Conformational Changes in Guanylyl Cyclase-activating Protein 1 (GCAP1) and Its Tryptophan Mutants as a Function of Calcium Concentration*

(Received for publication, March 4, 1999)


From the Departments of Ophthalmology, §§Chemistry, ¶¶Pharmacology, ¶¶Biological Structure, and ***Physiology and Biophysics, University of Washington, Seattle, Washington 98195 and the ‡‡Moran Eye Center, University of Utah Health Science Center, Salt Lake City, Utah 84132

Guanylyl cyclase-activating proteins (GCAPs) are 23-kDa Ca$^{2+}$-binding proteins belonging to the calmodulin superfamily. Ca$^{2+}$-free GCAPs are responsible for activation of photoreceptor guanylyl cyclase during light adaptation. In this study, we characterized GCAP1 mutants in which three endogenous nonessential Trp residues were replaced by Phe residues, eliminating intrinsic fluorescence. Subsequently, hydrophobic amino acids adjacent to each of the three functional Ca$^{2+}$-binding loops were replaced by reporter Trp residues. Using fluorescence spectroscopy and biochemical assays, we found that binding of Ca$^{2+}$ to GCAP1 causes a major conformational change especially in the region around the EF3-hand motif. This transition of GCAP1 from an activator to an inhibitor of GC requires an activation energy $E_a = 9.3$ kcal/mol. When Tyr$^99$ adjacent to the EF3-hand motif was replaced by Cys, a mutation linked to autosomal dominant cone dystrophy in humans, Cys$^99$ is unable to stabilize the inactive GCAP1-Ca$^{2+}$ complex. Stopped-flow kinetic measurements indicated that GCAP1 rapidly loses its bound Ca$^{2+}$ ($k_{-1} = 72$ s$^{-1}$ at $37^\circ$C) and was estimated to associate with Ca$^{2+}$ at a rate ($k_1 > 2 	imes 10^{10}$ M$^{-1}$ s$^{-1}$) close to the diffusion limit. Thus, GCAP1 displays thermodynamic and kinetic properties that are compatible with its involvement early in the phototransduction response.

The Ca$^{2+}$-binding motif termed EF-hand, introduced by Neckold et al. (1), refers to the helix-loop-helix structure responsible for selective high affinity ($K_a < 10^{-9}$ M) Ca$^{2+}$ binding. EF-hand motifs, reliably predictable based on primary sequence and present in one to eight copies in some polypeptides, have been identified in over 500 Ca$^{2+}$-binding proteins. Neuronal Ca$^{2+}$-binding proteins (NCBPs)$^1$ are a subset of the EF-hand-containing proteins that are found predominantly in neurons. The function of most of these proteins is largely unknown. The topography of NCBPs is based on four EF-hand motifs, some of which may be functional and some nonfunctional in Ca$^{2+}$ coordination (2). The sequence similarity among members of the NCBP family varies from −25% between calmodulin (CaM) and visinin to ~60% between GCAP1 and GCAP3 (3). NCBPs are acidic and similar in length, with CaM and CaM-like proteins being the shortest (149–150 amino acids; $M_e$, 16,837), and other members of this family being ~200 amino acids in length ($M_e$, ~23,000) (2, 4). GCAPs, a subgroup of NCBPs, activate guanylyl cyclase (GC) in their Ca$^{2+}$-free form. Three mammalian GCAPs, GCAP1 (5), GCAP2 (6, 7), and GCAP3 (3), have been characterized to date. Recently, we have identified a fourth photoreceptor Ca$^{2+}$-binding protein closely related to GCAPs (8). This novel protein does not stimulate GC at nanomolar [Ca$^{2+}$], but inhibits GC at micromolar [Ca$^{2+}$], and is therefore termed guanylyl cyclase inhibitory protein.

A defect in exon 3 of the GCAP1 gene leading to a Y99C missense mutation has recently been linked to autosomal dominant cone dystrophy (9). The Y99C mutation has been shown to alter Ca$^{2+}$ sensitivity of GCAP1, leading to the constitutive activity of GC1 at high [Ca$^{2+}$] where normal GCAP1 is an inhibitor (10, 11). The mutant residue is adjacent (position −1) to the EF3-hand motif, which is important in inactivation of GCAP1 by Ca$^{2+}$ (12).

Ca$^{2+}$-binding proteins may significantly change their conformation upon Ca$^{2+}$ coordination. Multiple EF-hand motifs may allow the Ca$^{2+}$-binding protein to respond cooperatively to changes in [Ca$^{2+}$]. The three-dimensional structures of NCBPs are known for both Ca$^{2+}$-free and Ca$^{2+}$-bound CaM and recoverin (13, 14), Ca$^{2+}$-bound GCAP2, and Ca$^{2+}$-bound unmyristoylated neurocalcin (16). GCAP2, recoverin, and neurocalcin are compact proteins (radius of gyration 16–18 Å) made of two domains separated by a variable linker and are different from a dumbbell arrangement found in CaM and troponin C (reviewed in Ref. 17). The N- and C-terminal domains of NCBPs contain a pair of EF-hands each composed of 29 amino acids. Whereas structural studies give precise answers on conformational changes upon Ca$^{2+}$ chelation, much less is known about the dynamics of individual EF-hand motifs during this transition. Ca$^{2+}$ (occasionally also Mg$^{2+}$) is coordinated via oxygen

This paper is available on line at http://www.jbc.org

* This work was supported by United States Public Health Service Grants EY08061 and EY08123, unrestricted grants from Research to Prevent Blindness (New York) to the Departments of Ophthalmology at the University of Washington and the University of Utah. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ These authors contributed equally to this work.

