α Helix Content of G Protein α Subunit Is Decreased upon Activation by Receptor Mimetics*

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To elucidate the mechanism whereby liganded receptor molecules enhance nucleotide exchange of GTP-binding regulatory proteins (G proteins), changes in the secondary structure of the recombinant G\textsubscript{i1} α subunit (G\textsubscript{i1}α) upon binding with receptor mimetics, compound 48/80 and mastoparan, were analyzed by circular dichroism (CD) spectroscopy. Compound 48/80 enhanced the initial rate of GTP\textsubscript{b}G binding to soluble G\textsubscript{i1}α 2.6-fold with an EC\textsubscript{50} of 50 μg/ml. With the same EC\textsubscript{50}, the mimetic decreased the magnitude of ellipticity, which is ascribed to a reduction in α helix content of the G\textsubscript{i1}α by 7%. Likewise, mastoparan also enhanced the rate of GTP\textsubscript{b}G binding by 3.0-fold and decreased the magnitude of ellipticity of G\textsubscript{i1}α similar to compound 48/80. In corresponding experiments using a K349P-G\textsubscript{i1}α, a G\textsubscript{i1}α counterpart of the urea mutant in G\textsubscript{i1}α in which Pro was substituted for Lys\textsubscript{349}, enhancement of the GTP\textsubscript{b}G binding rate by both activators was quite small. In addition, compound 48/80 showed a negligible effect on the circular dichroism spectrum of the mutant. On the other hand, a proteolytic fragment of G\textsubscript{i1}α lacking the N-terminal 29 residues was activated and showed decreased ellipticity upon interaction with the compound, as did the wild-type G\textsubscript{i1}α. Taken together, our results strongly suggest that the activator-induced unwinding of the α helix of the G protein α subunit is mechanically coupled to the enhanced release of bound GDP from the α subunit.

The central role played by trimeric GTP-binding regulatory proteins (G proteins)\textsuperscript{1} in signal transduction in membranes has received considerable research attention (reviewed in Refs. 1–3). Upon ligand binding, a G protein-coupled receptor promotes the release of GDP from inactive trimeric Goβγ, which allows binding of cytosolic GTP to the remaining Go subunit, thereby resulting in dissociation of trimeric Go(GTP)βγ complex into active Go-GTP and a βγ subunit complex. In this activation process of G protein, the release of bound GDP is of particular interest as it is the rate-limiting step (4). The analyses of x-ray crystallographic structures of the α subunit of G\textsubscript{i1} and G\textsubscript{a} have indicated the presence of two domains, i.e. a GTPase (or Ras-like) domain comprised of α helices and β strands and a highly α helical domain. In addition, the conformational changes induced in the α subunit by nucleotide exchange (GDP → GTP-S) and the mechanism of GTP hydrolysis have been determined (5–9). Conformational changes in the α subunit upon binding with a βγ subunit complex have been determined as well (10, 11). However, the mechanism whereby liganded receptor molecules enhance the GDP release from the α subunit remains unclear, as pointed out previously (3, 12). Likewise, the conformational change of the α subunit upon receptor binding is unknown. The use of physicochemical methods to gain further insight into these key reactions presents difficulties due to the facts that (i) only small amounts of G protein-coupled receptor proteins are expressed in cells, and (ii) no method exists for suitably analyzing the structure of a protein complexed with a large membrane protein.

We considered that mastoparan (MP), a 14-residue peptide discovered in wasp venom as the agent that induces histamine release from mast cells (13), might provide some important clues because it activates G\textsubscript{a} and G\textsubscript{i1} in a similar manner; its activation is both Mg\textsuperscript{2+}-dependent and blocked by ADP-ribosylation of G proteins (13, 14). In addition, it has an amphipathic sequence, as do putative G protein-binding sites of many receptors, namely, second and third intracellular loops and C-terminal tail (15). In fact, peptide fragments corresponding to the third intracellular loop of β adrenergic receptors were found to activate G\textsubscript{a} (Refs. 16–18, reviewed in Ref. 19). Also of interest, G\textsubscript{a} and G\textsubscript{i1} are known to be activated by another histamine releaser that is also amphiphilic, i.e. compound 48/80 (C48/80) (20).

