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Rhodopsin Chromophore Formation and Thermal Stabilities in the Opsin Mutant E134Q/M257Y

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Rhodopsin, a dim light photoreceptor, has been regarded as one of the model systems for the structural and functional study of G protein - coupled receptors (GPCRs). Constitutively active mutant GPCRs leading to the activation of heterotrimeric GDP/GTP-binding protein signaling in the absence of ligand binding are of interest for the study of the activation mechanism in GPCRs. The present study focused on the opsin mutant E134Q/M257Y, which showed a moderate level of constitutive activity and the formation of two distinct rhodopsin chromophores with absorption maxima of 500 nm and 380 nm, depending on the presence of an inverse agonist, 11-cis-retinal, and an agonist, all-trans-retinal, respectively. Reconstitution of the mutant rhodopsin upon incubation with different ratios of 11-cis-retinal and the all-trans-retinal, as well as upon sequential binding of the two retinals, indicated its preferential binding to 11-cis-retinal. The thermal stability of the 11-cis-retinal-bound form of the E134Q/M257Y mutant was lower than that of the mutants containing a single replacement but higher than that of the all-trans-retinal-bound forms. The mutant also showed a lower stability in its opsin state as compared with that of the wild-type opsin but had little effects on the binding affinity to 11-cis-retinal. Information obtained in this study will be helpful for analyzing the structural changes associated with the activation of rhodopsin and GPCRs.

Key words : G protein-coupled receptor (GPCRs), rhodopsin, opsin, constitutive activation, thermal stability

Introduction

G protein coupled receptors (GPCRs) represent the largest membrane protein family transducing a wide variety of signals including hormones, neurotransmitters and physiological (olfactory, taste, and visual) stimuli from outside to inside the cells. Despite of a diverse array of signal molecules recognized by GPCRs, all GPCRs share a common structural motif, seven-transmembrane (TM) helices and conserved amino acid sequences. GPCR has been regarded as one of the major targets for drug discovery due to its involvement in a diverse signal transduction and its association with many diseases in human [6]. Therefore, efforts have been focused to solve the inactive state as well as the active state structures of GPCRs to develop a compound modulating the specific GPCR signaling through a structure-based approach.

Rhodopsin is a dim light photoreceptor composed of an apoprotein, opsin, and an inverse agonist, 11-*cis*-retinal. Upon photoactivation, isomerization of 11-*cis*-retinal to an agonist, all-*trans*-retinal, triggers a series of conformational changes in rhodopsin initiating enzymatic cascade in visual signal transduction. Rhodopsin has been served as the prototypic system for the structural and functional study of GPCRs [3,7] for its easier preparation and biophysical analysis. Rhodopsin was the first GPCR of which the structure was solved by X-ray crystallography [13,15] and recently solved crystal structures of an agonist-bound form of rhodopsin [2,22] and other GPCRs [18,24] provided structural insights on the conformational changes associated with GPCRs activation.

Constitutive activities in GPCRs leading to an activation of G protein without ligand binding (in case of mutant rhodopsin without light activation) have been found in many GPCRs [1,16]. Constitutively active mutants are of interest for the structural study of the GPCRs assuming conformational changes occurred in the mutants may mimic the active state conformation even in the absence of agonist stimulation. There have been studies on constitutively active mutants using biochemical and spectroscopic as well as crystallographic technologies [3]. Among the classes of constitutively active rhodopsin mutants, opsin mutant containing E134Q/M257Y replacements formed two types of rho-

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dopsin chromophore depending on the presence of an inverse agonist or an agonist. While the mutant rhodopsin regenerated with 11-*cis*-retinal exerted a moderate level of constitutive activity [5,8], rhodopsin pigment formed in the presence of all-*trans*-retinal showed a stronger constitutive activity close to the level of the light-activated rhodopsin. Therefore, the rhodopsin mutant with distinguishable characteristics including UV/Vis spectra and constitutive activity could be a good candidate for the study of detailed aspects in conformational changes associated with activation. In this study, rhodopsin chromophore formation under the competition of 11-*cis*-retinal and all-*trans*-retinal mixtures and their thermal stabilities were analyzed to examine the effect of the mutations on the binding of an agonist and antagonist.

