A Site on Transducin α-Subunit of Interaction with the Polycationic Region of cGMP Phosphodiesterase Inhibitory Subunit*

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Activation of cGMP phosphodiesterase (PDE) by the rod G-protein transducin is a key event in visual signal transduction in vertebrate photoreceptor cells. Interaction between the GTP-bound form of the α-subunit of transducin (α*,) and the PDE inhibitory γ-subunit (Py) is a major component of PDE activation. The central polycationic region of Py, Py-24-45, has been implicated as one of the sites involved in α*,Py interaction. Here we determine the site on transducin labeled with the fluorescent probe 4-(N-maleimido)benzophenone (where ACM indicates acetamidomethyl group) and Cys-Py-24-45 were linked to NCS, N-chlorosuccinimide; ACM, acetamidomethyl group; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography. When the photoprobe was attached to the COOH terminus of the peptide, a specific high yield cross-linked product (80%) was formed between the peptide and α,GTPγS (guanosine 5'-O-(thiotriphosphate)). A lower yield of cross-linking (35%) was seen between the peptide and α,GDP. The site of cross-linking between Cys(ACM)Tyr-Py-24-45-Cys and α,GTPγS was localized to within α306-310 using a variety of chemical and proteolytic cleavages of the cross-linked product, analysis of the fragments with SDS-polyacrylamide gel electrophoresis, and matrix-assisted laser desorption ionization mass spectrometry.

In photoreceptor cells of vertebrates, the GTP-bound α-subunit of the rod G-protein transducin (α*,) activates cGMP phosphodiesterase (PDE) and thus controls levels of the second messenger cGMP (reviewed by Chabre and Deterre (1989) and Stryer (1991)). PDE becomes activated because α*, relieves an inhibitory constraint imposed by two identical inhibitory subunits of PDE (Py) on the αβ catalytic subunits (Paβ) (Hurley and Stryer, 1982; Deterre et al., 1988; Wensel and Stryer, 1990). Strong evidence has been accumulated showing that α*, binds directly to the Py subunits (Deterre et al., 1986; Fung and Griswold-Prenner, 1989; Artemyev et al., 1992). In the absence (or at low concentrations) of photoreceptor membranes, the α*,Py complex mainly dissociates from Paβ (Deterre et al., 1986; Yamazaki et al., 1990; Wensel and Stryer, 1990). In the presence of sufficient concentrations of ROS membranes, the complex α*,Py-Paβ remains membrane-bound (Clerc and Bennet, 1992; Catty et al., 1992). Until recently, all structural information available on α*,Py interaction was related to the central polycationic region of Py (Py-24-45), which had been implicated as the site of interaction with α*, (Lipkin et al., 1988; Morrison et al., 1989; Brown, 1992). Using synthetic peptides as probes, Rarick et al. (1992) have shown that, on α*, the region corresponding to residues 293-314 mediates PDE activation. Further analysis revealed that this region interacts with the COOH-terminal region of Py, Py-46-57 (Artemyev et al., 1992), which is an important site for PDE inhibition (Artemyev and Hamm, 1992). Additionally, it has been demonstrated that a second distinct site of interaction involves the central region of Py and an unknown site on α*, (Artemyev et al., 1992).

This paper presents a study directed toward identification of the site on α*, that interacts with the Py-24-45 region. A specifically designed synthetic peptide, Cys(ACM)Tyr-Py-24-45-Cys, was labeled with 4-(N-maleimido)benzophenone (MBP) and then efficiently photo-cross-linked to α,GTPγS. For simplicity we abbreviate this peptide as Py*-24-45-Cys. Cleavage of the cross-linked product followed by analysis of the fragments using SDS-PAGE and matrix-assisted laser desorption ionization (MALDI) mass spectrometry localized the site of cross-linking to within the region α306-310.

EXPERIMENTAL PROCEDURES

Materials—GTP, GTPγS, and endoproteinase Gly-C (protease V8, sequencing grade) were products of Boehringer Manheim. Trypsin and soybean trypsin inhibitor were obtained from Worthington. Blue-Sepharose CL-6B was a product of Pharmacia LKB Biotechnology, Inc. N-Chlorosuccinimide and 4-(N-maleimido)benzophenone were products of Sigma. The matrix material, α-cyano-4-hydroxycinnamic acid, was purchased from Aldrich. All other chemicals were from Sigma or ICN.

