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Coupling an α_2-Adrenergic Receptor Peptide to G-Protein: A New Photolabeling Agent

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TAYLOR, J. M., G. G. JACOB-MOSIER, R. G. LAWTON AND R. R. NEUBIG. *Coupling an α_2-adrenergic receptor peptide to G-protein: A new photolabeling agent.* PEPTIDES **15**(5) 829–834, 1994.—A photoreactive derivative of a tetradecapeptide G-protein activator (peptide Q) derived from the α_2-adrenergic receptor was designed and used to label purified G-protein (G_o/G_i). *N*-bromoacetyl-*N*'-(3-diazopyruvoyl)-*m*-phenylene-diamine (Br-DAP) was conjugated to the *C*-terminal cysteine of peptide Q. The DAP-modified peptide Q (DAP-Q) specifically incorporated into the a subunit of G_o. The incorporation of DAP-Q into α_o was blocked by unmodified Q peptide (IC_{50} = 15 ± 6 μM; n = 4). Photolysis of sixfold higher concentrations of DAP-Q with ovalbumin or bovine albumin failed to produce cross-linked products. Br-DAP should prove useful in detecting mutual contact sites between peptides and their binding proteins.

G-protein α_2-Adrenergic receptor Synthetic peptide Photoaffinity agent

THE plasma membrane-bound $\alpha_2$adrenergic receptor (α_2-AR) is coupled to its cytoplasmic effector enzymes through GTP binding proteins (heterotrimeric proteins that can shuttle information from the receptor to the effector). Sequence analysis of G-protein-coupled receptors (GPCR) predicts a receptor model consisting of seven hydrophobic regions spanning the membrane with three cytoplasmic loops and an intracellular *C*-terminus (13). Site-directed mutagenesis (19,27) and competition studies using synthetic peptides (3,6,9,14,20) have determined that the third cytoplasmic loop and possibly the second cytoplasmic loop and *C*-terminal tail are important in coupling members of this family of receptors to G-proteins. However, specific residues in the G-protein that participate in coupling to these sites of the receptors remain to be identified.

Direct physical detection of protein–protein interactions in membrane has proven to be difficult. To obtain such evidence, we have designed peptides corresponding to specific sequences in the α_2-AR to map the sites of receptor–G-protein interaction. We and others have shown that the α_{2A}-receptor-derived peptides from the second and third cytoplasmic loops modulate receptor–G-protein coupling (2,3,9). Peptide Q is a 14 amino acid peptide corresponding to the carboxy-terminal region of the third cytoplasmic loop of the α_{2A}-AR (aa 362–373 with an additional *C*-terminal cysteine). Peptide Q inhibited binding of the α_2-AR agonist *p*-iodoclonidine to platelet membranes, suggesting that this peptide is capable of disrupting α_2-AR–G-protein coupling (3). More directly, we have shown that peptide Q activates GTPase activity of purified bovine brain G-protein (G_o/G_i) (2,30). This evidence suggests that peptide Q can mimic receptor activation by binding to relevant receptor binding sites on the G-protein.

The ability of peptide Q to directly activate G-protein is consistent with data of others demonstrating the ability of certain small peptides to have G-protein activating activity. Higashigima et al. showed that mastoparan (a tetradecapeptide from wasp venom) is a potent stimulator of G-protein (8). Also, small synthetic peptides derived from a number of G-protein-coupled receptors including the β_2-adrenergic receptor, insulin growth factor II receptor, and rhodopsin directly stimulate G-protein (14,17,18). Selective G-protein agonists or antagonists could prove therapeutically useful in treating a growing number of disorders associated with aberrant receptor–G-protein coupling (21,25).