Materials and Methods

Materials

Detergent n-dodecyl-β-D-maltopyranoside (DM) was obtained from Anatrace (Maumee, OH). Anti-rhodopsin monoclonal antibody 1D4 [11] was coupled to cyanogen bromide-activated Sepharose^{TM}4B. Frozen bovine retinae were from J. A. Lawson Co. (Lincoln, NE) and used for transducin (Gt) preparation [14]. 11-*cis*-retinal and all- *trans*-retinal were purified by HPLC using 6% ethyl ether in hexane as a solvent. 1X PBS buffer contains 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, and 10 mM Na_2HPO_4, pH 7.2. BTP buffer used for opsin purification contains 10 mM 1,3-bis-[tris(hydroxymethyl)-methylaminopropane, pH 6.0]/140 mM NaCl/1% (wt/vol) CHAPS/1% DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine).

Expression of opsin mutants and rhodopsin purification

Opsin gene containing double replacements E134Q/M257Y was constructed from combination of its constituent mutations in pMT4 [8]. Fifteen μg of plasmid DNA were used for transfection of a plate (150x25 mm) of confluent Cos-1 cells. Transfected cells were harvested at 50 hr from the incubation of the cells with DNA [12]. DNA cloning procedures including plasmid isolation and subsequent analysis were carried out as described [21].

For rhodopsin purification, harvested cells were resuspended in 2 ml of 1X PBS per plate followed by incubation with 20 μM of 11-*cis*-retinal or all-*trans*-retinal for 2 hr at 4°C. After solubilization of the cells in the presence of 1% DM at 4°C for 1 hr followed by centrifugation at 35,000 rpm (Beckman Ti 50 rotor) at 4°C for 30 min, rhodopsin were purified by using 1D4 sepharose 4B affinity chromatography as described [12]. Upon washings with 50 column volumes of buffers containing 2 mM sodium phosphate (pH 6.0) and 0.05% DM, rhodopsin were eluted in the same buffer containing 50 μM C′1-9 peptide [12].

Purification of opsin mutants and reconstitution of rhodopsin chromophore

For opsin purification, transfected cells were solubilized in 2 ml of BTP buffer containing 1% DMPC/1% CHAPS and 0.1 mM PMSF (phenyl methyl sulfonyl fluoride)/plate at 4°C for 1 hr followed by centrifugation at 35,000 rpm (Beckman Type 50 Ti rotor) at 4°C for 30 min [19]. The supernatant was incubated with rho-1D4 sepharose at 4°C for 1 hr and passed through Millipore Ultrafree-CL filters by centrifugation followed by washing with 50 bed volumes of BTP buffer. Opsins were eluted by using BTP buffer containing 50 μM peptide, quickly frozen by liquid nitrogen, and stored at -80°C until use.

Stability of the mutant opsin was analyzed by the level of rhodopsin chromophore formed upon incubation in the presence of 1% DMPC/1% CHAPS. Reconstitution kinetic analysis of rhodopsin was carried out with mutant opsin diluted to an absorbance at 280 nm of 0.04 (0.7 μM) in BTP buffer upon incubation with 1/100 volume of 1.5 times molar excess of 11-*cis*-retinal. UV/Vis spectra were taken by using a Perkin-Elmer λ-6 spectrophotometer programmed for repeated scans with a scan speed of 480 nm/min at 20°C. Regeneration kinetics were calculated from an increase in the absorbance at 500 nm.

Measurement of rhodopsin stability and G protein activation

Thermal stability analysis of the mutant rhodopsin was carried out with rhodopsin purified in 2 mM sodium phosphate (pH 6.0) and 0.1% DM [13]. UV/Vis spectra of the mutant rhodopsin were taken upon incubation at temperatures 20°C, 37°C and 55°C in the dark. Stability of the mutants forming a rhodopsin with an absorption maximum of 380 nm was analyzed by the level of 440 nm species appeared upon acidification (30 mM H_2SO_4).