Preparation of α,GTPγS, α,GDP, α(19-310)GTPγS, and α(26-350)GTPγS—Bovine ROS membranes were prepared by the method of Pavlides and Dreyer (1974) with some modifications (Matzoni et al., 1991). The α,GTPγS subunit was extracted from ROS membranes using GTPγS and purified by chromatography on Blue-Sepharose CL-6B essentially as described by Kleuss et al. (1987). The α,GDP was prepared and purified according to Yamazaki et al. (1986). The purified proteins were stored in 40% glycerol at -20 °C. To obtain α(19-310)GTPγS, α,GTPγS was prototyped with trypsin essentially as described by Fung and Nash (1983). α,GTPγS (0.5 mg/ml) in buffer A (10 mM HEPS, pH 7.8, 100 mM NaCl, 1 mM MgCl₂, 1 mM dithiothreitol) was incubated with trypsin (0.1 mg/ml) at room temperature for 30 min, and the mixture was loaded onto Blue-Sepharose CL-6B. The α,GTPγS was eluted with 0.5 M NaCl and dialyzed against buffer A.
incubated with tosylphenylalanyl chloromethyl ketone-treated trypsin (0.05 mg/ml) for 60 min at 0 °C. The reaction was terminated by addition of 10-fold excess trypsin inhibitor. α2(26–350)GTPγS (prepared using limited proteolysis of α2GTPγS with endoproteinase Lys-C and purified on a MonoQ column) was a generous gift from Dr. J. Sondek.

Peptide Synthesis—A peptide corresponding to residues 24–46 of Py (Ovchinnikov et al., 1986), as well as the peptides Cys(ACM)Tyr-Py-24-45 and Cys-α2GTPγS, were synthesized by the solid-phase Merrifield method on an Applied Biosystems automated peptide synthesizer. The sequence of Py-24–46 is Asp-ArgGly-Pro-Phe-Ser-Pro-Asp-Leu-Asp-Asp-Lys-Glu-Lys-Leu-Leu-Asp-Lys. Cys was added to the COOH and NH2 termini of the Py-24–45 sequence as a site for the introduction of the cross-linking probe MBP. Cys, protected with an acetamidomethyl group, was placed at the NH2 terminus of the peptide as a potential site for labeling with MBP. Tyr in the front of the Py-24–45 sequence was introduced as a potential site for photo-activation.

RESULTS

In order to determine the site on α2GTPγS that interacts with the central residue of Py, a cross-linking approach was used in which a Cys residue on the Py-24–45 peptide was labeled with a photo-activatable group and cross-linked to α2GTPγS. For this purpose, a peptide analog of Py-24–45, Py*24–45, was synthesized using the solid-phase Merrifield method on a customized Merrifield resin. The purity and chemical formula of each peptide were confirmed by fast atom bombardment mass spectrometry and analytical reversed-phase HPLC.

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Prior to labeling with MBP, the ability of the peptide Py*24–45 to bind α2GTPγS was compared with that of its parent peptide, Py-24–45, and α2GTPγS using the fluorescent assay described by Artemyev et al. (1992). It was shown that Py*-24–45 binds to α2GTPγS with KD of 0.7 μM (Artemyev et al., 1992). Py*24–45 Cys also reversed the fluorescent increase of lucifer yellow-labeled Py (PyLY) caused by α2GTPγS with a similar IC50 (data not shown).

The α2GTPγS (5 μM) and MBP-labeled peptide Py*24–45-CysMBP (10 μM) were mixed, and, following photolysis, proteins were analyzed by SDS-PAGE. Fig. 2A (lanes 1 and 2) demonstrates that photo-cross-linking was very efficient and produced a product with a molecular mass of ~42 kDa, which corresponds to the α2 cross-linked to a single molecule of the peptide. A typical yield of the product determined by scanning of the gels was 70–80%. Addition of an excess of Py* before photooxidation effectively blocked cross-linking, indicating

**Fig. 1. A, chemical structure of the photo-activatable probe 4-(N-maleimidomethyl)benzophenone. B, COOH-terminal sequence of α2, with a summary of the cleavage sites. H, hydroxylamine; T, tryptophan; C, CysMBP; G, Glu-C protease.**
that cross-linking was specific (Fig. 1B, lane 3), indicating 31- and 12-kDa polypeptides. C, a,GTPyS (lane 1); the cleaved a,GTPyS (a,19-310)GTPyS is shown before (lane 2) and after cross-linking (lane 3) with Py*-24-45-CysMBP. T, trypsin; I, trypsin inhibitor.

To determine whether the amino-terminal end of the Py*-24-45-Cys peptide also could cross-link to a,GTPyS, the ACM protecting group on the amino-terminal Cys was removed. However, conditions for removing this group with concurrent labeling with MBP at high yield were not found. Therefore, another peptide, Cys-Py-24-45, was synthesized and labeled with MBP. Cross-linking of this peptide to a,GTPyS occurred with very low efficiency (5-7% yield), suggesting that the probe was oriented away from the binding site with a,GTPyS.

