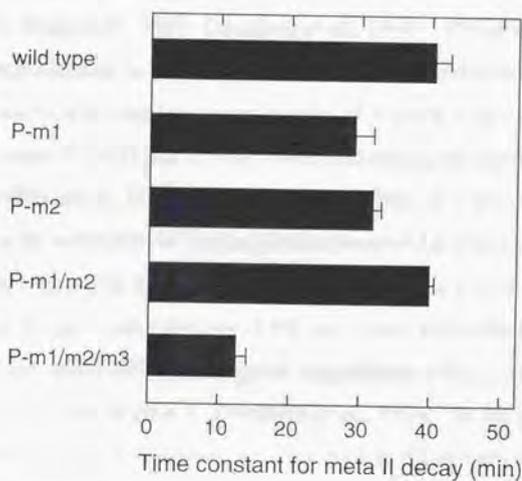


Figure 17. Comparison in time constant for the meta II decay process among wild-type pinopsin and mutants. The mean time constants for the meta II decay process were 41 min (wild type), 29 min (P-m1), 32 min (P-m2), 40 min (P-m1/m2), and 13 min (P-m1/m2/m3), respectively. The standard deviations were estimated from three (P-m1, P-m2 and P-m1/m2/m3) or four (wild type and P-m1/m2) independent experiments, each of which was performed by using independently expressed proteins.

#### 4.4 Discussion

In the present study, I showed that the deletion of two amino acid residues between Ser190 and Asn191 at the second extracellular loop of pinopsin significantly contributes to the meta II stability of pinopsin whereas Ser171 and Asn184 did little. In rhodopsin, similar site-directed mutagenesis in the loop region has been performed extensively (Karnik *et al.*, 1988; Doi *et al.*, 1990; Sung *et al.*, 1991; Davidson *et al.*, 1994). These studies suggested that several residues in the loop region are cooperatively involved in forming the specific folded structure at the intradiscal surface, and that a disulfide bond between Cys110 and Cys187 of bovine rhodopsin stabilizes this structure (Doi *et al.*, 1990; Davidson *et al.*, 1994). It is also shown that many mutants with substitutions in the second extracellular loop fail to regenerate pigments, suggesting that a particular structure on the intradiscal surface is important for the correct folding of the membrane-embedded helices. Furthermore, the disulfide bond gives a significant effect on the thermal stability of metarhodopsin II (Davidson *et al.*, 1994). In the present study, I show that the lack of two amino acid residues in the second extracellular loop of pinopsin plays an important role in the stabilization of metapinopsin II. The fact that mutant P-m3 failed to bind 11-*cis*-retinal may indicate that the deletion is also required for the correct folding of pinopsin. The characteristic sequence in the second extracellular loop could be involved in forming a certain overall structure of pinopsin, and this could play an important role in stabilizing the meta II intermediate.

The deletion of two amino acid residues in the second extracellular loop has been found not only in pinopsin but also in the other non-visual-type vertebrate opsins such as RGR (Jiang *et al.*, 1993), VA-opsin (Soni and

Foster, 1997), peropsin (Sun *et al.*, 1997), parapinopsin (Blackshaw and Snyder, 1997), melanopsin (Provencio *et al.*, 1998), encephalopsin (Blackshaw and Snyder, 1999) and VAL-opsin (Kojima *et al.*, in press). It is possible to speculate that meta II intermediates of these non-visual-type opsins may be more stable than those of cone visual pigments. Interestingly, all the known invertebrate opsins also lack the two amino acid residues, suggesting that the insertion of the two residues took place during the molecular evolution of vertebrate visual pigments from its common ancestral form. In the case of invertebrate rhodopsin, acid metarhodopsin, a physiological active intermediate (Suzuki *et al.*, 1995), is stable even at room temperature (Kropf *et al.*, 1959). Taken together, I can speculate that the evolution of cone pigments from an ancestral pigment accompanied the acquirement of the two amino acid insertions to shorten the lifetime of the meta II intermediate.

## 5. General discussion

In this study, I showed that the decay of metapinopsin II is relatively slow (rod-type) in spite of several cone-type features. What is the physiological significance of the relatively long lifetime of metapinopsin II? A simple speculation is that pinopsin might have been developed so as to detect changes in the environmental light conditions by stabilizing its active state for mediating circadian photoregulation in the pineal gland. The cellular structure of the pinealocytes may suggest another role of the thermal stability of metapinopsin II: The outer segment of the retinal photoreceptor cell is a specialized unit composed of highly stacked multi-lamellar structure capable of detecting light effectively. On the other hand, the outer segments of avian pinealocytes are generally degenerated without regular structures (Ohshima and Matsuo, 1991; Okano *et al.*, 1997). Thus it is likely that pinopsin acquired the long-lived active state to effectively activate G-protein (and/or other signaling molecules) at the expense of time resolution. In addition to the long lifetime of metapinopsin II, a reduced number of phosphorylation sites in pinopsin C-terminal tail (Okano *et al.*, 1994) might be also important in extending the time period for activation of the phototransduction cascade.