∥∥Recipient of a Senior Investigator Award from Research to Prevent Blindness.

§§Recipient of a Jules and Doris Stein Research to Prevent Blindness Professorship. To whom correspondence should be addressed: University of Washington, Dept. of Ophthalmology, Box 356485, Seattle, WA 98195-6485. Tel: 206-543-9074; Fax: 206-543-4414; E-mail: palczewse@u.washington.edu.

$^1$The abbreviations used are: NCBP, Neuronal Ca$^{2+}$-binding protein; GC, guanylyl cyclase; GCAP, GC-activating protein; GCAP1 (W21F, W51F, W94F), rGCAP1(w); ROS, rod outer segment(s); CaM, calmodulin; PBR, polymerase chain reaction; bp, base pair(s); MOPS, 4-morpholinopropanesulfonic acid.

atoms typically located on a consecutive sequence of 12 amino acids of the loop within helix-loop-helix structure (18). Importantly, the -1 positions of the EF-hand loops are most frequently occupied by a large hydrophobic amino acid (Ile, Leu, Val, Trp, Tyr, and Phe) (19). This unique position close to the EF-hand loop allows substitution by Trp without major disruption of conformation. Trp fluorescence techniques can then be used to monitor contributions of individual loops to conformational changes that occur during Ca \(^{2+}\) coordination.

In this study we used Trp-GCAP1 mutants to identify conformational changes in GCAP1 from the Ca \(^{2+}\)-bound to the Ca \(^{2+}\)-free form. We asked what role do the endogenous Trp residues play in the function of GCAP1, particularly in its conformational changes coupled to Ca \(^{2+}\) binding and switching from activator to inhibitor of GC? Can functional protein variants be produced that provide spectroscopic signals specifically coupled to individual Ca \(^{2+}\)-binding sites? If so, what are the contributions of each of these binding sites to the functionally important conformational changes? What are the kinetics of Ca \(^{2+}\) binding and release and associated conformational and functional switching? We found that Ca \(^{2+}\) binding at the EF3-hand motif evokes the largest conformational change, compared with other EF-hand motifs. Thus, EF3-hand region may act as a molecular [Ca\(^{2+}\)] switch between activating or inhibiting GC conformations. These findings provide mechanistic insights on how the mutation GCAP1(Y99C) is linked to autosomal dominant cone dystrophy.

**MATERIALS AND METHODS**

**Site-directed Mutagenesis, Removal of Intrinsic Trp from Wild-type GCAP1**—The three mutants, GCAP1(W21F), GCAP1(W51F), and GCAP1(W94F) were generated by inserting mutant cassettes into wild-type GCAP1 expressing GCAP1 in insect cells. For GCAP1(W21F), sense primer T1 (5'-AGG ACC GAC GAG TGC CAC CAG TTC TAC AAG) carrying a DraIII site (underlined) and the TGG to TTC mutation and antisense primer W213A (5'-CCA GTG AAA CAG CAG GCA CCA CCG TCA CAC A C) were used to amplify the mutant GCAP1 product with pVL941bGCAP1 as template (2 ng/50 \(\mu\)l PCR mixture, 100 ng of each primer). Standard concentrations of MgCl\(_2\) and dNTP and regular Taq polymerase (Promega) were used. PCR conditions were 94 °C for 1 min, 69 °C for 1 min, 72 °C for 2 min, cycled 35 times. The product of 638 bp was purified (Qiagen) and digested with DraIII at 37 °C for 1 h and then SfiI was added and restriction continued for an additional hour at 50 °C. For ligation, pVL941bGCAP1 was digested with DraIII and SfiI. For generation of GCAP1(W51F), sense primer T2 (5'-CCT GCG CAG GGG TGG ACT TCA) and antisense primer W513a (5'-CCG TGG CAA GCG TCA) were used to amplify a 188-bp PCR product. For ligation, pVL941bGCAP1 was digested with PmlI/Stul for 4 h at 37 °C. For generation of GCAP1(W94F), the intronic AlwNI site in pVL941 had to be deleted first. Briefly, pVL941 was linearized by digestion with AlwNI, blunt-ended, and re-ligated. Then bGCAP1 in pBluescript-SK was digested with EcoRI/Xhol and the fragment introduced into pVL941 (AlwNI) and digested with the same enzymes. A 418-bp PCR product was amplified with sense primer T3 (5'-TGG AAG AGA AGC TGC GTT TCT ACT TCA-3') (carrying the mutation) and antisense primer W943A. The PCR product and vector pVL941bGCAP1/AlwNI were cut at AlwNI at 37 °C and then SfiI at 50 °C. To isolate the mutant constructs, mutant PCR cassettes were ligated (T4 ligase at 16 °C for 12 h) to the corresponding vectors from which the nonmutant product had been removed by gel electrophoresis. The ligation mixture was transformed in XL1-Blue competent cells. Clones and DNA samples were isolated by standard procedures, and inserts were completely sequenced. DNA vectors containing the correct mutation in GCAP1 were purified using CsCl centrifugation, verified by sequencing, and transfected into HighFive insect cells (PharMingen).