To determine the receptor binding site(s) for the α_2-AR-derived peptide Q on the G-protein, we prepared a photoaffinity derivative of peptide Q using a heterobifunctional diazopyruvoyl (DAP) cross-linking agent. Photoactivatable cross-linking agents are particularly useful for identifying the regions of interaction between proteins and receptors (23,31). Generally, these probes are heterobifunctional, consisting of a chemically reactive site that is coupled to a protein of interest through a side chain and a photochemically reactive site that links the protein to its binding protein or receptor. Lawton and colleagues recently reported that the DAP moiety is a specific, photochemically activatable cross-linking agent (5) and described the ability of *p*-nitrophenyl 3-diazopyruvate (DAPpNP) to efficiently cross-link calmodulin

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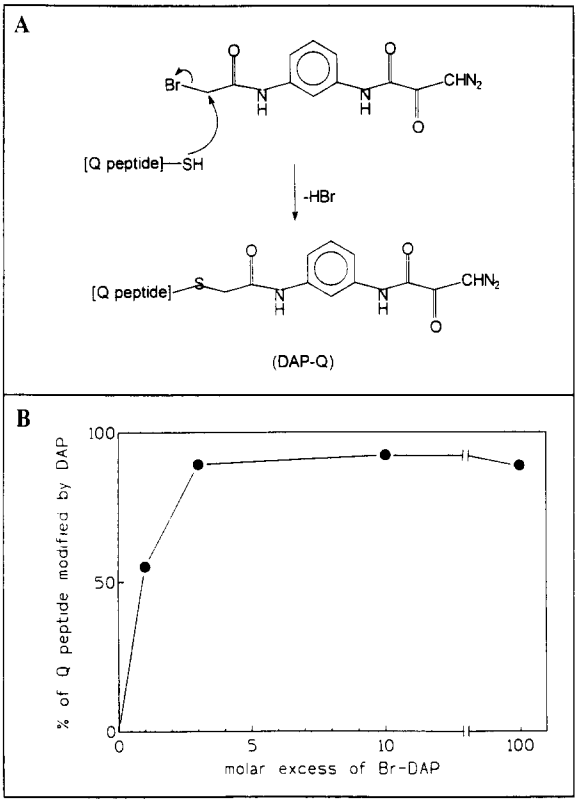


FIG. 1. Schematic illustrating the coupling of Br-DAP to the Q peptide. (A) Br-DAP is a heterobifunctional photoaffinity reagent; one end attaches covalently to a protein and upon photolysis the other end reacts with a nearby nucleophilic residue of a protein or other macromolecule. The coupling of Br-DAP to the Q peptide involves a nucleophilic attack of the sulfur from the *C*-terminal cysteine of the Q peptide followed by the release of bromine from the DAP molecule. (B) Peptide Q was incubated with 1:1, 3:1, 10:1, and 100:1 molar excess Br-DAP for 3 h in 20 m*M* Tris, pH 8.0. The percentage of Q peptide modified by DAP was assessed by HPLC as described in the Method section. The data are representative of two separate experiments.

to purified preparations of adenylate cyclase (7). In this study we use a new heterobifunctional cross-linking agent, *N*-bromoacetyl-*N*-(3-diazopyruvoyl)-*m*-phenylene-diamine (Br-DAP), a DAPpNP derivative, to study the molecular interactions between a synthetic peptide for which the sequence was derived from the α_2-AR with the target of this receptor, a purified G-protein. We describe the coupling of the sulfhydryl-selective cross-linking agent Br-DAP to peptide Q and the specific photocross-linking of the DAP-modified peptide Q (DAP-Q) to purified G-protein.

METHOD

Materials

The product of the reaction between *m*-phenylenediamine and DAPpNP was isolated and treated with bromoacetyl bromide and *N*-methylmorpholine to yield Br-DAP. The full details of this synthesis are described elsewhere (10). The GC/2 anti-α_o antibody was purchased from Dupont (Boston, MA). HPLC solvents were purchased from J. T. Baker (Phillipsburg, PA). All other chemicals and reagents were reagent grade or better and were purchased from Sigma Chemical Corp. (St. Louis, MO).