Rod outer segment (ROS) was prepared from bovine retinae and used for purification of Gt by using DE52 column chromatography as described [14]. Transducin activation as-

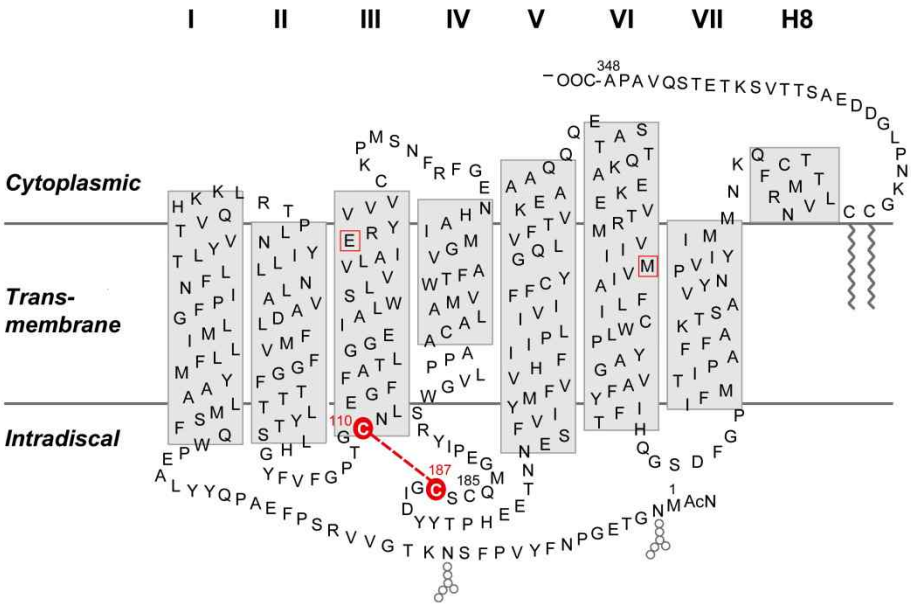

Fig. 1. A secondary structure model of bovine rhodopsin shows the sites of point mutations examined in this study. Positions of the mutations E134Q and M257Y are marked as squares.

say was carried out in a mixture containing 10 mM Tris.Cl (pH 7.4), 100 mM NaCl, 0.5 mM $MgCl_2$, 0.015% DM, 2 nM rhodopsin, 0.25 μM Gt and 3 μM GTP-γ^{35}S (specific activity ~ 5,000 cpm/pmole) as described [9].

Results and Discussions

Expression and purification of mutant rhodopsin

Opsin mutants containing a single replacement such as E113Q, E134Q, and M257Y alone or with combination were constructed as described [5,9,17]. Opsins were expressed from Cos-1 cells upon transient transfection and purified by using 1D4-immunoaffinity chromatography as described [12]. The mutant E134Q/M257Y formed a rhodopsin chromophore with an absorption maximum of 500 nm (Fig. 2A; spectra a) in the presence of 11-*cis*-retinal as similar to those of its constituent mutations E134Q and M257Y and wild-type rhodopsin [5,8]. Upon reconstitution with all-*trans*-retinal, the mutant formed a rhodopsin chromophore with an absorption maximum of 380 nm which is stable enough to be purified (spectra b) although wild type opsin and mutants with a single replacement showed little formation of the all-*trans*-retinal-bound form under the condition (data not shown). Its shift to 440 nm chromophore upon acidification indicated a Schiff base formation between all-*trans*-retinal and opsin (data not shown). While rhodopsin mutants regenerated with 11-*cis*-retinal exerted a moderate level (~3%) of constitutive activity in the dark and a light-dependent activation [9], all-*trans*-retinal-bound form showed a higher level of constitutive activity in the dark (Fig. 2B).

Rhodopsin chromophore formation in the presence of 11-*cis*-retinal and all-*trans*-retinal

In order to examine the effects of the mutations on ligand binding, reconstitution of mutant rhodopsin was carried out with mixtures of 11-*cis*-retinal and all-*trans*-retinal in which the molar ratios between 11-*cis*- and all-*trans*-retinals ranged from 100:1 to 1:100 but keeping the total retinal concentration (20 μM) constant. Fig. 3A showed UV/Vis spectra of the purified mutant rhodopsin reconstituted in the presence of 11-*cis*-retinal (spectra a) or all-*trans*-retinal (spectra g) forming a chromophore with absorption maxima of 500 nm and 380 nm, respectively. Upon reconstitution with 100:1 (spectra b), 10:1 (spectra c), and 1:1 (spectra d) ratios of 11-*cis*-:all-*trans*-retinals, only rhodopsin chromophore with an absorption maximum of 500 nm were detected. Higher level of 11-*cis*-retinal-bound chromophore formation was evident under the condition of 10-fold molar excess of all-*trans*-retinal as compared to that of the all-*trans*-retinal-bound form (spectra e). All-*trans*-retinal-bound form was the major species formed under the condition of 1:100 molar excess of all-*trans*-retinal (spectra f). The results indicated a preferential binding of the mutant E134Q/M257Y with 11-*cis*-retinal as compared to that of all-*trans*-retinal.