**Generation of GCAP1(W21F,W51F,W94F) or GCAP1(W21F,W51F,W94F,F63W)**—To obtain this construct, first, a double mutant GCAP1(W21F,W51F) fragment was generated. T1 sense and W233A antisense primers were used to generate a mutant fragment by PCR on pVL941bGCAP1(W51F) as template (see description for W21F). The resulting 650-bp PCR product carrying W21F and W51F mutations was ligated into a vector carrying the W94F mutation. The vector pVL941bGCAP1(W94F) was treated with AlwNI and DraIII, and a 335-bp piece was excised and replaced by the (W21F,W51F) fragment. Ligation, transformations, sequencing, and transfections were performed as described above.

**Introduction of Trp into Positions Adjacent to EF2, EF3, and EF4 in GCAP1**—These mutations were generated with a site-directed mutagenesis kit (Qickchange, Stratagene). For generation of W2-GCAP1(W21F,W51F,W94F,F63W) was used as template (10 ng) in a PCR amplification, the primer pair W4a (5'-ATG AGA CCT GGT GAG TCA) and W4s (5'-AGG ACT TCA) was employed. The PCR conditions were 95 °C for 30 s, 49 °C for 1 min, 68 °C for 8 min repeated 16 times. The PCR product had been removed by gel electrophoresis. The ligation mixture was transformed in XL1-Blue competent cells. Clones and DNA samples were isolated by standard procedures, and inserts were completely sequenced. DNA vectors containing the correct mutation in GCAP1 were purified using CsCl centrifugation, verified by sequencing, and transfected into HighFive insect cells (PharMingen).

**Generation of GCAP1(W21F,W51F,W94F) or GCAP1(W21F,W51F,W94F,F63W)**—To obtain this construct, pBluescript-SKIIbGCAP1(W21F,W51F,W94F,F63W) was used as template (10 ng) in a PCR reaction in two steps. In the first step, primers W3a and W3s (to introduce Y99F) were used. In the second step, the PCR product from this amplification step was used as template with primers W4s and W4a to introduce I143W. pVL941bGCAP1 and the mutant constructs after PCR were digested with Stul and XhoI, religated, transformed, purified, and finally transfected into HighFive insect cells.
Expression of GCAP1 Mutants in Insect Cells—The transfer vector pVLbGCAP1 was constructed by subcloning a full-length DNA fragment encoding bovine GCAP1 mutants into pVL1393 vector (Invitrogen). High Five insect cells (2–3×10⁶) derived from the cabbage looper (PharMingen) were cotransfected with 0.5 µg of BaculoGold DNA and 5 µg of pVLbGCAP1 mutants, and assayed as described previously (7). Sixty-four % of the reaction mixture contained 50 mM HEPES, pH 7.8, containing 60 mM KCl, 20 mM NaCl, 10 mM MgCl₂, 0.4 mM EGTA, 2 mM isobutylmethylxanthine, 0.4 mM ATP, 0.16 mM CaCl₂ (45 nM [Ca²⁺]), and washed ROS (80 µg of rhodopsin). [Ca²⁺] was calculated using the computer program “Chelator 1.0” (20) and adjusted to higher concentrations in some assays by increasing the amount of CaCl₂. The reaction was initiated by the addition of 1.3 mM [α-³²P]GTP (5,000–9,000 cpm/nmol). The activity is expressed as the amount of cGMP formed per min per 100 µg of ROS proteins used in the assay. All assays were repeated at least twice.

Fluorescence Measurement—Fluorescence measurements of GCAP1 and its mutants were carried out on a Perkin-Elmer LS 50B spectrophotometer using a 1 × 1-cm quartz cuvette. Emission spectra were recorded with excitation at 280 nm and 290 nm and at 5-nm slit widths. Spectra were determined in 50 mM HEPES, pH 7.0, containing 60 mM KCl, 30 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol, and 10⁻⁸ to 10⁻⁶ M CaCl₂. [Ca²⁺] was calculated as noted previously (20).

Kinetic Measurements—The kinetics of Ca²⁺ binding to GCAP1 mutants were performed on an Applied Photophysics stopped-flow spectrophotometer. The dead time of the stopped-flow apparatus was determined to be 1.1 ms. The light source consisted of a 150-watt xenon lamp. Excitation and emission wavelengths were 290 and 340 nm, respectively. The slit widths were 5 and 5 nm for excitation and emission lights, respectively. Spectra were done in 20 mM MOPS, pH 7.0, containing 70 mM KCl, 30 mM NaCl, 1 mM dithiothreitol, and 0.1 mM EGTA. [Ca²⁺] was adjusted by the addition of CaCl₂ and EGTA (20). In a typical experiment, dissociation of Ca²⁺ from GCAP1 was measured at 1–3 mM protein in the presence of 0.15 mM CaCl₂. This solution (50 µl) was mixed in the stopped-flow apparatus with an equal volume of buffer containing 0.5–2.1 mM EGTA. Data were expressed as a function of free (EGTA) just after mixing, considering that each protein molecule binds three Ca²⁺ molecules, and each EGTA molecule binds one Ca²⁺ molecule in this buffer.

