The Major Site of Photoaffinity Labeling of the γ-Aminobutyric Acid Type A Receptor by [3H]Flunitrazepam Is Histidine 102 of the α Subunit*

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The α subunit of the γ-aminobutyric acid type A (GABA_A) receptor is known to be photoaffinity labeled by the classical benzodiazepine agonist, [3H]flunitrazepam. To identify the specific site for [3H]flunitrazepam photoaffinity labeling in the receptor subunit, we have subjected photoaffinity labeled GABA_A receptors from bovine cerebral cortex to specific cleavage with cyanogen bromide and purified the resulting photolabeled peptides by immunoprecipitation with an anti-flunitrazepam polyclonal serum. A major photolabeled peptide component from reversed-phase high performance liquid chromatography of the immunopurified peptides was resolved by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The radioactivity profile indicated that the [3H]flunitrazepam photoaffinity labeling is covalently associated with a 5.4-kDa peptide. This peptide is glycosylated because treatment with the enzyme, peptide-N-glycosidase F, reduced the molecular mass of the peptide to 3.2 kDa. Direct sequencing of the photolabeled peptide by automated Edman degradation showed that the radioactivity is released in the twelfth cycle. Based on the molecular mass of the peptides that can be generated by cyanogen bromide cleavage of the GABA_A receptor α subunit and the potential sites for asparagine-linked glycosylation, the pattern of release of radioactivity during Edman degradation of the photolabeled peptide was mapped to the known amino acid sequence of the receptor subunit. The major site of photoaffinity labeling by [3H]flunitrazepam on the GABA_A receptor is shown to be α subunit residue His^102 (numbering based on bovine α1 sequence).

The γ-aminobutyric acid type A (GABA_A) receptor mediates the majority of rapid inhibitory synaptic transmission throughout the mammalian central nervous system. As a member of the superfamily of ligand-gated ion channels (1), the GABA_A receptor is believed to be a hetero-pentameric protein that spans the neuronal membrane to create a chloride conducting pore. The homologous subunits that assemble to form the receptor-chloride channel complex are encoded by distinct but related genes (2). Six α, four β, four γ, one δ, and two ρ subunit isoforms plus splice variants for many of the genes have been identified and classified by sequence similarity. However, the precise stoichiometry of subunit isoforms that comprise native receptors remains unknown.

A multiplicity of neuroactive drugs have been shown to interact specifically with the GABA_A receptor complex to modulate inhibitory neurotransmission throughout the brain (3). These include the benzodiazepines, barbiturates, some steroids and general anaesthetics, and possibly alcohol (4). Because of the clinical usefulness of the benzodiazepines as anxiolytics, hypnotics, and anticonvulsants, their interaction with the GABA_A receptor has been extensively studied. The benzodiazepines are known to be allosteric modulators of GABA_A receptors in that the classical agonists potentiate whereas the inverse agonists reduce GABA-mediated chloride conductances.

An area of particular interest has been the identification of protein domains involved in the interaction of benzodiazepines with the GABA_A receptor. Using multidisciplinary approaches, several groups have identified structural features of subunit isoforms that are important for ligand recognition and for the modulatory effects of the benzodiazepines (reviewed in Ref. 5). To date, site-directed mutagenesis has identified the amino acids Gly^225 of the α1 subunit (6), His^102 of the α1 subunit (7), and Thr^142 of the γ2 subunit (8) as residues that play a role in conferring the differential binding affinities of benzodiazepine ligands for the GABA_A receptor. Biochemical approaches have shown that the site of photoaffinity labeling by the classical agonist, [3H]flunitrazepam, is associated with the α subunit of the GABA_A receptor (9, 10) within the large extracellular amino-terminal domain (11, 12). In addition, partial sequences of proteolytic fragments from photoaffinity labeled receptors have indicated that the [3H]flunitrazepam site occurs within amino acid residues 8–297 of the α1 subunit (13), and using subunit specific antibodies, the site has been predicted to occur within residues 59–158 of the α1 sequence (14). We have mapped the [3H]flunitrazepam photoaffinity labeled peptides generated by hydroxylamine deavage to known GABA_A receptor sequences and have demonstrated that the site of photolabeling occurs within amino acids 1–103 of the α1 subunit (15). Considering these results, together, limit the predicted site of labeling to within residues 59–103 of the α1 subunit or within homologous segments of other α subunit isoforms. In the present study, we have employed immunoprecipitation and HPLC techniques to...
purify a [3H]flunitrazepam photoaffinity labeled peptide that was generated by cyanogen bromide cleavage of labeled GABA receptors from bovine cerebral cortex. It is shown by peptide mapping and microsequence analysis that the major site of [3H]flunitrazepam photoincorporation by the GABA receptor is likely to be the amino acid His102 of the bovine α1 subunit.

