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Regulatory Mechanism for the Stability of the Meta II Intermediate of Pinopsin[1]

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Pinopsin is a chicken pineal photoreceptive molecule with a possible role in photoentrainment of the circadian clock. Sequence comparison among members of the rhodopsin family has suggested that pinopsin might have properties more similar to cone visual pigments than to rhodopsin, but the lifetime of the physiologically active intermediate (meta II) of pinopsin is rather similar to that of metarhodopsin II, which is far more stable than meta II intermediates of cone visual pigments [Nakamura, A. *et al.*, (1999) *Biochemistry* 38, 14738–14745]. In the present study, we investigated the amino acid residue(s) contributing to this unique property of pinopsin by using site-directed mutagenesis to pinopsin-specific structural features, (i) Ser171, (ii) Asn184, and (iii) the second extracellular loop two-amino acids shorter than that of cone visual pigments. The meta II stability of the 171/184 double mutant of pinopsin (S171R/N184D) is almost the same as that of wild-type pinopsin. In contrast, the meta II lifetime is markedly shortened (one third) by introduction of the third mutation (replacement of a six-amino acid stretch, 188–193, by the corresponding eight residues of chicken green-sensitive cone pigment) to the 171/184 double mutant of pinopsin. Consistently, meta II of the green-sensitive pigment mutant, in which the eight-amino acid stretch is inversely replaced by the corresponding six residues of pinopsin, is more stable than meta II of the wild-type pigment. These results strongly suggest that the specific sequence and/or the number of residues at amino acids 188–193 in pinopsin play an important role in the stabilization of the meta II intermediate.

Key words: chicken pinealocyte, circadian clock, meta II intermediate, pinopsin, rhodopsin.

The chicken pineal gland has a circadian clock and its phase is reset by an environmental light signal, which is captured by an endogenous photoreceptive molecule (*1*). A candidate molecule responsible for this photosensitivity is pinopsin, a blue-sensitive pigment classified into a novel subtype of the vertebrate rhodopsin family (*2*). Pinopsin is a relatively unstable protein, and only a limited amount of pinopsin is expressed in the pineal gland (*3*). For functional studies on pinopsin, our efforts had been directed to establishing an overexpression system (*4*, *5*), which enabled us to investigate the photochemical and biochemical properties of pinopsin. In a previous report (*4*), we studied in detail the thermal behavior of the physiologically active intermediate (meta II) of pinopsin. This issue is particularly important for understanding pineal cell physiology, because, in the retina, the difference in meta II behavior between rod and cone visual pigments has been implicated in cellular photoresponses characteristic of rod and cone photoreceptor cells (*6*), while the rate of phosphorylation of meta II and its subsequent binding to arrestin also seem to be important (*7*). On the basis of both its evolutionary background (*2*) and its structural similarity to cone pigments (*8*), we first expected that pinopsin would be functionally similar to cone pigments. Pinopsin has, however, been shown to have the chimeric feature of cone and rod pigments (*4*), that is, the meta II formation of pinopsin is relatively rapid (cone-type) while, in contrast, its decay is relatively slow (rod-type). Previously, some of the authors have demonstrated that the amino acid residue at position 122 in retinal opsins (chicken rhodopsin numbering) is the major determinant for the meta II decay rates characteristic of rod and cone pigments (*9*). Almost all sequenced rhodopsins have a glutamate at this position, whereas most of cone visual pigments have a neutral amino acid. In pinopsin, despite the presence of a neutral residue, isoleucine, at this position, the meta II intermediate shows a slow decay process as in rhodopsin, and hence the "122 rule" for the meta II decay rate (*9*) is not simply applicable to pinopsin. It is most likely that the unexpectedly long lifetime of metapinopsin II is regulated

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Abbreviations: DM, dodecyl-β-D-maltoside; loop E2, the second extracellular loop; λ_{max}, absorption maximum in the visible region.

by a mechanism distinct from that determining the meta II lifetime of retinal rod/cone pigments.