Fluorescence Quenching—Steady state fluorescence measurements were carried out in an SLM 8000C fluorescence spectrometer in the ratio mode by photon counting. Fluorescence quenching was measured after addition of small aliquots of an acrylamide solution to the protein.
sample. Protein samples were excited at 290 nm, and emission intensities were recorded at 340 nm. These fluorescence intensities were corrected for the inner filter effect resulting from the addition of acrylamide to the protein solution (21). Fluorescence quenching data were then analyzed according to the following modified Stern-Volmer equation,

\[ \frac{F_0}{F} = 1 + (K_{SV} + V)[Q] + 1 + K_{eq}[Q] \]  

(Eq. 1)

where \( F_0 \) and \( F \) are the fluorescence intensities in the absence and presence of quencher, \( Q \), respectively. \( K_{SV} \) is the Stern-Volmer quenching constant (also called dynamic quenching constant) for the collisional quenching process. \( V \) is the static quenching constant in relation to a certain effective volume in the immediate vicinity of the fluorophore. The above modified Stern-Volmer equation is derived from the Stern-Volmer equation \( F_0/F = (1 + K_{SV}[Q])x_{sv}^{0.5} \), as \( [Q] \) becomes very small.

**Fluorescence Anisotropy**—Fluorescence anisotropy was determined with the SLM 8000C fluorimeter equipped with a Glan-Thompson polarizer assembly and a micro-magnetic stirrer. The anisotropy is defined as follows,

\[ A = (I_1 - I_3)/(I_1 + 2I_3) \]  

(Eq. 2)

where \( I_1 \) and \( I_3 \) are the fluorescence intensities observed parallel and perpendicular to the direction of polarization of the exciting light, respectively. Standard methods were applied to correct for unequal sensitivities of the detector system for vertically and horizontally polarized light (22). The measurements were done in 20 mM MOPS, pH 7.0, containing 50 mM KCl, 30 mM NaCl, and 3 \( \mu \)M W3-GCAP1 (w−) at 15 ± 0.1°C (\( \lambda_{em} = 290 \) nm, \( \lambda_{ex} = 340 \) nm). [Mg\(^{2+}\)] was 0.4 mM, and [Ca\(^{2+}\)] was 0.5 mM in the sample with cations. The calculations of the rotational correlation time was carried out as described previously (23).

**Homology Model of GCAP1**—A homology model of GCAP1 was created on the basis of the crystal structure of unmyristoylated recoverin (Protein Data Bank entry: 1REC (13)), taking advantage of the sequence alignment (5, 7). The model was generated with the HOMOLOGY module of the INSIGHTII software (Molecular Simulations, Inc., San Diego, CA) using established homology modeling protocols (24). In short, protein backbone coordinates were taken from recoverin for all helices, strands, EF-hand motifs, and loops with identical lengths. Coordinates for other loops were transplanted from appropriate Protein Data Bank entries. Coordinates of conserved side chains were kept. Nonconserved side chains were built from a rotamer database. Finally, 2,000 steps of conjugate gradient energy minimization were executed to alleviate small irregularities in the structure. This model is C-terminally truncated after Thr\(^{170}\) because of the lack of sequence identity with the recoverin structure. Although the structure of GCAP1 is unknown, superposition of the main chain structures of unmyristoylated, Ca\(^{2+}\)-bound GCAP-2, recoverin, and neurocalcin indicates that the root mean square deviation of the main chain atoms (in the EF-hand motifs) is 2.2 Å in comparing GCAP-2 to recoverin and 2.0 Å in comparing GCAP-2 to neurocalcin.\(^2\)

**RESULTS AND DISCUSSION**

**Trp Residues in Native and Mutant GCAP1**—Trp fluorescence of GCAP1 and its mutants was employed to explore conformational changes within these proteins evoked by Ca\(^{2+}\) binding. Native GCAP1 contains three Trp residues in positions 21, 51, and 94 (Fig. 1) that would interfere with fluorescence measurements of mutant GCAP1. These residues were replaced by Phe or Tyr residues in GCAP1(W21F), GCAP1(W51F), GCAP1(W94F), and GCAP1(W94F) (solid line), respectively. The dotted line represents the Ca\(^{2+}\) titration profile of GC activity in washed ROS membranes in the presence of GCAP1(W21F), GCAP1(W51F), or GCAP1(W94F) (solid line), respectively. The emission spectra of GCAP1(W21F) were recorded using excitation at 290 nm. There is no significant change in spectral shape for GCAP1 mutants in different [Ca\(^{2+}\)]

**TABLE I**

<table>
<thead>
<tr>
<th>Mutants</th>
<th>The maximal activity of ROS GC</th>
<th>The maximal activity of ROS GC</th>
<th>IC(_{50}) for Ca(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/min % nmol</td>
<td>nmol/min % nmol</td>
<td>nmol/min % nmol</td>
</tr>
<tr>
<td>GCAP1</td>
<td>115 ± 21</td>
<td>100</td>
<td>260 ± 25</td>
</tr>
<tr>
<td>GCAP1(W21F)</td>
<td>51 ± 15</td>
<td>44</td>
<td>360 ± 11</td>
</tr>
<tr>
<td>GCAP1(W51F)</td>
<td>96 ± 12</td>
<td>84</td>
<td>290 ± 20</td>
</tr>
<tr>
<td>GCAP1(W94F)</td>
<td>65 ± 18</td>
<td>56</td>
<td>320 ± 21</td>
</tr>
<tr>
<td>GCAP1(W94Y)</td>
<td>60 ± 20</td>
<td>52</td>
<td>230 ± 15</td>
</tr>
<tr>
<td>GCAP1 (W−)</td>
<td>32 ± 6</td>
<td>28</td>
<td>190 ± 20</td>
</tr>
<tr>
<td>W2W3W4-GCAP1(W−)</td>
<td>39 ± 10</td>
<td>34</td>
<td>210 ± 50</td>
</tr>
<tr>
<td>W2-GCAP1(W−)</td>
<td>60 ± 14</td>
<td>52</td>
<td>310 ± 52</td>
</tr>
<tr>
<td>W3-GCAP1(W−)</td>
<td>30 ± 8</td>
<td>26</td>
<td>290 ± 43</td>
</tr>
<tr>
<td>W4-GCAP1(W−)</td>
<td>52 ± 2</td>
<td>45</td>
<td>260 ± 92</td>
</tr>
</tbody>
</table>