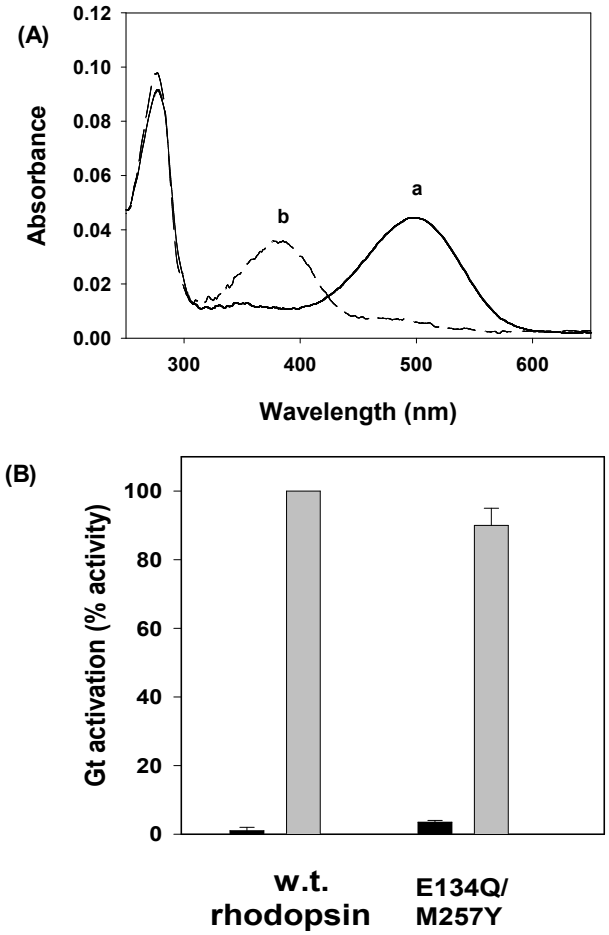

Fig. 2. UV/Vis spectra of E134Q/M257Y mutant rhodopsin (A) and constitutive activity (B). (A) UV/Vis spectra of E134Q/M257Y mutant rhodopsin regenerated with 11-*cis*-retinal (a, dark line) and all-*trans*-retinal (b, dotted line) were taken in the dark. (B) Transducin (Gt) activation analysis was carried out by using GTP-γ-^{35}S filter binding assay and rates were calculated from the slope for Gt activation during initial 5 minutes of the reaction. Analysis was carried out with wild type rhodopsin in the dark (black bar) and upon photoactivation (gray bar). Rhodopsin mutant E134Q/M257Y regenerated with 11-*cis*-retinal (black bar) and all-*trans*-retinal (gray bar) were tested for Gt activation in the dark.