In this study, we searched for characteristic sequence features common to all the known cone pigments that are different in pinopsin, because we expected that such residue(s) would contribute to the difference in the meta II stability between pinopsin and cone visual pigments. This comparison showed that two amino acid residues, Ser171 and Asn184, in pinopsin are replaced by Arg and Asp, respectively, in all known cone pigments (Fig. 1). The other feature of pinopsin structure is a lack of two residues at the position between residues 190 and 191 (Fig. 1), as pointed out originally by Okano *et al.* (*2*). Interestingly, all of these structural features are found in the second extracellular (E2) loop. In the present study, we designed a series of site-directed mutants of pinopsin and a green-sensitive cone pigment (green). In both, the residues at these positions were mutated to those of the other pigment to evaluate their roles comparatively. Spectroscopic analyses of these mutants showed that differences between the six residues (188–193) of pinopsin and the corresponding eight residues of green have a notable effect on their meta II stabilities, and imply an important role of residues 188–193 in the stabilization of metapinopsin II.

MATERIALS AND METHODS

Preparation of Mutant Pigments—The cDNAs of chicken pinopsin and green were modified so as to have additional six histidine residues at the N-terminus for affinity purification as described in the previous report (*4*). Site-directed mutations were introduced into the modified constructs using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's manual. The entire coding regions of the modified cDNAs were sequenced by the dideoxy termination method. Each mutated cDNA in pBluescript II KS+ (Stratagene) was excised with *Hin*dIII and *Eco*RI and subcloned into the *Hin*dIII and *Eco*RI sites of the mammalian expression vector pUSRα (*10*), which is a derivative of pUC-SRα (*11*). Each mutant was expressed by transient transfection in 293S cells, reconstituted, solubilized, and purified as described (*4*, *12*).