Peptide Synthesis

Peptide Q (RWRGRQNREKRFTC) was synthesized by a Biosearch 9600 Peptide Synthesizer using Fmoc (fluorenylmethoxycarbonyl) chemistry and was purified by a preparatory HPLC Beckman System Gold on a reverse-phase column. Peptide Q corresponds to the carboxy-terminal region of the third intracellular loop of the α_{2A}-AR (residues 361–373) with an additional cysteine attached to the *C*-terminus of the native receptor sequence. The purity and identity of this peptide were confirmed by electrospray mass spectrometry using a Vestec single quadrople mass spectrometer with electrospray interface by the Protein and Carbohydrate Structure Core at the University of Michigan.

General Chemical Procedures

Ultraviolet visible absorption spectra were obtained on a Perkins Elmer spectrophotometer (model #LS-5) using the absorption of ddH_20 as a standard reference. Analytical thin-layer chromatography was carried out on silica gel 60 F254 plates (EM Science, Gibbstown, NJ). Plates were visualized with UV light, photographed, and quantitated using the IMAGE QUANT program (Molecular Dynamics) as described previously (29).

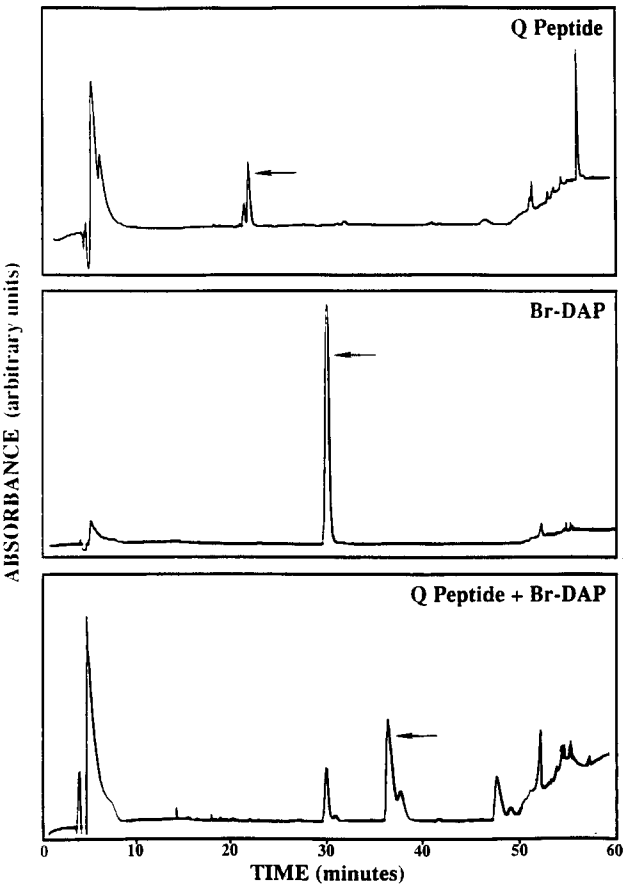


FIG. 2. HPLC analysis of Q peptide, Br-DAP, and Q peptide + Br-DAP. Samples were applied to a Vydac C-18 reverse-phase column (0.46 × 25 cm) and eluted with a 16.5–19% gradient of acetonitrile + 0.1% TFA. Absorbance was monitored at 220 nm. The Q peptide (A) displayed a peak with the retention time of 22 min; Br-DAP (B) displayed a peak at 30 min and the diazopyruvoyl-Q-peptide conjugate displayed a peak at 37 min.

TABLE 1

EVIDENCE OF DAP INCORPORATION INTO PEPTIDE Q AT Cys 14

1. Absorbance spectra:	
Q peptide; 220 and 280 nm	
DAP; 256 and 310 nm	
DAP-Q peptide; 220, 256, 280, and 310 nm	
2. Amino acid analysis:	
Q peptide; cysteine	
DAP-Q peptide; *carboxy-methyl cysteine*	
3. Mass spectrometry (Da):	
2137.5 (38%) = DAP + Q peptide	
2110.0 (47%) = DAP ($-N_2$) + Q peptide	
2125.0 (15%)	

Determination of free sulfhydryl groups in the Q peptide as well as in the DAP-Q conjugates was measured using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as described by Ellman and Lysko (4).