In order to localize the site of cross-linking of Py*-24-45-CysMBP on a, preliminary experiments were performed using cleavage of the cross-linked product with N-chlorosuccinimide (NCS) as described under "Experimental Procedures." NCS cleaves a, (350 amino acid residues) at Trp127 and Trp207, thus producing fragments of ~8 kDa (128-207), 14 kDa (1-127), and 16 kDa (208-350), as well as products of partial cleavage. Analysis of the silver-stained gels after cross-linking revealed a decrease of the 16-kDa band and appearance of a ~19-kDa band, suggesting that the a,208-350 fragment is cross-linked with the peptide (data not shown).

To further localize the Py*-24-45 binding site, the cross-linked product was cleaved with hydroxylamine. The a, subunit has a single peptide bond sensitive to hydroxylamine (Asn287-Gly288) (see Figs 1B for site of hydroxylamine cleavage). Fig. 2B (lane 4) shows that cleavage of a, with hydroxylamine resulted in the formation of 31-kDa (1-287) and 8-kDa (288-350) fragments. When the cross-linked product of a,GTPyS and the Py peptide was cleaved with hydroxylamine, the 31-kDa fragment appeared with the same intensity as after cleavage of a,GTPyS alone (Fig. 2B, lane 3). An additional band of ~12 kDa, corresponding to the cross-linked product of 31-kDa a, fragment with the Py peptide, appeared. No band corresponding to the cross-linked product of 31-kDa a, fragment with the Py peptide was observed. We could not achieve a more efficient cleavage of a, cross-linked with Py*-24-45-CysMBP, since hydroxylamine partially cleaved the Py-peptide from a, (the ratio of the cross-linked product to a, in lane 3 is 4:1 and in lane 4 is ~1:1; Fig. 2B). This experiment localizes the site of cross-linking of Py*-24-45 to the a,288-350 region.

It was previously demonstrated that limited proteolysis of a,GTPyS with trypsin results in cleavage of a, at Lys18 and Arg310 (Hurley et al., 1984). On SDS-gels, a,19-310 migrates as a 32-kDa band. The 32-kDa fragment retains the ability to activate PDE (Fung and Nash, 1983). Based on these data we performed limited proteolysis of a,GTPyS with trypsin and tested the functional ability of a,(19-310)GTPyS to bind to Py using a fluorescent assay. No difference was observed between a,(19-310)GTPyS and a,GTPyS in their ability to bind PyLY. Limited proteolysis of a,GTPyS produced a 32-kDa fragment and a faint 5-kDa fragment (Fig. 2C, lane 2) as expected. Results of the cross-linking of a,(19-310)GTPyS and Py*-24-45-CysMBP are presented in Fig. 2C (lane 3). This experiment clearly shows that the Py peptide mainly cross-links to a,19-310. However, it does not rule out low yield cross-linking to the 5-kDa COOH-terminal fragment. Taken together, the above experiments suggest that the a, region 288-310 is a major site of a,GTPyS cross-linking with the Py peptide.

Final analysis of the cross-linking site was performed using MALDI mass spectrometry to determine the masses of fragments of the cross-linked product, obtained after cleavage with CNBr and Glu-C protease. In these experiments we used a,(26-350)GTPyS purified after limited proteolysis of a,GTPyS with Lys-C protease (Mazzoni et al., 1991). NH2-terminal truncation removes heterogeneity of the a, subunit due to heterogeneous fatty acylation (Neubert et al., 1992; Kokame et al., 1992) and thus simplifies mass spectrometric analysis. Additionally, we found that this truncation allows reversed-phase purification of a, which otherwise irreversibly sticks to the C-4 protein column. Such purification enables us to prepare high purity samples for chemical or enzymatic cleavage after cross-linking of a,(26-350)GTPyS and the Py peptide. Typical results of cross-linking of a,(26-350)GTPyS and the Py peptide is presented in Fig. 3. After cross-linking and reversed-phase HPLC, the peak containing the cross-linked product and a small amount of uncross-linked a,26-350 was lyophilized, and the denatured protein was cleaved with CNBr or Glu-C protease. The peptide fragments were separated on a C-4 narrow bore column and analyzed with MALDI mass spectrometry.
MALDI molecular weight mapping of the CNBr products revealed approximately 70% of the α,-26–350 sequence; however, no cross-linked products were observed. A comparison of the α,-26–350 map and the cross-linked α,-26–350 map indicated a significant decrease in the intensity of m/z 1386.8, putatively assigned as residues 309–319 (predicted mass 1385.7). The cross-linked product of peptide 309–319 (expected mass 4842) was not observed. The absence of a cross-linked product may be due to cleavage of the cross-link by the conditions of CNBr treatment.