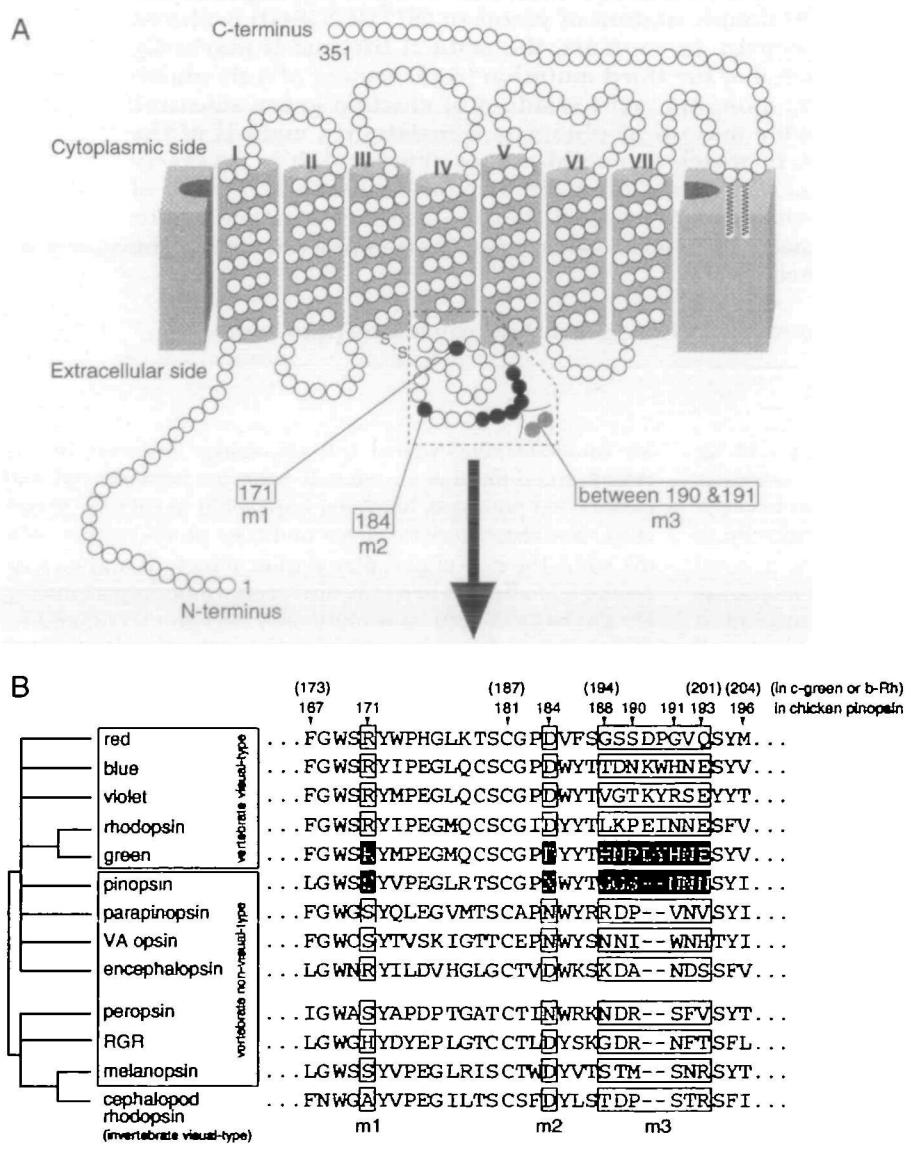

Fig. 1. **Selection of amino acid residues for site-directed mutagenesis.** (A) The heptahelical model of pinopsin. Solid circles represent the positions of residues that were mutated in this study. (B) Phylogenetic relationship among members of the rhodopsin family and their amino acid sequences in the loop domain (E2) between helices IV and V. The sequences of red (chicken), blue (chicken), violet (chicken), rhodopsin (chicken), green (chicken), pinopsin (chicken), parapinopsin (catfish), VA opsin (salmon), encephalopsin (human), peropsin (human), RGR (human), melanopsin (xenopus), and cephalopod rhodopsin (octopus) were obtained from GenBank™, and the residues of pinopsin and green mutated in this study are shown as white characters on a black background. This phylogenetic tree is a schematic drawing and the branch lengths are not meaningful.

Briefly, cells expressing the recombinant opsin were harvested for incubation 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-β-D-maltoside (DM; Dojindo Laboratories). The mutant pigments were purified by three steps of column chromatography on DEAE-Sepharose (Amersham Pharmacia Biotech), Probond nickel-charged agarose (Invitrogen), and SP-Sepharose (Amersham Pharmacia Biotech). The purified pigments were dialyzed against buffer containing 50 mM HEPES-NaOH (pH 6.6), 50 kallikrein inhibitor units/ml aprotinin, 4 μg/ml leupeptin, and 0.02% (w/v) DM. For spectroscopic analysis of the green mutants, glycerol was mixed with the sample at a final concentration of 56% (v/v) in order to determine precisely the rate constant for meta II decay, which was much faster than those of pinopsin and its mutants.

Spectrophotometry—The absorption spectra of purified pigments were recorded with a Shimadzu Model MPS-2000 spectrophotometer, from which the data were transferred to an NEC PC 9801VX computer as reported previously (*13*). The sample in an optical cell (volume, 0.2 ml; width, 2 mm; light path, 1 cm) was kept at 0 or 2°C in a thermostated cell holder in the spectrophotometer. The sample was irradiated with light from a 1 kW tungsten halogen lamp (Rikagaku Seiki). The wavelengths of the irradiated light were selected with a glass cutoff filter (VY-50, VO-54; Toshiba): a 5-cm water layer was placed between the light source and filter to remove the heat from the irradiation light.

RESULTS AND DISCUSSION

We prepared two kinds of pinopsin mutants each with a single amino acid substitution, S171R and N184D (termed P-m1 and P-m2, respectively). A double mutant P-m1/m2 with the combined substitutions (S171R/N184D) was also prepared to assess any effect of a possible electrostatic interaction between Arg and Asp residues on meta II stability. In the case of bovine rhodopsin, the corresponding residues (R177 and D190) are close to each other (*14*), and they seem to interact *via* T193 (Okada, T., personal communication). In designing the third type mutant to evaluate the effect of the insertion of the two residues in the second extracellular loop (loop E2), it was difficult to determine the position for the insertion because of the remarkable divergence in amino acid sequence at 188–193 (Fig. 1B; the two amino acids are tentatively depicted at the position between 190 and 191 of pinopsin according to Ref. *2*). Noticeably, the sequences within this region do not align adequately with any combination of pigments shown in Fig. 1B, and all vertebrate visual pigments have insertions. Due to such ambiguous alignment, we prepared a mutant P-m3, in which the six-amino acid stretch "GGSNNN" (188–193, m3 region in chicken pinopsin) was replaced by the corresponding eight-amino acid stretch of chicken green, "HNPDYHNE" (194–201). These pinopsin mutants and their combined mutant, P-m1/m2/m3, were incubated with 11-*cis*-retinal to reconstitute photosensitive pigments. All the mutants except P-m3 formed photopigments, and their λ_{max} values (465–469 nm) were almost the same as that of wild-type pinopsin (Table I). Moreover, as in the case of wild-type pinopsin, all the reconstituted mutants were stable in the presence of 10 mM NH_2OH for at least 30 min at 2°C (data not shown). These observations suggest that the mutations had only a slight, if any, effect on the conformation near the chromophore of pinopsin.