Conjugation of Q Peptide to Br-DAP

Br-DAP was combined with the Q peptide in a 3:1 molar ratio in 20 m*M* Tris (pH 8.0) + 10% acetone for 3–18 h at ambient temperature under low light conditions. Typically, 1.5 μmol of Br-DAP and 0.5 μmol of Q peptide were combined in 1 ml. The diazopyruvoyl–Q-peptide conjugates (DAP-Q) were isolated by HPLC (rabbit HP, Ranin) using a semipreparative (1.0 × 25 cm) Vydac 218TP C-18 reverse-phase column. The mobile phase consisted of A: 0.1% TFA in ddH_20; and B: 0.1% TFA in acetonitrile. A 30-min gradient from 16.5–19% acetonitrile was run at a flow rate of 2.5 ml/min. Detection was achieved by monitoring absorption at 220 nm using a Spectroflow 75 detector.

G-Protein Purification and Preparation

The G_o/G_i was purified from synaptosomal membranes of bovine brain cortex by the method of Sternweis and Robishaw (26) as modified by Kim and Neubig (12). Protein was quantitated using the method of Shaffener and Weisman (24). [^{35}S]GTPγS binding was measured as described by Sternweis and Robishaw (26).

DAP-Q Photolysis

The DAP-Q was incubated on ice for 10 min with purified G_o/G_i from bovine brain, ovalbumin, or bovine serum albumin at the indicated concentrations in buffer A (50 m*M* Na-HEPES, pH 8.0, 1 m*M* EDTA, 1.3 m*M* $MgCl_2$, 0.1% Lubrol, and 60 μM GTP). A 366 nm mineral light (model UVGL-25) was then placed 4.5 cm from the samples for 10 min. Sodium dodecylsulfate-polyacrylamide gels (SDS-PAGE) sample buffer was then added and cross-linked products were prepared for electrophoresis.

Western blots

Cross-linked products were separated on 10% SDS-PAGE prepared according to Laemmli (15). Proteins were transferred to Immobilon, and a Western blot was performed with G-protein antisera (Dupont) for the subunit (GC/2) at 1/1000 dilution. Blots were then incubated with biotin–IgG complex and ExtrAvidin at 1/2000 dilutions, followed by visualization by dye precipitation (nitro-blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate).

RESULTS

It has been previously established that DAPpNP reacts, cleanly and with high yield, with primary and secondary amines to yield three diazopyruvamide derivatives (5). Further exploration of the chemistry of DAPpNP led to the synthesis of *N*-(3-aminophenyl)-3-diazopyruvamide. As shown in Scheme 1, the free amine functionality of this latter compound permits facile condensation with bromoacetyl bromide, in the presence of *N*-methylmorpholine, to yield the α-bromo amide derivative Br-DAP (10). A schematic of the coupling of Br-DAP to Q peptide resulting in the DAP-modified Q peptide (DAP-Q) is shown in Fig 1(A). The bromoacetyl group serves as a cysteine-specific modifier by forming a stable thioether linkage with the free sulfhydryl group. The concentration dependence of the DAP peptide