**Fig. 3.** Characterization of GCAP1s with individually mutated Trp residues. A, C, and E. Ca\(^{2+}\) titration of GC activity in washed ROS membranes in the presence of ~2 nmol of GCAP1(W21F), GCAP1(W51F), or GCAP1(W94F) (solid line), respectively. The dotted line represents the Ca\(^{2+}\) titration profile of GC activity in washed ROS membranes in the presence of GCAP1. The insets show bar graphs depicting GC activity with and without respective mutant GCAP1 at distinct Ca\(^{2+}\) concentrations as described in the legend to Fig. 2. B, D, and F, Fluorescence emission spectra of GCAP1(W21F), GCAP1(W51F), and GCAP1(W94F), respectively, at Ca\(^{2+}\) concentrations as indicated in the legend to Fig. 2. The emission spectra of GCAP1(W21F) were recorded using excitation at 290 nm. There is no significant change in spectral shape for GCAP1 mutants in different [Ca\(^{2+}\)].
Ca$$^{2+}$$ Binding to GCAP1

**Fluorescence properties of GCAP1 and its mutants**

The fluorescence measurements were carried out as described under “Materials and Methods.”

<table>
<thead>
<tr>
<th>Mutants</th>
<th>$$\lambda_{\text{max}}$$ ($$10^{-10} \text{ M}$$)</th>
<th>$$\lambda_{\text{max}}$$ ($$10^{-5} \text{ M}$$)</th>
<th>$$\Delta \lambda_{\text{max}}$$</th>
<th>$$\Delta F$$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCAP1</td>
<td>345 ± 0.5</td>
<td>347 ± 1.0</td>
<td>2.0</td>
<td>2</td>
</tr>
<tr>
<td>GCAP1(W21F)</td>
<td>345 ± 0.5</td>
<td>345 ± 0.5</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>GCAP1(W51F)</td>
<td>344 ± 1.0</td>
<td>345 ± 0.5</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>GCAP1(W94F)</td>
<td>346 ± 1.0</td>
<td>346 ± 0.5</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>GCAP1(W94Y)</td>
<td>343 ± 0.5</td>
<td>344 ± 0.5</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>GCAP1(w-)</td>
<td>ND$$^d$$</td>
<td>ND$$^d$$</td>
<td>ND$$^d$$</td>
<td>ND$$^d$$</td>
</tr>
<tr>
<td>W2W3W4-GCAP1(w-)</td>
<td>345 ± 0.5</td>
<td>343 ± 0.5</td>
<td>2.0</td>
<td>2</td>
</tr>
<tr>
<td>W2-GCAP1(w-)</td>
<td>343 ± 0.5</td>
<td>341 ± 1.0</td>
<td>2.0</td>
<td>2</td>
</tr>
<tr>
<td>W3-GCAP1(w-)</td>
<td>345 ± 1.0</td>
<td>336 ± 0.5</td>
<td>2.0</td>
<td>2</td>
</tr>
<tr>
<td>W4-GCAP1(w-)</td>
<td>345 ± 1.0</td>
<td>343 ± 1.0</td>
<td>2.0</td>
<td>2</td>
</tr>
</tbody>
</table>

$^a$ The wavelength of the maximum fluorescence at the indicated [Ca$$^{2+}$$]. Excitation at 290 nm.

$^b$ The difference between the maximum fluorescence at [Ca$$^{2+}$$] = $$10^{-10} \text{ M}$$ minus the maximum fluorescence at [Ca$$^{2+}$$] = $$10^{-5} \text{ M}$$ Excitation at 290 nm.

$^c$ The change in the intensity of fluorescence was defined as $$\Delta I = [I_{[\text{Ca}^{2+}]} = 10^{-5} \text{ M}] - I_{[\text{Ca}^{2+}]} = 10^{-10} \text{ M}$/([Ca$$^{2+}$$] = 10$$^{-10} \text{ M}) \times 100$.

Excitation at 290 nm.

$^d$ Not determined.

**Fig. 4. Characterization of W2-GCAP1(w-).** A. Ca$$^{2+}$$ titration of GC activity in washed ROS membranes in the presence of W2-GCAP1(w-) (~2 µg). The dotted line represents the Ca$$^{2+}$$ titration profile of GC activity in washed ROS membranes in the presence of GCAP1. The inset shows a bar graph depicting GC activity with and without mutant GCAP1 at distinct Ca$$^{2+}$$ concentrations as described in the legend to Fig. 2. B. Fluorescence emission spectra of W2-GCAP1(w-) using excitation at 290 nm at [Ca$$^{2+}$$] as indicated in Fig. 2. C, changes in fluorescence at $$\lambda_{ex}$$ = 290 nm and $$\lambda_{em}$$ = 343 ± 3 nm as a function of [Ca$$^{2+}$$]. D, fluorescence difference spectra for W2-GCAP1(w-) measured at [Ca$$^{2+}$$] = $$10^{-10} \text{ M}$$ minus [Ca$$^{2+}$$] = $$10^{-10} \text{ M}$$.

cells, immunoaffinity-purified to apparent homogeneity, and their properties stringently characterized in enzymatic assays and by fluorescence spectroscopy.