These compounds are particularly useful for analyzing G protein activation when employed as low molecular weight mimetics of receptors. As such, the present study uses circular dichroism (CD) spectra to analyze conformational changes in G\textsubscript{i1}α upon interaction with these two compounds. Analysis of CD spectra is an ideal method for determining overall structural changes in proteins. For example, CD measurements of a DNA-binding domain of yeast transcription activator GCN4 estimated that its α helix content increases from 70–73% to

Received for publication, October 29, 1997
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95–100% upon interaction with DNA containing its binding site (AP-1 site) (21). This estimation was later confirmed by the x-ray crystallographic analysis of the structure in a DNA-bound state (23). In the present study, the CD analysis of His10-Giα allowed us to determine how the interaction affects the α-helicity of Giα with the resultant conformational changes leading to the enhancement of GDP release.

EXPERIMENTAL PROCEDURES

Materials—The following materials were used: N123-NTA agarose (Qiagen), GTP-S and sequencing grade endopeptidase Lys-C (Boehringer Mannheim), [α-32P]GTP and [35S]GTP-S (New England Nuclear), C48/80 (alloymer of p-methoxy-N-methylphenethylamine) (Sigma), Lubrol PX (Nacalai Tesque, Kyoto, Japan), 5'-GDP sodium salt (Seikagaku Corp., Tokyo, Japan), standard bovine serum albumin (2 mg/ml) solution (Pierce), and BSA5 nitrocellulose filter (Schleicher & Schuell). GTP-S was purified by Mono Q (Pharmacia) anion exchange chromatography. MP was synthesized by standard solid-phase methodology and purified as described previously (24). All other reagents were of analytical grade (Wako Pure Chemicals, Osaka, Japan).

Preparation of G Protein α Subunits—Because histidine-tagged proteins can easily be purified by affinity purification on Ni2+-NTA agarose, we prepared His10-Giα tagged with 10 histidine residues (His10-Giα) as well as non-tagged full-length Giα (FL-Giα) as a control. We also prepared a lysate—Pro mutant in His10-Giα (K349Q-Giα), a mutant corresponding to the N-terminus of Giα (25, 26) and expected to be both activators, and a 325-amino acid proteolytic fragment of Giα lacking the N-terminal 29 residues (ΔN-Giα), which allowed us to investigate whether the protein’s N-terminal segment is involved in activation.

FL-Giα was expressed in Escherichia coli BL21(DE3) cells harboring the pQE60/Giα plasmid (27) and purified as described (28), and His10-Giα was prepared as follows. After cloning cDNA of Giα using polymerase chain reaction with appropriate primers and QUICK-Clone cDNA (CLONTECH) as a template, the product cDNA was ligated into the NdeI and BamHI sites of pET19b (Novagen), which we modified beforehand by adding an adapter sequence giving a sequence encoding Met-Gly-(His)10-(Ser)2-(Gly-His-Ile)-(Asp)4-Lys-His at the N terminus of Giα. Next, the complete coding sequence, the XhoI-BamHI fragment of pET19b-Giα, was ligated into the XhoI and BamHI sites of pET24a(+) (Novagen) to produce pET24a(+)-His10-Giα, which was transformed into E. coli BL21(DE3) cells. Finally, His10-Giα protein was expressed and purified as described (29). K349P-Giα was prepared by subcloning into the BamHI and SalI sites of pUC19 a BglII-SalI fragment of pET24a(+)-His10-Giα corresponding to the 3’ terminal 490 bp of the entire Giα sequence. Then, to substitute Pro for Lys490, the generated plasmid was subjected to site-directed mutagenesis (27) using 5’-GAGGACACAGTCTGGATGTATT3’- as a mutagenic primer; the mutations were confirmed by DNA sequencing. The entire coding sequence was subsequently obtained by ligating the DraI-SalI fragment of pUC19 possessing the mutated sequence of the 3’ terminal of the Giα sequence into the DraI-SalI sites of pET24a(+)-His10-Giα. The mutant protein was correspondingly expressed and purified like wild-type His10-Giα.