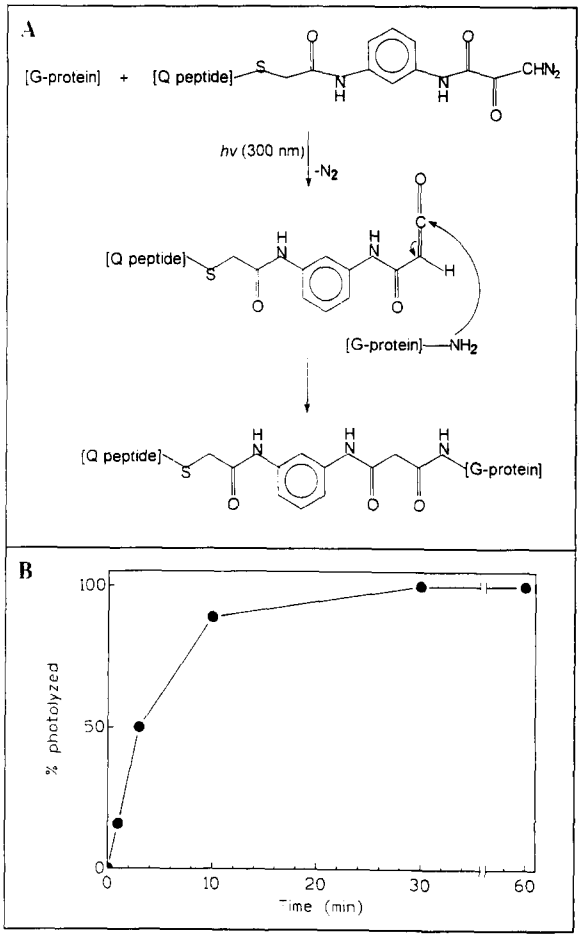


FIG. 3. Schematic illustrating the cross-linking of DAP-modified Q peptide to G-protein. (A) Exposure of DAP-Q peptide to UV light (λ max 366 nm) releases N_2 from the DAP moiety, which then undergoes rearrangement to the ketene amide. A nucleophile from the G-protein then reacts with the ketene amide and cross-links the Q peptide to G-protein. (B) Br-DAP (*modified with 2-mercaptoethanol at a 1:1 molar ratio*) was photolyzed with a UV light (λ max 366 nm) for 0, 1, 3, 10, 30, and 60 min and the products were analyzed by thin-layer chromatography (2:1 hexane:ethylacetate). The data are presented as the amount photolyzed as a percentage of control.

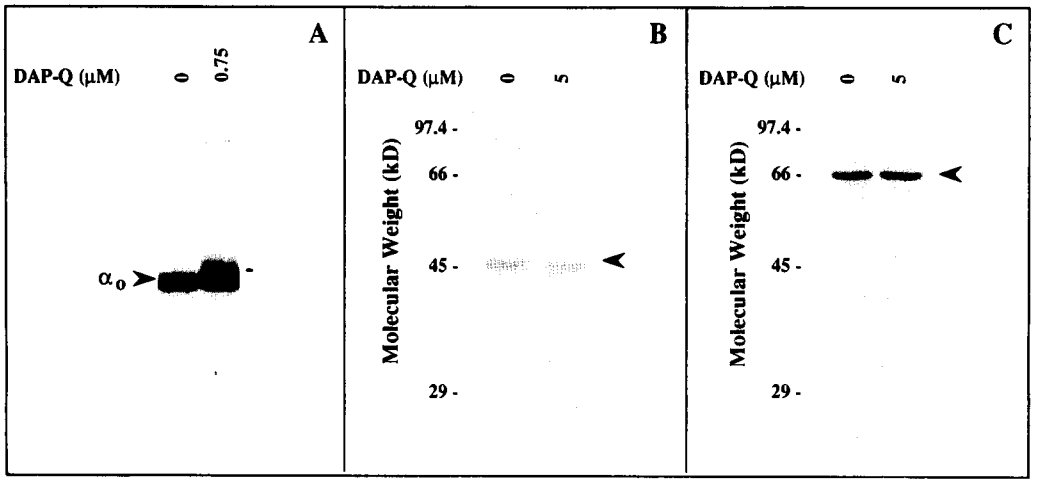


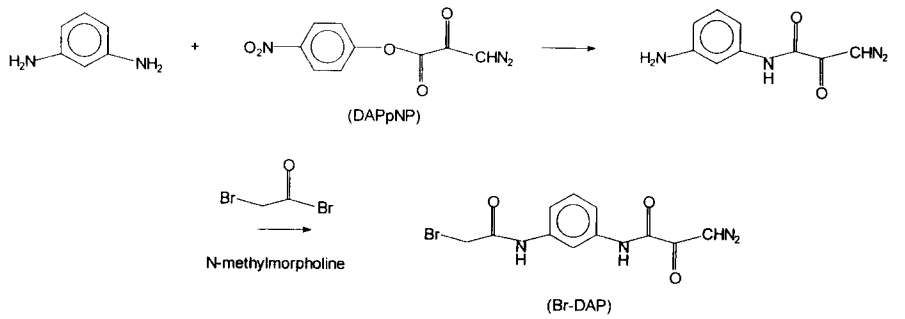
FIG. 4. Specificity of DAP-Q peptide cross-linking to G-protein. Q peptide was coupled to Br-DAP and then cross-linked to G-protein, ovalbumin, or bovine serum albumin as described in the Method section. (A) 250 n*M* purified bovine brain G_o/G_i was photolyzed in the absence or presence of 750 n*M* DAP-Q peptide. The cross-linked products were analyzed by 10% polyacrylamide gel electrophoresis followed by Western blot analysis using an anti-α_o subunit antibody. (B,C) 100 n*M* ovalbumin (B) or 100 n*M* bovine serum albumin (C) was photolyzed in the absence or presence of 5 μ*M* DAP-Q peptide. The cross-linked products were analyzed by 10% polyacrylamide gel electrophoresis and visualized by staining with Coomassie Blue R250. The data are representative of at least two separate experiments.