The MALDI map of the Glu-C protease digest products revealed 59.7% of the α,-26–350 sequence as shown in Table I. An ion at m/z 4613.8 ± 3.8 was observed in the digest of cross-linked α,-26–350 and is in good agreement with the predicted mass (4616.4) of the peptide 306–314 (m/z 1161.4) cross-linked to Cys(ACM/Tyr-Py-24–45-CysMBP (m/z 3455). The oxidized (Ox) peptide 306–314(Ox) was also observed at m/z 1178.6 (predicted mass 1177.4) as was the oxidized cross-linked product at m/z 4631.8 ± 2.8 (predicted mass 4632.4). Oxidation of methionine residues is commonly observed, and, in the case of the α,-26–350 map, peptides 112–118 (Met115) and 306–314 (Met309) appear as oxidized products having mass shifts of 16 daltons relative to their predicted masses. An additional COOH-terminal peptide was observed at m/z 3203.9 corresponding to the 315–342 region (predicted mass 3204.7). Although this peak was abundant in the α,-26–350 map, a cross-link to this region was not observed in the cross-linked α,-26–350 map. The unmodified peptides 306–314 and 306–314(Ox) were also present in the cross-linked α,-26–350 map suggesting that the cross-linking was incomplete. Overall, MALDI mass spectrometric analysis localized the cross-linking site to the COOH-terminal peptide was observed at m/z 4631.8 ± 2.8 (predicted mass 4632.4). Oxidation of methionine residues is commonly observed, and, in the case of the α,-26–350 map, peptides 112–118 (Met115) and 306–314 (Met309) appear as oxidized products having mass shifts of 16 daltons relative to their predicted masses. An additional COOH-terminal peptide was observed at m/z 3203.9 corresponding to the 315–342 region (predicted mass 3204.7). Although this peak was abundant in the α,-26–350 map, a cross-link to this region was not observed in the cross-linked α,-26–350 map. The unmodified peptides 306–314 and 306–314(Ox) were also present in the cross-linked α,-26–350 map suggesting that the cross-linking was incomplete. Overall, MALDI mass spectrometric analysis localized the cross-linking site to the region α,-306–314, which overlaps with the α,-288–310 region, mapped in the preceding experiments.

**DISCUSSION**

Our previous report demonstrated that there are at least two distinct sites of interaction between the rod G-protein α-subunit and the PDE inhibitory γ-subunit. We showed that the α peptide 293–314, which activates PDE (Rarick et al., 1992), binds within the COOH-terminal region of Py, Py-46–87 (Artemyev et al., 1992). We also concluded that the Py-24–46 region, which had previously been implicated as important for the Py-24–46 interaction with α, Py-24–46 and PyLV, is critical for PDE activation (Artemyev et al., 1992). SDS-PAGE and mass spectrometric analysis of the fragments obtained after chemical and proteolytic cleavage of the cross-linked product between αGTPγS and Py-46–45-CysMBP provided evidence for cross-linking of the region α,-306–310. Taking into account the size of the photoprobe (from the reactive carbonyl to the -SH reactive bond) plus the extra Cys (~12 A), we may speculatively place Lys44 and Lys45 of Py near any of the residues within the region α,-302–314. It is likely that these lysine residues form salt bridges with acidic residues on αGTPγS. Residues Glu306, Asp311, and Glu314 appear to be preferable candidates for such interaction.

The results of this work raise an interesting question. We have shown that the binding of fluorescently labeled Py to αGTPγS is actually enhanced by ~3-fold in the presence of 20 μM Py-24–46. This suggests that the sites on αGTPγS of interaction with Py-24–46 and Py-46–87 are not competitive, but adjacent. The most likely explanation of our cross-linking experiments is that the region of αGTPγS that binds to Py-24–46 overlaps with the COOH-terminal part of the α,-293–314 region, but this overlap is not significant enough to produce the α,-293–314 Py-24–46 interaction that could be detected in the competition assay. We suggest that an additional interaction, which accounts for the observed affinity of αGTPγS-Py-24–46 binding (Kd 0.7 μM) (Artemyev et al., 1992), is located in another region on α, which is adjacent on the three-dimensional structure to α,-302–314.

An interesting general conclusion from our studies is that the two major Py regions of interaction with α, Py-24–46 and Py-46–87, interact with non-competitive sites within α,“s “effector activating region.” Two observations suggest that the
$\alpha_{-293-314}$ region undergoes a conformational change upon GDP/GTP exchange that is necessary for PDE activation. First, this region on $\alpha_\text{t}$ interacts with Py and in the GTPyS-bound form, but not the GDP-bound form, causes a fluorescent increase of PyLY (Artemyev et al., 1992). Second, the peptide Py-24-45 cross-links to $\alpha_{-306-310}$ more effectively in the nal part of at-293-314. These data leave open the possibility that another region(s) of Py-24-45 region interacts with the COOH-terminus of Py-24-45 region interacts with the COOH-terminal part of $\alpha_{-293-314}$. These data leave open the possibility that another region(s) of $\alpha_\text{t}$ (close to this region on the three-dimensional structure) interact(s) with Py.

REFERENCES