Alternatively, the long-lived active state of pinopsin might be favorable to interact simultaneously with two kinds of G-proteins. In chicken pinealocytes, light causes two distinct effects; one is the acute suppression of

melatonin synthesis, and the other is the phase-shifting of the circadian rhythm of melatonin synthesis (Zatz and Mullen, 1988; Takahashi *et al.*, 1989). The acute light-signaling pathway is sensitive to pertussis toxin-treatment, whereas the phase-shifting pathway is insensitive (Zatz and Mullen, 1988; Takahashi *et al.*, 1989). The long lifetime of metapinopsin II might be required to trigger the two pathways simultaneously or with a time-delay by activating both transducin and a certain pertussis toxin-insensitive G-protein such as  $G_{z\alpha}$  (Max *et al.*, 1998) and  $G_{11\alpha}$  (Matsushita *et al.*, in press).

Recently, *Drosophila* blue-light photoreceptor, cryptochrome (CRY) is implicated as a circadian photoreceptor (Emery *et al.*, 1998; Stanewsky *et al.*, 1998). However, the locomotor behavior of the *cry* mutant fly can be entrained to the light-dark cycle (Stanewsky *et al.*, 1998), suggesting an involvement of the other circadian photoreceptor(s). In behavioral entrainment study of *Drosophila*, dietary depletion of carotenoids caused a decrease in light sensitivity of the flies' entrainment to light-dark cycles (Ohata *et al.*, 1998). This suggests that an opsin-type photoreceptive molecule also play an important role as a circadian photoreceptor in *Drosophila*. In mammals, two cryptochrome genes (*cry1* and *cry2*) have been isolated (Todo *et al.*, 1996; van der Spek *et al.*, 1996; Hsu *et al.*, 1996; Miyamoto and Sancar, 1998). Even in the mice lacking *cry1* and *cry2*, the apparent behavioral rhythmicity is observed under light/dark conditions, but the behavior immediately became arrhythmic when the mice are transferred to constant dark condition (van der Horst *et al.*, 1999; Vitaterna *et al.*, 1999). Moreover, CRY1 and CRY2 interact with clock component proteins in light-independent manner (Griffin Jr. *et al.*, 1999). These studies suggest that, in

mammals or at least in mice, cryptochromes do not retain the photoreceptive function responsible for the photoentrainment of clock (Griffin Jr., *et al.*, 1999). In vertebrates, opsin-type photoreceptive molecule may play a major role in the photo-entrainment of the oscillator.

Recently, it was reported that light regulates the activity of MAPK in the chicken pineal gland (Sanada *et al.*, 2000) and in the SCN of mouse (Obrietan *et al.*, 1998). Especially in the chicken pineal gland, MAPK seems to play a pivotal role in the clock oscillator (Sanada *et al.*, 2000). Since many G-protein-coupled receptors were shown to regulate the MAPK cascade (reviewed by Luttrell *et al.*, 1999), it is possible that light-signal captured by pinopsin in the chicken pineal gland regulates the MAPK cascade and resets the phase of the endogenous circadian pacemaker.

In the present study, I have developed an over-expression system providing a sufficient amount of purified pinopsin. This system enabled me to investigate for the first time the photobiochemical properties of pinopsin in detail, and I showed that pinopsin has chimeric nature between rod and cone visual pigments. Because the physiological contribution of this interesting property to the chicken pineal phototransduction pathway remains a matter of speculation, it is necessary to study the interaction of pinopsin with signaling molecules such as G-protein, G-protein-coupled receptor kinase, arrestin or other unidentified molecules by using purified pinopsin in future studies. Such an analysis would also provide information about pineal-specific photon-signaling mechanisms including a possible photo-regulation of the endogenous circadian pacemaker in the chicken pineal gland.