To prepare ΔN-Giα, His10-Giα protein was subjected to limited digestion with endopeptidase Lys-C as described (6). The cleavage site was confirmed by amino acid sequencing (Applied Biosystems 477A Protein Sequencer), and the integrity of the C terminus was determined by Western blot analysis using antiserum specific to the C-terminal 10 residues of Giα.

In addition to these proteins, which are bound by GDP, we also prepared His10-Giα in GTP-S- and GDP-AIF2- bound forms to investigate the effect of bound nucleotide on the secondary structure of the protein. The GTP-S-bound form was prepared by incubating 10 mg/ml of His10-Giα in the GDP-bound form at 30 °C for 5 h in a buffer of 100 mM sodium phosphate (pH 7.0), 1 mM EDTA, 10 mM DTT, 10 mM MgSO4, and 2 mM GTP-S; the complete conversion was confirmed by demonstrating trypsin resistance (30). His10-Giα in the GDP-AIF2- form was correspondingly prepared using a buffer containing 30 mM AlCl3 and 10 mM NaF in place of GTP-S; the complete conversion was confirmed by verifying that this form did not bind [35S]GTP-S (7). The concentration

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2 We found that some lots of C48/80 give noisy spectra in 200–210 nm; when large noises are observed in its CD spectrum, another lot should be tested.

3 H. Itoh, unpublished results.
RESULTS

Enhancement of GDP Release from G_{11α}—The effects of MP and C48/80 on GTPγS binding to FL- and K349P-G_{1α} are illustrated in Fig. 1, A and B, respectively, and Table I summarizes the initial binding rates and fold enhancement of each G protein examined. Note that (i) all four proteins show similar initial GTPγS binding rates in the absence of the activators; (ii) MP and C48/80 similarly enhance the initial binding rate of FL- and His_{10G}G_{1α}; (iii) enhancement is very weak for K349P-G_{1α}; (iv) the affinity of a GDP molecule for G_{1α} is not altered by the addition of the His_{10} tag, substitution of Pro for Lys_{349}, or the deletion of the N-terminal 29 residues. However, the substitution of Pro for Lys_{349} substantially weakened activation by MP and C48/80.

Intactness of His_{10G}G_{1α} during CD Measurements—To confirm that His_{349}G_{1α} is not denatured in the presence of C48/80, the amount of GDP bound to His_{349}G_{1α} was determined under the CD measurement conditions. In the CD buffer that did not contain guanine nucleotides, dissociation of GDP did not occur in either the absence or the presence of 100 μg/ml C48/80 up to 50 min (Fig. 2). When free GDP was included in the buffer, marked release of GDP was observed, and its release rate was increased in the presence of C48/80 (data not shown). GTPγS binding activity of His_{349}G_{1α} in the presence of 100 μg/ml C48/80 was also determined with different preincubation times with C48/80. The GTPγS binding activity did not change significantly (<5%) up to 50 min (data not shown).