TABLE I. **λ_{max} of mutant opsins reconstituted with 11-*cis*-retinal.**

Pinopsin	λ_{max} (nm)[a]	Green	λ_{max} (nm)[a]
Wild type	468	wild type	507
P-m1[b]	468	G-m1[d]	—[f]
P-m2[c]	468	G-m2[e]	—[f]
P-m3	—[f]	G-m3	506
P-m1/m2	469	G-m1/m2	—[f]
P-m1/m2/m3	465	G-m1/m2/m3	—[f]

[a]Difference absorption maximum before and after complete bleaching of the sample with light (> 520 nm: for pinopsin or its mutants, > 540 nm: for green or its mutant) in the presence of 10 mM NH_2OH (pH 6.6) at 2°C. [b]S171R of pinopsin. [c]N184D of pinopsin. [d]R177S of green. [e]D190N of green. [f]No photosensitivity formed with 11-*cis*-retinal.

Each pinopsin mutant preparation was subjected to spectrophotometric analysis to measure the lifetime of its meta II intermediate after exposure to yellow light (Fig. 2). In the thermal reaction of photoactivated wild-type pinopsin at 2°C (Fig. 2, A and F), the absorbance at ~380 nm decreased with a concomitant increase in the absorbance at ~450 nm, representing the conversion of metapinopsin II to metapinopsin III (*4*). At this temperature, similar conversions of the meta II to meta III intermediate were observed for all pinopsin mutants examined (Fig. 2, B, C, D, E, G, H, I, and J). As indicated in Fig. 3, the meta II decay processes of P-m1 and P-m2 (time constants, 29 and 32 min, respectively) were slightly faster than that of wild-type pinopsin (41 min). These effects were, however, canceled in the double mutant P-m1/m2, which had a time constant (40 min; Fig. 3) almost the same as that of wild-type pinopsin. Thus, the S171/N184 pair in pinopsin and the R177/D190 pair (with or without electrostatic interaction) in wild-type green seem to contribute little to the difference in meta II decay between pinopsin and cone pigments. In contrast, the meta II intermediate of the triple mutant P-m1/m2/m3 decayed much more rapidly (time constant, 13 min; Fig. 2J) than the double mutant P-m1/m2 (Fig. 3). These results support the idea that a specific sequence and/or the length in the m3 region of the loop E2 plays an important role in stabilizing metapinopsin II.

Our experimental data on pinopsin mutants does not exclude the possibility that the m3 region in pinopsin might play a relevant role only on the background of the double mutant m1/m2. At present, we can not address this issue directly, because the P-m3 recombinant protein does not form a pigment with 11-*cis*-retinal. To evaluate further the functional role of the m3 region, we instead prepared reverse mutants of green. For comprehensive studies, we prepared six kinds of "inverse mutants" of green, in which residues at positions corresponding to the pinopsin mutations were inversely mutated to those of pinopsin. Unfortunately, most of the mutants failed to bind 11-*cis*-retinal (see Table I), but we were able to reconstitute a photosensitive pigment G-m3, in which the eight-amino acid stretch "HNPDYHNE" (194–201 in chicken green) was replaced by the corresponding six amino acid residues of chicken pinopsin, "GGSNNN" (188–193). The λ_{max} of G-m3 (506 nm) was nearly the same as that of wild-type green (507 nm), indicating that the substitution had little, if any, effect on the