**EXPERIMENTAL PROCEDURES**

Materials—[N-Methyl-[3H]flunitrazepam (85.8 Ci/mmol) was from DuPont. Sheep anti-flunitrazepam polyclonal serum (1:17,680 titre, final dilution) was from Biodis International; protein G-linked Sepharose fast flow, flunitrazepam, CHAPS and Sigma.

Preparation of Membranes from Bovine Cerebral Cortex and Photoaffinity Labeling with [3H]Flunitrazepam—Adult bovine brain was obtained from a local slaughterhouse. The cerebral cortex was dissected out and was immediately frozen on dry ice and stored at -80°C. Brain membranes were prepared from approximately 100 g of partially thawed cortex as described previously (16). For the photoaffinity labeling reaction, membrane aliquots were thawed and diluted to a final protein concentration of 2 mg/ml in 20 mM Tris citrate, pH 7.4 buffer containing 1 mM EDTA, 1 mM benzamidine, 0.5 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 20 μg/ml bacitracin, and 0.02% NaN3. [3H]Flunitrazepam, isotopically diluted 5-fold to a specific activity of 17.2 Ci/mmol, was added to a final concentration of 10 nM. The mixture was incubated for 45 min in the dark on ice with constant shaking, before irradiating with long wave-length ultraviolet light for 45 min using a Spectroline ENF 260C lamp at a distance of 6 cm. The membranes were subjected to repeated cycles of centrifugation (150,000 g, 45 min) and resuspension until the radioactivity in the supernatant fell to close to the background, at which point the membranes were finally resuspended at a concentration of 15 mg/ml in the above buffer.

Cyanogen Bromide Cleavage of the Photoaffinity Labeled Receptor—Photoaffinity labeled membranes and Protein Preparation—Photoaffinity labeled membranes were stirred on ice at 4°C, and an equal volume of solubilization buffer containing 20 mM Tris citrate, pH 7.5, 0.5 mM KCl, 3% CHAPS, 0.3% asolectin, and protease inhibitors as noted above was added dropwise. After being stirred for 60 min at 4°C, the mixture was centrifuged for 75 min at 100,000 g. To the supernatant containing the photoaffinity labeled receptor, a volume of trichloroacetic acid was added to a final concentration of 12% (w/v). Following incubation on ice for 15 min, the solution was centrifuged at 10,000×g for 15 min, and the protein pellets were washed twice with acetone.

Cyanogen Bromide Cleavage of the Photoaffinity Labeled Receptor—CNBr cleavage (17) of the photolabeled receptor was carried out by dialysis against 0.4 M formic acid solution to achieve a final amount of CNBr equal to that of the total protein (mg per mg basis). Following 24 h of incubation at room temperature in the dark, the reaction mixture was diluted into 15 volumes of distilled water, freeze dried twice, and resuspended to about 10 mg protein/ml in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.02% NaN3 buffer for subsequent immunopurification.