Helix Content of G_{1α} in the Absence of Activators and Effect of the Bound Nucleotide on the Secondary Structure of G_{1α}—Fig. 3A illustrates the CD spectrum of FL-G_{1α} in the GDP-bound form. CCA and SELCON analysis of the spectrum gave α helix values of 50.6 and 55.9%, respectively; these values were in good agreement with that obtained by x-ray crystallographic analysis of the G_{1α}GDP structure (9), which should be expected because both CCA and SELCON are known to show high accuracy in estimating α helix content (38). Fig. 3A also illustrates the CD spectra of His_{10G}G_{1α} in the GDP-, GDP-AlF_{4}−, and GTPγS-bound forms. The magnitude of ellipticity is greater in FL-G_{1α}GDP than in His_{10G}G_{1α}GDP. This is presumably due to the absence of an ordered structure in the His_{10} tag segment. There were few spectral differences among the three forms of His_{10G}G_{1α}, indicating that the secondary structure of G_{1α} does not substantially change irrespective of the chemical structure of the phosphate moiety of the guanine nucleotides bound (see Fig. 3, B and C, for expansion around 210 and 220 nm, respectively). This is consistent with the x-ray analysis results indicating the presence of few differences among the secondary structure contents of G_{1α} in the GDP-, GDP-AlF_{4}−, and GTPγS-bound forms and among corresponding...
In agreement with the observation that GDP molecules are not released from His₁₀-Gᵢ₁α in the absence of free guanine nucleotides even in the presence of 100 μg/ml C₄₈/₈₀ (Fig. 2), the addition of 50 μM GDP did not affect the CD spectra in either the absence or the presence of C₄₈/₈₀ (data not shown). These data confirm that the difference spectrum in the presence of this activator (Fig. 3D) reflects the secondary structure of Gᵢ₁α-GDP in the activated state rather than the structure of guanine nucleotide-free Gᵢ₁α.

Fig. 3E illustrates the dependence of [θ]₂₂₂ on the concentration of C₄₈/₈₀ in FL-Gᵢ₁α. With an EC₅₀ value of 31.8 ± 2.6 μg/ml, the spectral change reaches a plateau at about 100 μg/ml. At this saturation concentration, CCA and SELCON analyses gave α helix values of 43.8 and 49.3%, respectively. Both of these values are 7% lower than the original values; this reduction corresponds to the unwinding of the α helix by 20 of 354 amino acid residues.

Effect of C₄₈/₈₀ on CD spectra of modified Gᵢ₁α proteins. Spectra were recorded as described under "Experimental Procedures." Shown are His₁₀-Gᵢ₁α (A), ∆N-Gᵢ₁α (B), and K₃₄₉P-Gᵢ₁α (C) in the absence (His₁₀, K₃₄₉P, and ∆N) or presence (His₁₀+ C₄₈/₈₀, K₃₄₉P + C₄₈/₈₀, and ∆N + C₄₈/₈₀) of 100 μg/ml C₄₈/₈₀. D, time courses of [θ]₂₂₂ of His₁₀-Gᵢ₁α in the absence or presence of 100 μg/ml C₄₈/₈₀. Data points indicate the average of duplicate measurements; the difference of the duplicate values was less than 0.5%.

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to 50 min (Fig. 4D).

In marked contrast to these wild-type proteins, K349P-Giα shows only a small decrease upon addition of C48/80 (Fig. 4C).

This observation indicates that the α helical structure in K349P-Giα is not unwound upon interaction with C48/80.

Secondary Structure Prediction of G i1—Table II shows the predicted secondary structure preferences of those sequences that are α helical in the GTPase domain of G i1α in the GDP form (9). Among six helices, the α5 helix is predicted by both the Garnier-Osguthorpe-Robson and NNPREDICT methods to possess the lowest propensity to form helices.

**Table II**

| Helix (residue number) | αN (11–17) | α1 (46–57) | α3 (242–255) | αG (271–280) | α4 (296–310) | α5 (329–350) |
|------------------------|------------|------------|--------------|--------------|--------------|--------------|
| Sequence               | AAVERSKa   | KSTIVKQMKIIH | HMESMKLFSIS1CN | KDLFEKIKK | YEAAAYIQCFEDL | TKNVQVFDAVTDVIIKNNLKDa |
| GOR                    | HHHHHHH    | THHHHHHHHEEEE | HHHHHHHHHHHHTTTT | HHHHHHHHHHHHHHH | HHHHHHHHHHHHHHH | HHHHHHHHHHHHHHH |
| NNPREDICT              | HHHHHHH    | TTHHHHHHHHEEEE | HHHHHHHHHHHHTTTT | HHHHHHHHHHHHHHH | HHHHHHHHHHHHHHH | HHHHHHHHHHHHHHH |

a The numbering of helices is according to Ref. 9.