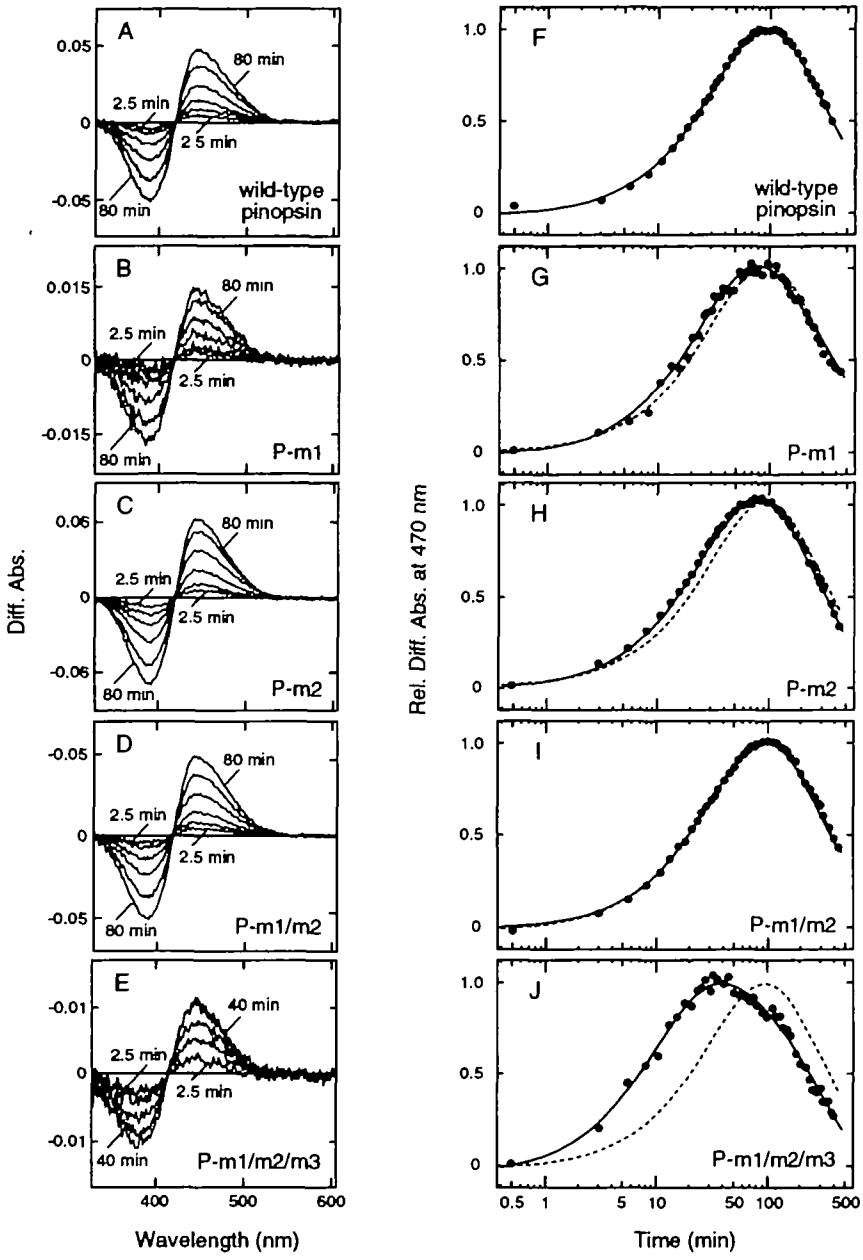

Fig. 2. **Thermal decay processes of meta II intermediates of pinopsin and its mutants at 2°C.** (A–E) Wild-type pinopsin and its mutants were irradiated with yellow light (> 480 nm) for 30 s at 2°C, and the samples were subsequently incubated in the dark at the same temperature. The curves are the difference spectra between the spectrum recorded immediately after irradiation and those recorded 2.5, 5, 10, 20, 40, and 80 min after irradiation [(A), wild type; (B), P-m1; (C), P-m2; (D), P-m1/m2], or those recorded 2.5, 5, 10, 20, and 40 min after the irradiation [(E), P-m1/m2/m3]. (F–J) The thermal conversion processes of meta intermediates of wild-type pinopsin (F), P-m1 (G), P-m2 (H), P-m1/m2 (I), and P-m1/m2/m3 (J) are shown by relative absorbance changes at 470 nm plotted against incubation time after 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 (F) is shown in each Panel (G–J) as a broken line.