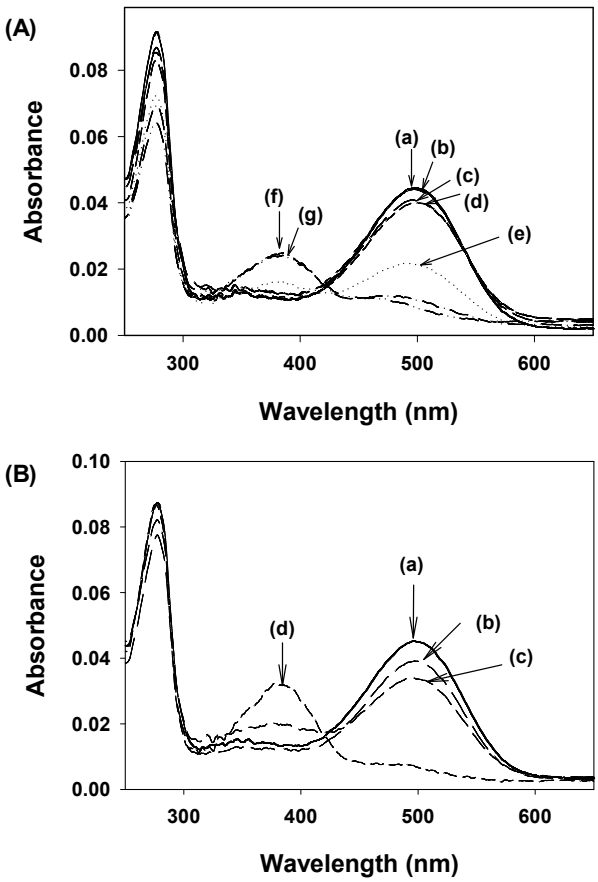

Fig. 3. UV/Visible spectra of the rhodopsin mutant E134Q/M257Y. (A) Cells expressing opsin mutant E134Q/M257Y were incubated with 20 mM of 11-*cis*-retinal (spectra a), 11-*cis*-retinal:all-*trans*-retinal mixtures with their ratios ranging from 100:1 (spectra b), 10:1 (spectra c), 1:1 (spectra d), 1:10 (spectra e) and 1:100 (spectra f), and all-*trans*-retinal (spectra g). (B) Cells expressing mutant E134Q/M257Y were first incubated with 10 mM of 11-*cis*-retinal (spectra a & b) or with all-*trans*-retinal (spectra c & d), and then further incubated with an additional 10 mM of 11-*cis*-retinal (spectra a & c) or all-*trans*-retinal (b & d). All the mutants were purified by using 1D4 sepharose chromatography and eluted in a buffer containing 2 mM sodium phosphate (pH 6.0), 0.05% DM, and 50 mM C'1-9 peptide.

To examine further the differences in the binding of the retinals, cells expressing opsin mutants were first incubated with 10 μM of 11-*cis*-retinal (spectra a & b) or all-*trans*-retinal (spectra c & d), respectively, for 1 hr. They were then incubated with an additional 10 μM of 11-*cis*-retinal (spectra a & c) or all-*trans*-retinal (spectra b & d) for 1 hr followed by purification (Fig. 3B). Almost exclusive formation of 11-*cis*-retinal-bound rhodopsin but no all-*trans*-retinal-bound form were observed in the mutant incubated first with 11-*cis*-retinal (spectra a) followed by all-*trans*-retinal (spectra b). A slight decrease in the level of 500 nm in the latter might result from a lower concentration (10 μM) of 11-*cis*-retinal available for regeneration as compared to the former (20 μM). In comparison to the level of rhodopsin regenerated in the presence of all-*trans*-retinal only (spectra d), a drastic decrease in the level of 380 nm chromophore but a predominant formation of 500 nm chromophore was found in the mutant incubated first with all-*trans*-retinal followed by

11-*cis*-retinal (spectra c). The results indicated that all-*trans*-retinal-bound form of the mutant is not as stable as 11-*cis*-retinal-bound form. The mutant rhodopsin might be dissociated into all-*trans*-retinal and opsin which is then able to form a more stabilized rhodopsin chromophore with 11-*cis*-retinal.

Thermal stability of rhodopsin mutants regenerated with 11-*cis*-retinal and all-*trans*-retinal

Preferential binding with 11-*cis*-retinal observed in the mutant might result from the effects on the stability of rhodopsin chromophores or on the binding affinity for 11-*cis*-retinal. Thermal stability of the mutants containing E134Q and/or M257Y replacement regenerated with 11-*cis*-retinal were analyzed by the level of 500 nm chromophore remained upon incubation at the indicated temperatures. Fig. 4A showed the slowest rate of chromophore decay in wild type rhodopsin, an intermediate level of chromophore decay in the mutants E134Q or M257Y, and the fastest decay in the mutant E134Q/M257Y.

Opsin mutants containing E113Q replacement formed a rhodopsin chromophore with an absorption maximum of 380 nm [5] which is overlapped with that of the free retinal. Therefore, thermal stabilities of the mutants containing E113Q replacements were analyzed by the level of 440 nm chromophore appeared upon acid treatment (Fig. 4B). In comparison to wild type and E134Q/M257Y mutant rhodopsins showing a little decrease in the level of rhodopsin chromophore upon incubation at 37°C, a faster rate of decay was observed in the mutant E113Q/M257Y rhodopsin with a higher constitutive activity [5,9]. The results indicated an inverse relationship between constitutive activity of the mutant and the thermal stability of 11-*cis*-retinal-bound form [9].