The electrostatic repulsion between the two negative charges on these residues would destabilize the helix structure if they were not neutralized by some positive charges. Actually, the two carboxylate groups of these residue form a bidentate salt bridge with the side chain amino group of Lys192 in the β2/β3 loop (9). This bidentate salt bridge seems to stabilize the potentially unstable α5 helix structure and, by bridging the α5 helix and the β2/β3 loop, the whole tertiary structure as well. Such a salt bridge is formed also in GI ααGDP (6) and is expected to occur in G i1α (50), G oα (51), and Gα α (52), as well. Because both MP and C48/80 have multiple positive charges, interactions of each positive charge with either Asp337 or Asp441 may result in the destabilization and subsequent unwinding of the α5 helix. Such an interaction is compatible with the observation that [Tyr3,Cys11]MP is cross-linked with Cys3 of GoS (45).

Although the N-terminal eight residues are disordered and are not observed in the crystal structure of G i1αGDP (9), Asp9 is located very close to Asp341 and, therefore, to Asp337.

Coupling of Helix Unwinding and Enhanced GDP Release—C48/80 enhanced GTPαS binding to G i1α with nearly the same EC50 as it decreased [3H]222 of G i1α, i.e., 33.0 ± 1.5 μg/ml (Fig. 1C) and 31.8 ± 2.6 μg/ml (Fig. 3E), respectively. In addition, the K349P mutant in G i1α, which is activated minimally by C48/80 and MP (Fig. 1B), demonstrated an insignificant decrease in the magnitude of ellipticity in the presence of C48/80 (Fig. 4C). Taken together, these results strongly suggest that the decrease in the α helix content of G i1α is coupled to the enhanced GTPαS binding, i.e., enhanced GDP release in other words, the unwinding of α helical residues (presumably in the α5 helix) upon binding with C48/80 (or MP) is considered to be propagated to the guanine nucleotide binding sites of G i1α such that the affinity of the protein to a bound GDP molecule is lowered. The disruption of the bidentate salt bridge would release the β2 sheet (residues 185–191) from the α5 helix and result in the dislocation of guanosine-binding residues (Leu175 and Arg176) positioned to the N terminus of the β2 sheet; ultimately, this would decrease the affinity to the bound GDP.

In support of this postulation, the affinity of GDP to Gα α is known to decrease remarkably by the removal of the C-terminal 14 residues of the protein (including Asp341) (51), which adopt an α helical conformation in G i1αGDP (9).

In summary, the present study reports for the first time the conformational change of G protein α subunit upon activation by receptor mimetics. Additional studies should prove MP and C48/80 useful in elucidating the interaction between receptor and G protein α subunits in more detail.

**Acknowledgments**—We are grateful to Prof. A. G. Gilman (University of Texas Southwestern Medical Center) for the generous gift of E. coli cells harboring pQE60/G i1α plasmid, Dr. A. Omori and S. Yoshida (Mitsubishi Kasei Institute of Life Sciences) for amino acid sequencing of αN-Giα, Dr. G. D. Fasman (Brandeis University) for the use of CCA, Dr. R. W. Woody (Colorado State University) for the use of SELCON, and Dr. N. J. Greenfield (UMDNJ-Robert Wood Johnson Medical School) for providing us with these programs.
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*J. Biol. Chem.* 1998, 273:3247-3252.
doi: 10.1074/jbc.273.6.3247

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