conformation near the chromophore of green. Then G-m3 and wild-type green were subjected to spectrometric analyses. As shown in Fig. 4A, we observed the conversion of meta II to meta III of photoactivated wild-type green at 0°C by monitoring the absorbance changes at 470 and 380 nm after irradiation with orange light. The meta II intermediate of the G-m3 mutant decayed significantly more slowly (time constant, 75 s, Fig. 4, B and C) than that of wild-type green (45 s, Fig. 4, A and C), indicating that the meta II intermediate of green was stabilized by the replacement of the eight amino acid residues in loop E2 with the corresponding six amino acid residues of pinopsin. This supports the important role of the m3 region of pinopsin in stabilizing metapinopsin II, and also eliminates the possibility that any mutation in loop E2 may cause a shortening of the meta II lifetime due to general destabilizing effects on the structure.

Because the formation and decay of the meta II intermediate involve relatively large conformational changes in the protein, only a limited combination of amino acid mutations may be insufficient to switch the thermal behaviors of meta II completely between two pigments that show little similarity in their amino acid sequences (*e.g.* pinopsin *vs.* green). Taking this into consideration, the present results that partly explain the difference in the meta II stability between pinopsin and cone visual pigments support an important role of the m3 region in loop E2. If this effect is attributable to any specific sequence in the m3 region, it would be common among the cone visual pigments (but diverged in pinopsin), because the relatively low stability (fast decay) of meta II is a common property of all cone visual pigments (*9*, *13*, *15–18*). However, the amino acid

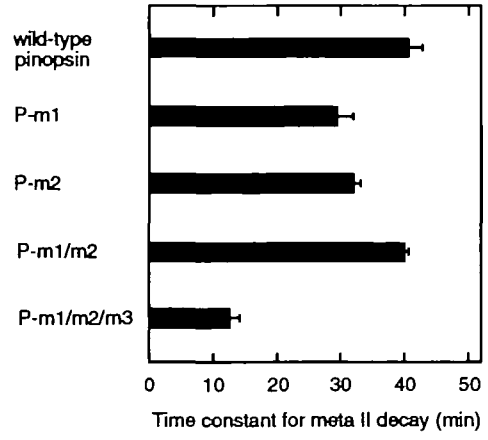

Fig. 3. **Comparison of the time constants for meta II decay among wild-type pinopsin and its mutants.** The mean time constants for meta II decay are 41 min (wild-type), 29 min (P-m1), 32 min (P-m2), 40 min (P-m1/m2), and 13 min (P-m1/m2/m3). Standard deviations were determined from three (P-m1, P-m2, and P-m1/m2) or four (wild type and P-m1/m2) independent experiments, each performed using independently expressed and purified proteins.