**Characterization of GCAP1 and GCAP1(w-)**—The fluorescence spectra of GCAP1 displayed complex changes as a result of Ca$$^{2+}$$ addition. First, addition of Ca$$^{2+}$$ caused a decrease in fluorescence intensity with a minimum at 100–200 nm [Ca$$^{2+}$$]. Further addition of Ca$$^{2+}$$ led to a 2% increase in fluorescence intensity (Ref. 25; Table II). This subsequent change in the fluorescence intensity correlates with a transition of GCAP1 from an activator to an inhibitor of photoreceptor GC. Ca$$^{2+}$$ also caused a 2-nm bathochromic shift in fluorescence $$\lambda_{\text{max}}$$ (Table II). In addition to 3 Trp residues, GCAP1 contains 7 Tyr and 12 Phe residues (~10% of all amino acids) located mostly in the N-terminal part of the protein (5). Tyr and Phe residues contribute to the suppressed fluorescence spectrum of GCAP1(w-), which displayed at $$\lambda_{ex}$$ = 280 nm maximal emission at 305 nm, typical for proteins lacking Trp residues. Relative to the signal from normal GCAP1, the fluorescence of this mutant further decreased in its intensity at $$\lambda_{ex}$$ = 290 nm. Ca$$^{2+}$$ had no effect on fluorescence properties of the mutant at either excitation wavelength (Fig. 2A).

GCAP1 activated photoreceptor GC at [Ca$$^{2+}$$] < 100 nm (Fig. 2B, inset, c) and inhibited at micromolar [Ca$$^{2+}$$] (Fig. 2B, inset, b; see also Ref. 12; for GCAP2, see Ref. 26). Maximal stimulation of ROS GC by GCAP1 was 115 nM (Fig. 2C). The maximal stimulation of ROS GC activity by GCAP1(w-) was about one-third as high as that of GCAP1. This mutant inactivated GC at micromolar [Ca$$^{2+}$$], and its IC$$^{50}$$ for Ca$$^{2+}$$ was lowered to ~190 nm (Fig. 2C, Table I). These data suggest that endogenous Trp residues, to some degree, affect overall inter-
action with GC and/or affinity for Ca\(^{2+}\), and when these residues are replaced by Phe, the mutant GCAP1 appears to be more efficiently inactivated by Ca\(^{2+}\) (no stimulation of GC above 300 nM). Importantly, endogenous Trp residues are non-essential for overall activity/structure of GCAP1, allowing kinetic and steady state analysis of conformational changes induced by Ca\(^{2+}\) in GCAP1 mutants lacking native Trp residues.

**Characterization of GCAP1 Mutants in Which Single Trp Residues Were Replaced by Phe**—Mutation of Trp\(^{231}\) to Phe in GCAP1 produced a protein that was similar to GCAP1, but exhibited slightly modified Ca\(^{2+}\) sensitivity, decreased the maximal stimulation of ROS GC (~50% of normal GCAP1) (Fig. 3A, Table I), similar affinity for ROS GC (Fig. 3A, inset), and decreased fluorescence without a shift in \(\lambda_{\text{max}}\) upon addition of Ca\(^{2+}\) (Table II and Fig. 3B). Changes in fluorescence at \(\lambda_{\text{ex}} = 290\) nm were similar to fluorescence changes at \(\lambda_{\text{em}} = 280\) nm (data not shown). Assuming that the fluorescence of Trp residues in GCAP1 is additive, these results suggest that Trp\(^{231}\) increases GCAP1’s fluorescence upon addition of Ca\(^{2+}\) and blue-shifts its fluorescence at \(~10^{-7}\) M [Ca\(^{2+}\)]. One of several interpretations would be that the N terminus around residue 21 is exposed to a more hydrophobic environment after conformational rearrangement upon addition of \(~10^{-7}\) M [Ca\(^{2+}\)]. This change in the N-terminal portion could be a part of inactivation of GCAP1 by Ca\(^{2+}\). This hypothesis is consistent with findings that deletion of the N-terminal 10 and 20 residues in GCAP1 leads to only partial inactivation by [Ca\(^{2+}\)] (~10\(^{-7}\) M) (25).

Mutation of Trp\(^{231}\) to Phe in GCAP1, located close to the EF2-hand motif, produced protein that was similar to GCAP1 in stimulation of GC and had a small increase in fluorescence upon Ca\(^{2+}\) addition (Tables I and II and Fig. 3, C and D). In contrast to other single mutants, Trp\(^{231}\) may decrease its fluorescence intensity upon addition of Ca\(^{2+}\) because it may become exposed. In normal GCAP1, this decrease may be compensated by an increase in fluorescence due to movements of Trp\(^{221}\) and Trp\(^{294}\) to more hydrophobic environments. Consistent with this idea is property of the next mutant, GCAP1(W94F) had Ca\(^{2+}\) sensitivity similar to GCAP1, a ~50% decreased maximal stimulation of ROS GC activity, and a ~27% decreased fluorescence intensity with a \(\lambda_{\text{max}}\) that does not change upon addition of Ca\(^{2+}\) (Tables I and II and Fig. 3, E and F).