conjugation is shown in Fig. 1(B). Peptide Q was incubated with one-, three-, 10-, and 100-fold molar excess of Br-DAP, and the DAP peptide conjugates were isolated from the reaction mixture by C-18 reverse-phase HPLC as described in the Method section. A 1:1 molar ratio of DAP:peptide Q results in the conjugation of approximately 55% of peptide Q, whereas a 3:1 molar ratio conjugates approximately 90% of peptide Q to DAP.

Figure 2 shows the reverse phase C-18 HPLC profiles of peptide Q, Br-DAP, and the DAP-Q derivative. The Q peptide [Fig. 2(A)] displayed a major peak with a retention time of 22 min during a 30-min gradient from 16.5–19% acetonitrile. Under the same conditions, Br-DAP [Fig. 2(B)] displayed a peak at 30 min. After conjugation of the Q peptide with Br-DAP, a major peak is observed at 37 min, which has been isolated and characterized as DAP-Q peptide. The minor shoulder peaks observed in the HPLC traces of Q peptide and DAP-Q peptide were of the same mass as the major peak. It is likely that the minor peaks are due to an isopeptide bond formation at the glutamic acid residue.

To ensure that we have isolated a Q peptide derivative with DAP incorporated into the *C*-terminal cysteine, we first tested for the presence of a free sulfhydryl group in the modified peptide. The Q peptide and the modified Q peptide were incubated with the sulfhydryl reagent DTNB (4). Under these conditions, the Q peptide reacted with DTNB at 0.8 mol/mol whereas no reaction was observed with the modified Q peptide (data not shown). This suggests that the modified Q peptide no longer has a free sulfhydryl group, as would be predicted after attachment of the bromoacetyl moeity of DAP compound to the *C*-terminal cysteine. Additional evidence that the Q peptide incorporated DAP into the *C*-terminal cysteine is presented in Table 1. The HPLC-purified DAP-Q was analyzed by UV-visible absorption spectroscopy. The spectrum indicated absorption peaks characteristic of the diazopyruvoyl at 256 and 310 nm and the peptide bond (210 nm) and tryptophan (280 nm). In addition, amino acid analysis for Q peptide and DAP-Q peptide were identical except that the modified peptide had a carboxymethyl-cysteine, a modification consistent with DAP incorporation at the *C*-terminal cysteine. Mass spectrometry of DAP-Q peptide revealed a major peak with the expected molecular weight of 2137.5 (Da) and a lower molecular weight peak of 2110 (Da). The loss of molecular nitrogen from DAP-Q is consistent with the second peak of mass 2110 (Da). It is likely that the mass spectral detection methods caused the loss of N_2.

A schematic illustrating the cross-linking of DAP-Q to G-protein is shown in Fig. 3(A). DAP-Q is combined with purified



SCHEME 1.