Opsin mutants containing double replacements E134Q/M257Y (Fig. 2A) and E113Q/M257Y [9] showed to form a rhodopsin chromophore with an absorption maximum of 380 nm upon regeneration with all-*trans*-retinal. Thermal stability analysis of the all-*trans*-retinal bound forms was also carried out by measuring the level of 440 nm appeared upon acidification. The results showing a faster rate of decay in the all-*trans*-retinal-bound form of the mutant E134Q/M257Y as compared to that of the 11-*cis*-retinal-bound form indicated a direct correlation between constitutive activity and the stability of rhodopsin bound with all-*trans*-retinal

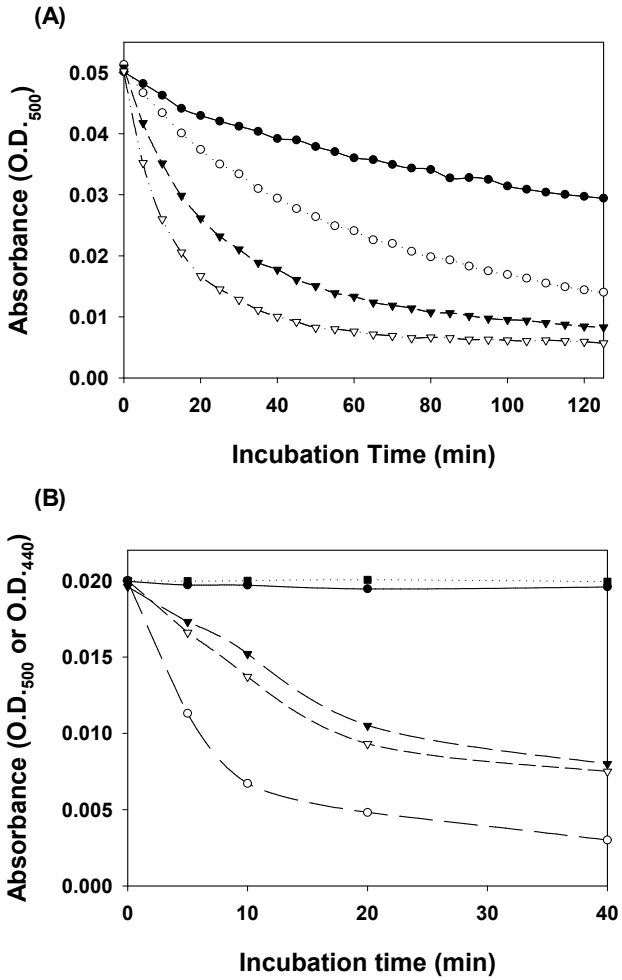

Fig. 4. Thermal stability of E134Q/M257Y mutant rhodopsin regenerated with 11-*cis*-retinal (A) and all-*trans*-retinal (B). (A) Chromophore decays in the mutants containing E134Q and/or M257Y replacements were calculated from the extent of 500 nm chromophore ($O.D._{500}$) remained upon incubation at 55°C. Tested were wild type rhodopsin (closed circle), E134Q (empty circle), M257Y (closed triangle), and E134Q/M257Y (empty triangle). (B) Chromophore decay in the mutants forming rhodopsin with 380 nm absorption maxima were analyzed by the level of 440 nm ($O.D._{440}$) upon incubation at 37°C for the time indicated followed by acidification. Tested were wild type rhodopsin (closed square) and mutants E134Q/M257Y (circle) and E113Q/M257Y (triangle) regenerated with 11-*cis*-retinal (closed symbols) and all-*trans*-retinal (open symbols), respectively.

(Fig. 4B). Overall, a higher stability of chromophore regenerated with an agonist, all-*trans*-retinal, but a lower stability of the chromophore regenerated with an inverse agonist, 11-*cis*-retinal, were found in the rhodopsin mutants with a higher constitutive activity.