Immunoprecipitation of [3H]Flunitrazepam Photoaffinity Labeled CNBr Peptides—Sheep raised anti-flunitrazepam polyclonal serum (approximately 1.25 μl of neat antiserum) was added per ml of the [3H]Flunitrazepam photoaffinity labeled CNBr peptides, and the mixture was incubated for 1 h at 37°C. About 1 μl of 50% protein A-Sepharose in 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.02% NaN3 buffer was added per ml of labeled peptide, and the slurry was incubated for 24 h at 4°C with constant mixing. The Protein G matrix was extensively washed by repeated cycles of centrifugation (10,000×g, 15 min) and resuspension in a series of different solutions; 1) 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.02% NaN3, 2) 10 mM Tris-HCl pH 7.4 buffer, 3) 20% (v/v) ethanol, and 4) distilled water. To specifically recover the labeled peptides while avoiding spurious contaminants that were present in the precipitation assay, a large excess of free unlabeled flunitrazepam was used to elute the immunoprecipitated peptides from the antibody-protein G complex. A volume of flunitrazepam dissolved in 10% ethanol was added to the protein G beads equivalent to 100 nmol of acceptor drugpmol of labeled peptide. The slurry was incubated for 24 h at 4°C with constant mixing, before the beads were pelleted by centrifugation and the supernatant containing the immunopurified peptides was removed and freeze-dried. The dried product was resuspended in distilled water, and the insoluble free flunitrazepam was removed by ether extraction. After gentle mixing with diethyl ether/distilled water (3:1) to solubilize the drug, the ether phase was discarded, and the aqueous phase containing the immunopurified peptide components was again freeze-dried. The [3H]flunitrazepam photoaffinity labeled CNBr peptides were finally resuspended in 0.1% trifluoroacetic acid and stored at 4°C for analysis by HPLC.

**RESULTS**

To characterize the site of photoincorporation by [3H]flunitrazepam on the GABA receptor, a photoaffinity labeled peptide component from bovine cerebral cortex was purified by immunopurification with a polyclonal antiserum raised against free flunitrazepam and with the precipitating reagent, protein G-linked Sepharose. In addition to quantitatively precipitating free [3H]flunitrazepam, the anti-flunitrazepam serum was shown to immunoprecipitate photoaffinity labeled peptides by specifically recognizing the [3H]flunitrazepam ligand covalently associated with the GABA receptor. After confirming (by SDS-PAGE) that the photoincorporation of [3H]flunitrazepam with the GABA receptor from bovine cerebral cortex was associated with a major 53-kDa protein, previously defined as the α subunit(s) (9, 10), and that there was no significant labeling of other species, the photoaffinity labeled
protein was solubilized and subjected to specific chemical cleavage at Met residues by treatment with CNBr. The concentration dependence of immunoprecipitation of \(^{[3}H\)flunitrazepam photoaffinity labeled CNBr peptides with anti-flunitrazepam serum was investigated to assess the optimal antibody concentration for use in large scale purification. A maximum immunoprecipitation of 70 ± 9% was achieved with less than 1 \(\mu\)l of neat antiserum/\(\mu\)mol of \(^{[3}H\)flunitrazepam photoaffinity labeled CNBr peptide. Nonspecific adsorption of labeled peptides to the protein G matrix was measured in the absence of antiserum and represented less than 1% of the total yield.