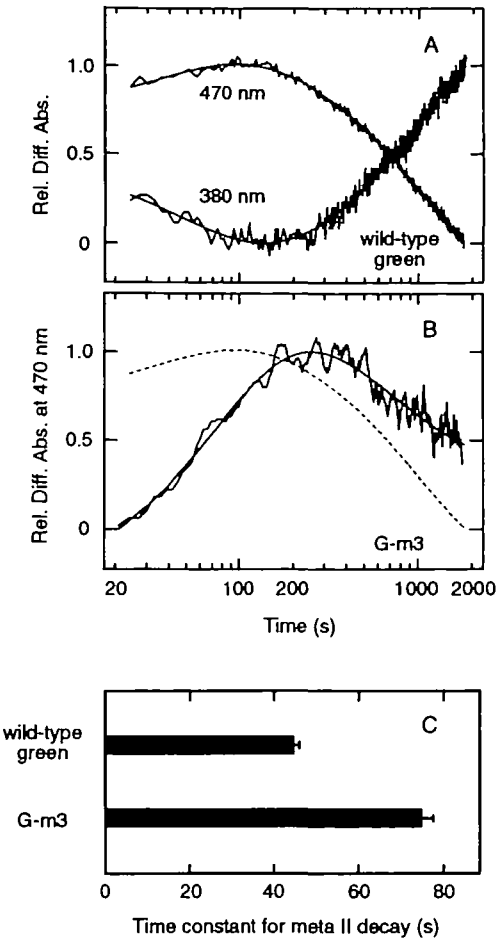

Fig. 4. **Comparison of the meta II decay between wild-type green and its mutant G-m3.** (A) Wild-type green was irradiated with orange light (> 520 nm) for 10 s at 0°C, and the meta II decay was monitored by temporal changes in the absorbance at 380 and 470 nm. These absorbance changes were simulated by a combination of two sequential single-exponential curves. The first phase of the transition represents the conversion of meta II to meta III, and the second phase represents the decay of meta III. (B) The meta II decay of G-m3 was investigated under the same conditions as in (A). The temporal change in the absorbance of G-m3 at 470 nm (solid line) was compared with that of wild-type green [broken line, reproduced from (A)]. (C) Time constants for the meta II decay in wild-type green and mutant G-m3. The mean time constants for the meta II decay were 45 s (wild-type) and 75 s (G-m3), respectively. The standard deviations were determined from three independent experiments, each performed using independently expressed and purified proteins.

sequence of the m3 region is highly diverged among cone visual pigments (Fig. 1), and we cannot find any specific residue(s) responsible for this property common to cone pigments. Instead, the number of residues in the region is consistently different between pinopsin (six residues) and all cone pigments (eight residues). It is likely that the number of residues in the m3 region, rather than the specific sequence, participates largely in determining the difference in the meta II lifetime between pinopsin and cone visual pigments. As shown in Fig. 1, rhodopsin also has a two–amino acid insertion in the m3 region, although its meta II is relatively stable like that of pinopsin. The mechanism stabilizing metarhodopsin II (the "122 rule" in Ref. *9*) should be different from that for metapinopsin II as described in the Introduction: It is predicted that rhodopsin has evolved from a cone pigment by acquiring Glu122, which stabilizes its meta II intermediate (*9*). Here we should note that the lack of two amino acid residues in loop E2 is found not only in pinopsin, but also in all other non-visual-type vertebrate opsins (Fig. 1), including RGR (*19*), VA-opsin (*20*), peropsin (*21*), parapinopsin (*22*), melanopsin (*23*), encephalopsin (*24*), and VAL-opsin (*25*). It is likely that physiologically active (meta II) intermediates of these non-visual-type opsins might be stable just as in the case of metapinopsin II. Interestingly, all known invertebrate opsins also lack two amino acid residues in the m3 region (Fig. 1), suggesting that the insertion of the two residues took place only in the course of the molecular evolution of vertebrate (cone) visual pigments from their common ancestral form. In the case of invertebrate visual pigments, a physiologically active intermediate, acid metarhodopsin (*26*), is stable even at room temperature (*27*). Taken together, we can speculate that the molecular evolution of cone pigments from an ancestral pigment accompanied the functionally important insertion of the two residues into loop E2, leading to a significant shortening of the meta II lifetime favorable to a higher time resolution of vision.

In the present study, we designed a total of ten mutants with amino acid replacements in loops E2 of pinopsin and green, but half of these failed to form photosensitive pigments in the presence of 11-*cis*-retinal (Table I). It has been reported that many rhodopsin mutants with substitutions in loop E2 fail to form pigments (*28–30*), suggesting that a particular structure on the intradiscal surface is important for the correct folding of the membrane-embedded helices. A recently reported 3D structure of rhodopsin (*14*) shows that loop E2 of rhodopsin contains an antiparallel β-sheet structure, and that one strand [β4 (*14*)] is in proximity to the chromophore. This structure might play a role in meta II stabilization, as disruption of the disulfide bond between Cys110 (in loop E1) and Cys187 (in β4 in loop E2) significantly reduces the thermal stability of metarhodopsin II

(*31*). Consistently, our present results demonstrate that the m3 region in loop E2 of pinopsin contributes to the stabilization of metapinopsin II. The accumulating data described above suggest that loop E2 generally plays important roles in both correct folding and meta II stability of a variety of pigments. Moreover, our study strongly suggests that the difference in the number of amino acid residues in the m3 region of pinopsin and visual pigments has a notable effect on their meta II stabilities, possibly due to the formation of different structures in the loop E2 region.

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