**Characterization of GCAP1 Mutants in Which Trp Residues Were Placed in Front of Ca\(^{2+}\) Loops**—Three Trp residues were introduced to the GCAP1(w) molecule in front of each of the EF2-, EF3-, and EF4-hand motifs (residues 63, 99, and 143). W2W3W4-GCAP1(w) had the maximal stimulation of ROS GC activity >one-third of that for GCAP1, but similar IC\(_{50}\) (210 nM) for Ca\(^{2+}\) inactivation (Table I). Addition of Ca\(^{2+}\) caused blue-shifted emission by 2 nm and ~13% decrease in fluorescence, opposite to that of GCAP1 (Table II). These results suggest that Trp residues placed in the front of EF-hand motifs do not inactivate GCAP1. To assess the contributions of individual EF-hand motifs into Ca\(^{2+}\) inactivation of GCAP1, mutants with a single reporter Trp residue at positions 63, 99, 143, respectively, were characterized (on GCAP1(w) background).

W2-GCAP1(w), with the Trp residue in front of the EF2-hand motif, exhibited properties similar to GCAP1, but had decreased maximal stimulation of ROS GC activity (~50% of that for GCAP1), a ~11% decreased fluorescence intensity, and a 2-nm blue-shifted \(\lambda_{\text{max}}\) upon addition of Ca\(^{2+}\) (Tables I and II and Fig. 4, A, B, and D). The fluorescence change was reduced by ~50% at ~200 nM [Ca\(^{2+}\)] (Fig. 4C).

W3-GCAP1(w), with the Trp residue in front of the EF3-hand motif, exhibited properties similar to GCAP1, but had a decreased maximal stimulation of ROS GC activity (~70% of that for GCAP1), a ~32% increased fluorescence intensity, and a large 9-nm blue-shifted \(\lambda_{\text{max}}\) upon addition of Ca\(^{2+}\) (Tables I and II and Fig. 5, A, B, and D). The fluorescence change was increased by ~50% at ~220 nM [Ca\(^{2+}\)] (Fig. 5C). These results suggest major conformational changes around the reporter Trp residue, consistent with this Trp moving into a more hydrophobic environment upon addition of Ca\(^{2+}\). These results are also similar to data obtained for GCAP1(W51F) with Trp residues present in positions 94 and 21.

W4-GCAP1(w), with a Trp residue in front of the EF4-hand motif had decreased maximal stimulation of ROS GC activity (~50% of that for GCAP1), a ~15% decreased fluorescence intensity, and 2-nm blue-shifted \(\lambda_{\text{max}}\) upon addition of Ca\(^{2+}\) (Tables I and II and Fig. 6, A, B, and D). The fluorescence change was increased by ~50% at ~190 nM [Ca\(^{2+}\)] (Fig. 6C). These results suggest some conformational changes around the reporter Trp residue, consistent with this Trp moving into a
more hydrophilic environment upon addition of Ca$^{2+}$. They point to the importance of this region in the interaction with ROS GC in sensing changes in [Ca$^{2+}$]. This hypothesis is supported further by 1) the truncation mutant of GCAP1-(1-164), which displayed Ca$^{2+}$ binding properties very similar to normal GCAP1, but does not activate ROS GC (data not shown) and 2) by inactivation of EF-hand motifs by mutagenesis of GCAP1, which showed that only EF-hand motif 3 and 4 are essential in inactivation of GCAP1 (12). It appears that the 164–183 segment of GCAP1 is an important region in GC activation, because a truncation mutant GCAP1-(1-183) containing EF4 is active and has properties indistinguishable from GCAP1 (data not shown). The C-terminal region (184–204) is disordered in the NMR structure of GCAP2.

Properties of Trp in W3-GCAP1(W21F,W31F,W94F) in the Presence and Absence of Cations—The emission anisotropy of fluorescence was measured for W3-GCAP1(w$^-$) in the presence or absence of Mg$^{2+}$ and Ca$^{2+}$. The anisotropy for this mutant was significantly higher in the absence of cations (0.144) than in the presence of cations (0.091). A decreased anisotropy indicates a decreased molecular size (or a more compact structure of GCAP1-Ca$^{2+}$), or an increased mobility. This is also consistent with the shorter rotational correlation times in the presence of cations for W3-GCAP1(w$^-$) (46.338 ± 1.943 in Ca$^{2+}$-free, 36.999 ± 1.257 in Ca$^{2+}$-bound).

Accessibility of the Trp residues to small, neutral acrylamide was addressed by steady state fluorescence quenching. The fluorescence emission and excitation maxima were unchanged with 0.1–1 mM EGTA (a typical result is shown in Fig. 7A) in a stopped-flow apparatus. The observed rates were independent of [EGTA] and [W3-GCAP1(w$^-$)] in the studied range (Fig. 7B). The EGTA-induced dissociation of Ca$^{2+}$ was fitted to the equation for the appearance of B [Ca$^{2+}$-free form of W3-GCAP1(w$^-$)] in a first-order reaction: $A \rightarrow B$, where A is the Ca$^{2+}$-loaded form of W3-GCAP1(w$^-$). The average rate for the conformational change induced by EGTA, $k_{1}$, was $-9 \text{ s}^{-1}$ at 7 °C, similar to that observed for Drosophila CaM (27). This conformational change must likely reflect Ca$^{2+}$ dissociation from W3-GCAP1(w$^-$). Since $K_{d} = 290 \text{ nM}$ (derived from the Ca$^{2+}$-dependent activity titration, Table I), and since $k_{1}$ at $37^\circ$C is $72 \text{ s}^{-1}$ (Fig. 7C), and the estimated dissociation rate constant is $k_{d} = 2 \times 10^{-8} \text{ M}^{-1} \text{s}^{-1}$ (with $K_{d} = k_{-1}/k_{1}$), close to the rate limited by diffusion (28). These calculations should be considered approximate, as they do not take into account cooperativity of Ca$^{2+}$ binding. Activity tests and fluorescence measurements presented in this study indicated a Hill coefficient of 1.5–2.2. This approximate rate could even be underestimated if the conformational rearrangement, measured by fluorescence change, is slower than the actual dissociation of Ca$^{2+}$.