Stability of the mutant in the opsin state

To determine whether the mutations affect the opsin stability and its regeneration kinetics, mutants were purified in the presence of 1% DMPC/1% CHAPS lipid-detergent micelles. Thermal stabilities of the mutant opsin were tested by its ability to bind with 11-*cis*-retinal upon incubation in the presence of DMPC/CHAP. Little difference in the levels of rhodopsin chromophore regenerated upon incubation of the opsin indicated that wild type opsin is stable under the condition (Fig. 5). However, levels of rhodopsin chromophore formation were gradually decreased in the mutants E134Q and M257Y as incubation without retinal continued. Opsin mutant E134Q/M257Y showed the steepest decrease in the level of rhodopsin chromophore formation indicating a higher instability in its opsin state. The results showed an inverse correlation between the constitutive activity and opsin stability.

To explore the effects of mutations on the binding affinity for 11-*cis*-retinal, *in vitro* regeneration kinetics of the mutant was analyzed with opsins purified in the presence of 1% DMPC/1% CHAPS. Upon addition of 11-*cis*-retinal, the rate of retinal binding was calculated from an increase in absorbance at 500nm. Mutant opsin E134Q/M257Y showed a rate of regeneration as similar to that of wild type opsin and the mutants containing a single replacement (Fig. 6). A lower extent of chromophore formation was detected in the mutant E134Q/M257Y probably due to a lower stability of the mutant opsin. The results indicated that mutations E134Q and M257Y have little effects on the binding affinity to 11-*cis*-retinal but on the stability of the opsin.

Structural changes associated with E134Q/M257Y mutations

Structural analysis of GPCRs are crucial in pharmaceutical industries to develop a more potent agonist and antagonist modulating GPCR signaling in the cell. Structural information obtained from the inactive state of rhodopsin [13] provided a framework of GPCR structure but limited information on activation mechanism. It took almost 10 years to solve the crystal structure of an agonist-bound form of GPCRs mainly due to transient nature of the active state conformation in GPCRs [2,22]. This was possible by the efforts for overcoming the inherent structural instability of the active state GPCRs such as a mutagenesis introducing a thermal stability to GPCRs [23] and a modification including a deletion of the region with a structural flexibility, and complex formation with G protein or antibody stabilizing the active state conformation [4]. Crystal structures of agonist-

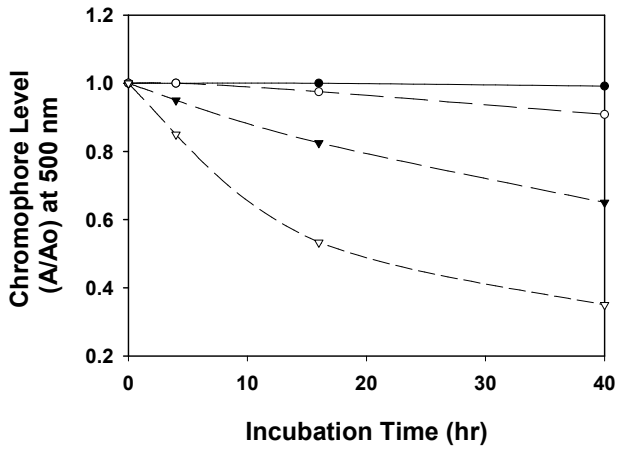

Fig. 5. Relative stability of opsin mutants in DMPC/CHAPS lipid-detergent micelles. Mutant opsin were incubated in 1% DMPC/1% CHAPS at 22°C for indicated times before the addition of 11-*cis*-retinal. Levels of rhodopsin chromophore formation were measured from the absorbance at 500 nm and normalized to that of rhodopsin chromophore formed without preincubation (A/Ao). Tested were wild type opsin (closed circle) and mutants E134Q (empty circle), M257Y (closed triangle), and E134Q/M257Y (empty triangle).