Batch immunoprecipitation was used to purify \(^{[3}H\)flunitrazepam photoaffinity labeled CNBr peptides in quantities sufficient for further characterization. About 50% of the total yield of immunoprecipitated product could be specifically eluted from the antibody-protein G complex by incubation with free flunitrazepam. The radioactivity profile of the immunopurified peptides resolved by Tricine SDS-PAGE (Fig. 1A) demonstrated that CNBr cleavage of the \(\gamma\) subunit \(\alpha_{102}\) receptor preparation generated a \(^{[3}H\)flunitrazepam photoaffinity labeled peptide of 5.5-kDa molecular mass, with a minor component that resolved as a 2.5-kDa species. Unfortunately, the inadequate amount of purified protein precluded the use of silver stain for protein detection. The elution profile from reversed-phase HPLC of the immunopurified peptides (Fig. 1B) displays three apparent peaks of radioactivity, arbitrarily marked (i), (ii), and (iii). Although the \(^{3}H\) peak marked (iii) invariably represented 55–60% of the total radioactivity loaded onto the column, the relative amount of radioactivity in the other two peaks varied between peptide preparations. The percentage of \(^{3}H\) that eluted with the peaks ranged from 17 to 35% for (i) and from 8 to 26% for (ii). However, the combined cpm in peaks (i) and (ii) routinely represented 40–45% of the total radioactivity.

The pooled fractions from each of the radioactive HPLC elution peaks were resolved by Tricine SDS-PAGE for determination of the molecular mass of the \(^{[3}H\)flunitrazepam labeled CNBr peptide components. In addition, the samples were treated with the deglycosylation enzyme, N-Glycanase, to assess whether the radiolabeled peptides contained asparagine-linked oligosaccharides. On SDS-PAGE, HPLC peak (iii), following treatment of the peptides eluted in the unlabeled peptides with \(\beta\)-Glycanase and this accounted for approximately 55–60% of the total radioactivity loaded onto the column, the relative amount of radioactivity in the two peaks varied between peptide preparations. The percentage of \(^{3}H\) that eluted with the peaks ranged from 17 to 35% for (i) and from 8 to 26% for (ii). However, the combined cpm in peaks (i) and (ii) routinely represented 40–45% of the total radioactivity.

When HPLC peak (ii) was resolved by SDS-PAGE, it ran as a broad band of radioactivity with apparent molecular mass ranging from 8.7 to 12.1 kDa. After digestion with N-Gly-
Several structural determinants required for the allosteric modulation of GABA$_A$ receptors by the benzodiazepines have been characterized by site-directed mutagenesis of recombinant receptors (see Introduction). Using this information, the benzodiazepine binding domain has been modelled as a composite of these structural features (5). However, the identity of specific amino acid residue(s) in native GABA$_A$ receptors that are directly involved in benzodiazepine binding has remained an area of intense interest. One approach used extensively has been to specifically and irreversibly label the benzodiazepine binding site with the photoactivatable agonist, [³H]flunitrazepam, as first described by Möhler et al. (21). Although strong evidence has been reported to show that the major site of [³H]flunitrazepam photoaffinity labeling occurs on the GABA$_A$ receptor α$_2$ subunit (5), the precise position of the photo label on the polypeptide has not previously been established.

To identify the [³H]flunitrazepam photoaffinity labeling site on the GABA$_A$ receptor, a purified preparation of labeled peptide was required in sufficient quantities to allow for characterization by conventional biochemical techniques. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immunoprecipitation assay has provided a highly specific method to purify the photoaffinity labeled peptides from a crude brain preparation. The development of an anti-flunitrazepam immuno precipitated radioactivity and was shown by SDS-PAGE to be...
associated with a peptide of apparent molecular mass 5.4 kDa. It was also shown that this photolabeled peptide contains asparagine-linked carbohydrate, because after N-Glycanase digestion, it migrated as a 3.2-kDa peptide. Recognizing that: 1) CNBr specifically cleaves peptide bonds on the carboxyl side of Met residues with high efficiency, except for Met-Thr and Met-Ser bonds, which are essentially resistant to CNBr and 2) asparaginyl-linked glycosylation exclusively occurs at the consensus sequence of Asn-Xaa-Thr or Asn-Xaa-Ser, the origin of the photolabeled peptide can be mapped to the known asparagine sequence of the NH2-terminal domain of the GABA_A receptor α1 subunit (see Fig. 5). Considering the potential sites for cleavage by CNBr and for asparaginyl glycosylation, the only peptide that could be generated from the α1 subunit to contain Asn-linked carbohydrate and to resolve by electrophoresis as described above is Ala^91^-Met^114. This peptide has a predicted molecular mass of 3.1 kDa without consideration for glycosylation, which is close to the estimates obtained by SDS-PAGE analysis. The other CNBr peptide that could be generated from the α1 subunit is the residue that was shown by point mutation to be required for the high affinity binding of benzodiazepines in recombinantly expressed GABA_A receptors (7).