The temperature dependence of the conformational change in W3-GCAP1(w$^-$) was elucidated by plotting ln $k_{obs}$ (Fig. 7B) against $1/T$ (in kelvin) according to the Arrhenius equation (Fig. 7C). $k_{obs}$ likely represents the $k_{-1}$ rate constant of Ca$^{2+}$ dissociation. An approximately linear relationship was found, and from the slope ($-E_{a}/R$) of the Arrhenius plot, an activation energy of $E_{a} = 9.3 \text{ kcal/mol}$ of the conformational change of W3-GCAP1(w$^-$) could be calculated.
CONCLUSIONS

Binding of Ca\(^{2+}\) converts GCAP1 from an activator to an inhibitor of photoreceptor GC (for GCAP1, Ref. 12; for GCAP2, Ref. 26). Using Trp fluorescence spectroscopy, we present evidence that GCAP1 undergoes major conformational changes during this transition. The region around the EF3-hand motif, specifically Tyr99, becomes more exposed to solvent in the Ca\(^{2+}\)-free form of GCAP1 on the basis of the following observations: 1) significant increase in the Trp fluorescence intensity of W3-GCAP1(w\(^2\)) upon addition of Ca\(^{2+}\); 2) hypsochromic shift of the maximum of the fluorescence upon addition of Ca\(^{2+}\); 3) similar accessibility to quenching of the fluorescence of this mutant by acrylamide in the presence and absence of cations, while the excited fluorophore in the presence of Ca\(^{2+}\) produce a significantly higher intensity fluorescence; and 4) increased accessibility of the Ca\(^{2+}\)-free form to limited proteolysis in contrast to the GCAP1 Ca\(^{2+}\)-bound form (12). In the model generated based on the structure of recoverin, it is apparent that the Tyr99 mutant is buried within a C-terminal \(\alpha\)-helical domain of GCAP1 (Fig. 8). The conformational changes in recoverin are more profound than those found around other Ca\(^{2+}\)-binding loops of GCAP1.

A Y99C mutation in GCAP1 is associated with autosomal dominant cone dystrophy (9). The biochemical explanation of this disease is that the mutated GCAP1 is constitutively active at high [Ca\(^{2+}\)] in conditions when normal GCAP1 inhibits GC (10, 11). In other words, this mutation changes the affinity for Ca\(^{2+}\) to the EF3-hand motif. From several other independent
is conceivable that the binding of Ca\(^{2+}\) to this loop causes Tyr99 to move from a solvent-exposed environment into a hydrophobic pocket stabilized by van der Waals interactions, requiring $E_a = 9$ kcal/mol as determined from the Arrhenius plot. When Tyr99 is replaced by Cys in the GCAP1(Y99C) mutant, the smaller and nonaromatic Cys may not provide sufficient contacts within this pocket, thus preventing stabilization of the Ca\(^{2+}\)-bound form of GCAP1. This phenomenon may be more general for this family of proteins, as typically this position is highly conserved, and large aromatic residues are present in this position. Indeed, such movement of a corresponding region was shown already for recoverin (29).

Mutations of the endogenous three Trp residues in GCAP1 to Phe residues provide complementary information about specific regions of the molecule that undergo conformational changes during Ca\(^{2+}\) binding. Because they are distributed evenly within the N-terminal domain, Trp\(^{21}\), Trp\(^{53}\) and Trp\(^{94}\), combined results from characterization of these mutants and W2- and W3-GCAP1(w\(^{-}\)) suggest that the relatively large region of protein around residues 94–99, and the N-terminal helix of EF3-hand motif, undergo structural rearrangement.

Another important point of this study is the kinetics of these conformational changes in relation to phototransduction. Current studies on the kinetics of phototransduction responses in knockout mice that had GCAP1 and GCAP2 inactivated pointed out the importance of activation of photoreceptor GC very early in the phototransduction response. To elicit a fast response of GC, Ca\(^{2+}\) dissociation from GCAP1 must be compatible with the rate of the initial phase of phototransduction. Indeed, GCAP1 loses its bound Ca\(^{2+}\) rapidly enough to account for its importance in the early phase of phototransduction (less than 200 ms) (Ref. 30 and references cited therein). The association of Ca\(^{2+}\) and inactivation of GCAP1 is largely limited by the diffusion rate. Thus, GCAP1 has thermodynamic and kinetic properties that fulfill a key role in photoreceptor GC activation during phototransduction response (15, 31).

Acknowledgments—We thank Drs. Tomasz Heyduk, Ted Wensel, and Jack C. Saari for their valuable comments and advice during the course of these studies and Darin Bronson for expert technical assistance in sequencing DNA and primer synthesis.

---


REFERENCES