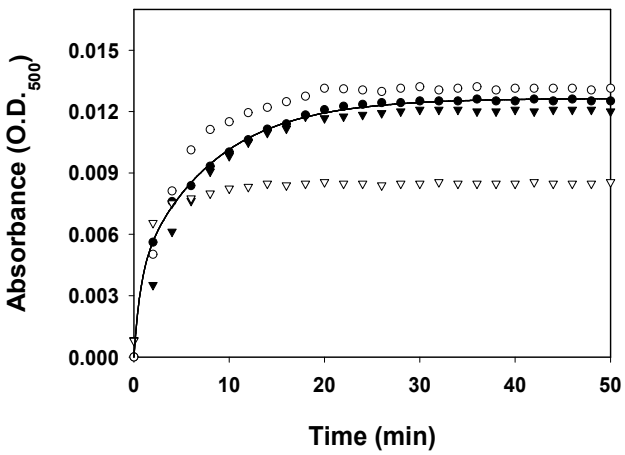

Fig. 6. Regeneration kinetics of mutants containing E134Q and/or M257Y mutations. *In vitro* reconstitution of rhodopsin was carried out with the mutant opsin purified in 1% DMPC/1% CHAPS. UV/Vis spectra were recorded at 20°C with 2 min intervals upon addition of 1 mM 11-*cis*-retinal. The level of 500 nm chromophore ($O.D._{500}$) was plotted against the incubation time. Tested were wild type opsin (closed circle), E134Q (open circle), M257Y (closed triangle), and E134Q/M257Y (empty triangle).

bound forms of beta2 adrenergic receptor and A2A adenosine receptor, together with those of rhodopsin obtained from its opsin state [15] as well as in the presence of 11-*cis*-retinal and all-*trans*-retinal [2,22] provided a structural insight on the activation mechanism of GPCRs [2,18,24]. In addition to the structural information on the inactive and active states of GPCRs, more detailed information on the structural changes associated with activation will be needed to develop a compound for the fine tuning of GPCR signaling. Therefore, the mutant E134Q/M257Y with a moderate level of constitutive activity is a good model system for the structural study.

Constitutive activity analysis of the mutants containing double replacements indicated an additive effect upon combination of E134Q and M257Y but a synergistic effect upon combinations with a mutation perturbing the salt bridge, E113Q [9,20]. Structural analysis using spin-label sensors indicated that E134Q mutation induces a photoactivated conformation around TM III and VI even in the dark state [9] but little change in TM VI which is known to undergo a dominant motion upon photoactivation. This seemed to be relevant to a low level of constitutive activity in the mutant. While a profound movement of both TM III and TM VI were noticed in the mutants including E113Q with a perturbed salt bridge [9,20], a related but distinguishable structural change was shown to occur in the mutant M257Y. The movement of TM VI and other changes are exaggerated in the mutant E113Q/M257Y as compared to those occurred in the single mutants E113Q and M257Y. Our results suggest that conformational changes occurred in E134Q and M257Y mutants might not be the same as the photoactivated rhodopsin as indicated by its lower level of constitutive activity. However, those changes are enough to affect the stability in the the opsin and rhodopsin states but not its binding affinity to 11-*cis*-retinal.

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초록 : 옵신 mutant E134Q/M257Y의 로돕신 형성과 열안정성 분석

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세포막 단백질 중 가장 큰 family를 형성하는 G protein-coupled receptor (GPCR)는 세포 외부의 다양한 신호를 세포 내 G 단백질의 활성화를 통하여 전달한다. 외부 신호자극이 없는 조건에서도 활성을 나타내는 항활성 돌연변이(constitutively active mutants, CAM)는 GPCR 신호전달 이상으로 인한 질병 치료나 GPCR의 활성화 구조연구에 좋은 대상이다. 본 연구는 시각수용체 로돕신에서 약한 항활성을 보이는 CAM의 하나인 E134Q/M257Y를 대상으로, inverse agonist와 agonist 존재 하에서 형성하는 두 가지 chromophore의 특성을 연구하였다. 이 CAM은 11-*cis*-retinal과 all-*trans*-retinal 존재 하에서 각기 최대흡광도가 500 nm와 380 nm인 로돕신을 형성한다. 두 가지 retinal을 다양한 비로 혼합한 조건과 연속적으로 결합하는 조건 하에서 각 형태의 로돕신 형성을 조사한 결과 E134Q/M257Y mutant는 11-*cis*-retinal과 우선적으로 결합함을 보여준다. E134Q/M257Y mutant는 wild type 옵신에 비해 11-*cis*-retinal에 대한 친화도는 별다른 차이가 없으나 옵신과 로돕신 상태의 안정성이 낮음이 확인되었다. 본 연구 결과는 GPCR의 활성화 시 일어나는 부분적 구조변화에 대한 정보를 제공하고, 구조정보에 기반한 GPCR신호를 미세하게 조절하는 물질의 발굴이나 개발에 유용하게 이용될 것이다.