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「松果体の光受容と概日リズム」

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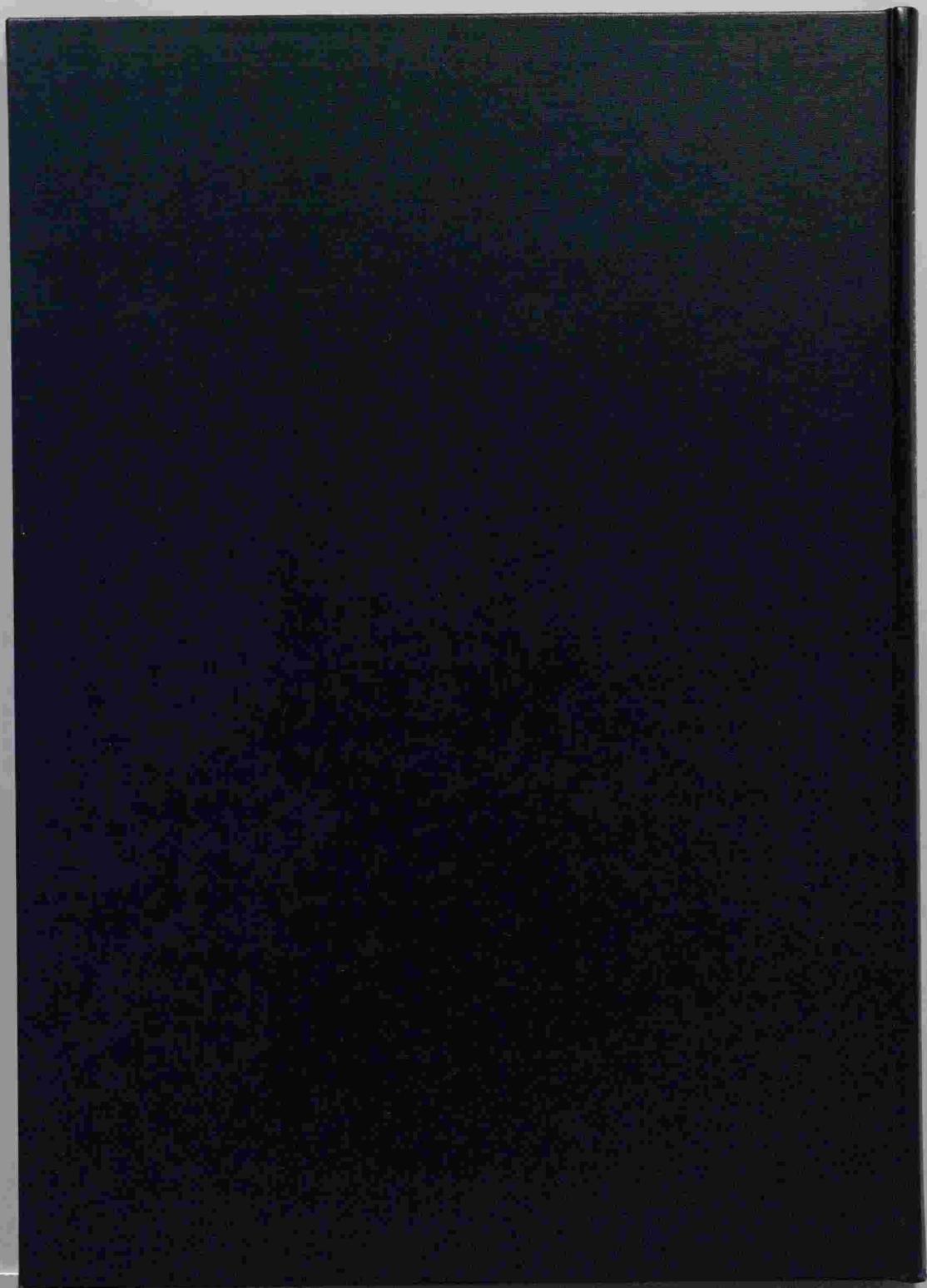
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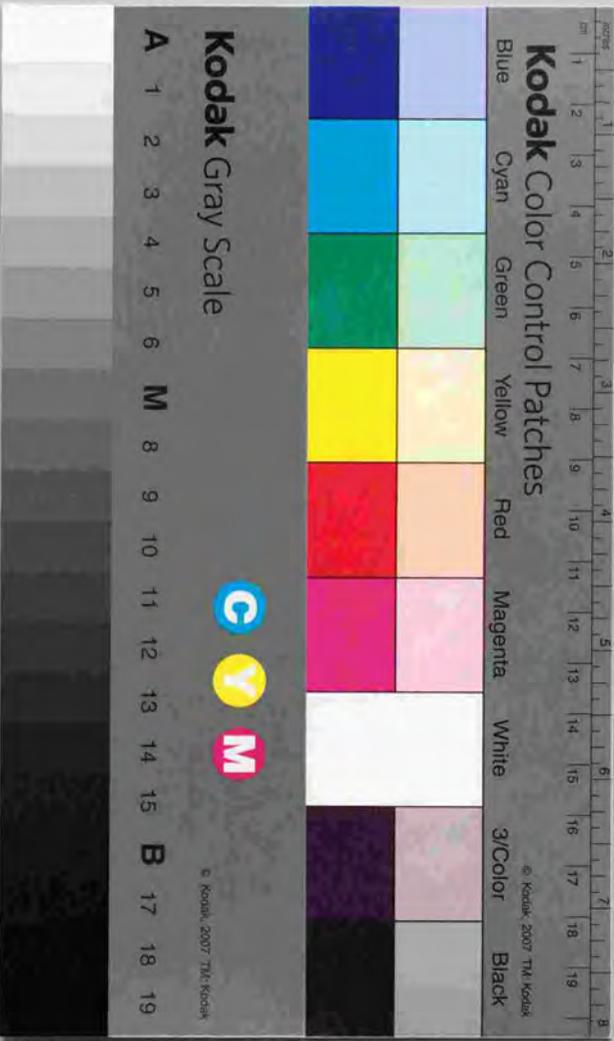
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