The chemical nature of the radioactivity present in HPLC peaks (i) and (ii) has not been established. HPLC peak (ii), which accounted for 10–25% of the total radioactivity, resolved by SDS-PAGE to a molecular mass ranging from 9 to 12 kDa, with deglycosylation causing a marginal shift in the gel profile that indicated a 9-kDa photolabeled peptide. The radioactivity profile obtained from direct sequencing of peak (ii) did not suggest the [3H]Flunitrazepam label was associated with a particular amino acid residue. Automated sequencing in the absence of PITC demonstrated that the release of radioactivity seen during the first few cycles of Edman degradation was not due to the generation of radiolabeled PTH amino acids resulting from NH2-terminal cleavage of a labeled peptide. Although other, less abundant, isoforms of the α subunit have been identified in cortical GABA_A receptors, the interpretation for the origin of the photolabeled peptide is not compromised, because the isoforms possess a high degree of sequence identity throughout this domain.

The pattern of release of radioactive PTH amino acids obtained from automated Edman degradation of the major photolabeled peptide (Ala^91^-Met^114) indicated the [3H]Flunitrazepam is covalently associated with the twelfth residue, which corresponds to His in position 102 of the α1 subunit. The photocoenrporation of [3H]Flunitrazepam with His^102 is consistent with the findings of hydroxylamine cleavage experiments that demonstrated photolabeling occurred prior to Asn^103, as well as previous reports that predicted the site occurred within limited subunit domains (see Introduction). The involvement of a histidine residue in the interaction of benzodiazepines with the GABA_A receptor was implicated in earlier studies that investigated the effects of chemical modification and the pH dependence of radioligand binding (23, 24). In addition, His^102 of the α1 subunit is the residue that was shown by point mutation to be required for the high affinity binding of benzodiazapine agonists in recombinantly expressed GABA_A receptors (7).

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β-subunit. Alternatively, the peptide may have been generated from a different domain of the α subunit or from another subunit subtype, thereby representing a minor fraction of photolabel incorporated with a residue other than His102. Recent studies have suggested that the benzodiazepine binding domain is made up of determinants from both the α and γ subunits. Site-directed mutagenesis has found that Thr142 of the γ subunit is involved in conferring the modulatory effects of benzodiazepine ligands (8), and previous photoaffinity labeling experiments have shown data that suggest some [3H]flunitrazepam label may incorporate with the γ subunit (14). The apparent inability to resolve the radioactivity present in HPLC peak (i) by electrophoresis suggested the [3H]flunitrazepam was not associated with a distinct peptide. In addition, the radioactivity profiles obtained from automated sequencing of peak (i) were not consistent with the release of labeled PTH amino acids from the Edman degradation reaction. It is possible that the peak (i) fraction of the immunopurified product may represent free [3H]flunitrazepam or ligand incorporated with carbohydrate or some other nonprotein molecule.

In conclusion, we have presented evidence that photoaffinity labeling of the GABA<sub>A</sub> receptor with [3H]flunitrazepam leads to the covalent association of the ligand with His<sup>102</sup> of the α subunit. Despite our intention to characterize the photolabeled peptide components by microsequence analysis, current technical limitations and insufficient yields of the purified peptides have precluded the identification of the PTH amino acids generated during each cycle. Therefore, although His<sup>102</sup> of the α subunit is likely to be the major site of photoincorporation, it is not possible to substantiate the possible existence of other amino acids that may be photolabeled by [3H]flunitrazepam.

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