G-protein and exposed to a UV light (λ max 366 nm) for 10 min. The photolysis efficiently generates, after loss of nitrogen and Wolff rearrangement, a highly reactive ketene amide that presumably reacts with a neighboring nucleophile from the G-protein to form a malonamide linkage between DAP-Q and the G-protein. Figure 3(B) shows the time course for the photolysis of DAP. The Br-DAP was modified with 2-mercaptoethanol at a 1:1 molar ratio. The probe was then photolyzed with a UV light (λ max 366nm) for 0, 1, 3, 10, 30, and 60 min and the products were analyzed by thin-layer chromatography (2:1 hexane:ethylacetate) as described in the Method section. Under these conditions, complete photolysis (loss of the reagent spot) was observed after 10 min.

Cross-linking G_o/G_i (250 n*M*) with DAP-Q peptide (750 n*M*) altered the electrophoretic mobility of the subunit of G-protein by the expected 2 kDa to produce a new band of 41 kDa on a Western blot probed with anti-α_o antisera [Fig. 4(A)]. A small amount of double cross-linking is also consistently observed (4 kDa shift). This cross-linking is specific in that the DAP-Q peptide at a sixfold higher concentration (5 μ*M*) fails to cross-link to ovalbumin (100 n*M*) or bovine serum albumin (100 n*M*) [Fig. 4(B,C)]. In addition, DAP-Q cross-linking to the α subunit can be blocked by unmodified Q peptide with an IC_{50} of 15 ± 6 μ*M* (28).

DISCUSSION

The applications of cross-linking agents are diverse. Cross-linking agents have been utilized to tether biological probes to antibodies, to identify amino acid residues in active enzyme sites, and to determine the proximity between subunits of oligomeric proteins (16). Heterobifunctional cross-linking agents are particularly useful for the conjugation of different proteins. Such agents have been useful in the identification of vasoactive intestinal peptide receptors in human lymphoblasts (31) and *N*-formyl peptide receptors in human polymorphonuclear cells (23).

We have described the application of a new photoactivatable heterobifunctional cross-linking agent Br-DAP. The different side chain reactivities of Br-DAP permits coupling to be carried out in a stepwise manner, allowing purification of the DAP peptide intermediate prior to conjugation. The bromoacetyl group serves as a cysteine-selective modifier by forming a stable thioether linkage with the free sulfhydryl group of the peptide. The ability to use a low molar ratio of Br-DAP to peptide minimizes the potential for side reactions during the coupling. We have shown that the desired DAP modification of the cysteine residue in our receptor-derived synthetic peptide was rapid and specific. Also, the DAP-modified peptide can be easily separated from the unreacted reagents by HPLC. This probe can be photolyzed rapidly due to the reactive ketene amide-based diazopyruvoyl moiety. We have shown complete photolysis of the DAP moiety in 10 min with a UV light (366 nm). Alternatively, photolysis of the diazopyruvoyl function can be achieved by irradiating the compound with three high-intensity flashes from a studio flash unit (7).

Other photoactivatble reagents upon photolysis can produce intermediates such as carbenes (1) and nitrenes (11), which can react with unactivated carbon–carbon or carbon–hydrogen bonds under ideal conditions. However, almost all reactions performed in the presence of nucleophiles result in the trapping of the nucleophile by the electrophilic reactive intermediates. Also, upon photolysis of these aryl azide compounds, many reactive intermediates are formed, causing difficulty in identification of the chemical links produced (22). An advantage of the diazopyruvoyl class of compounds is the ability to efficiently produce one intermediate that reacts with a nucleophile from a nearby protein.

Previous data from our laboratory and others suggest that an α_2-receptor-derived peptide (peptide Q) can mimic receptor activation by binding to and stimulating the G-protein directly (2,3,9). We have used Br-DAP as a tool to show that the Q peptide from the third cytoplasmic loop of the α_2-AR does bind directly to the α subunit of purified G-protein. This binding is specific in that the DAP-Q peptide does not react with ovalbumin or bovine serum albumin. Also, DAP-Q cross-linking to the α subunit can be blocked by unmodified Q peptide (28). The specificity of this probe should enable us to identify the mutual contact sites between peptide Q and the G-protein and to predict features of the native α_2-AR that are important in receptor–G-protein coupling. Determination of the precise amino acid sequence in the G-protein that binds this photoactivatable peptide probe should aid in the future development of more potent and selective G-protein agonists and antagonists.

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