Mapping the benzodiazepine photoaffinity-labelling site with sequence-specific γ -aminobutyric acid_A-receptor antibodies

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The γ -aminobutyric acid_A (GABA_A) receptor purified from adult bovine cerebral cortex was photoaffinity-labelled with the agonist benzodiazepine [³H]flunitrazepam and the radioactivity shown to be coincident with a band with M_r 53000 that was recognized by three anti-(GABA_A receptor α 1 subunit sequence)-specific antibodies. Complete and limited CNBr cleavage of the purified photoaffinity-labelled receptor was carried out. The products of this reaction were analysed for radioactivity, for immunoreactivity with anti-[α 1-(1–15)-peptide], anti-[α 1-(324–341)-peptide] and anti-[α 1-(413–429)-peptide] polyclonal antibodies and for carbohydrate by biotinylated concanavalin A lectin overlay. Complete CNBr cleavage gave a radioactive peptide with M_r 10000–12000 that was not recognized by the above-mentioned specific antisera. By using the deduced amino acid sequence of the α 1 subunit [Schofield, Darlison, Fujita, Burt, Stephenson, Rodriguez, Rhee, Ramachandran, Reale, Glencorse, Seeburg & Barnard (1987) *Nature* (London) 328, 221–227], it is proposed that the site of the benzodiazepine-agonist photoaffinity-labelling reaction does not lie within the amino acid sequences α 1 1–58 and α 1 149–429.

INTRODUCTION

The γ -aminobutyric acid_A (GABA_A) receptor of mammalian brain is a member of the ligand-gated ion-channel superfamily (Schofield *et al.*, 1987). It is a chloride-ion channel that is gated by the major inhibitory neurotransmitter GABA and it has been shown to contain multiple allosteric drug-binding sites for therapeutically important compounds, notably the anxiolytic benzodiazepines and the depressant barbiturates. The GABA_A receptor is an integral membrane glycoprotein with a heterologous oligomeric structure. It has been purified to apparent homogeneity from several vertebrate species by benzodiazepine affinity chromatography and it was shown to consist of two polypeptide chains, α with M_r 53000 and β with M_r 58000 (reviewed by Stephenson, 1988). The primary structures of the α 1 and the β polypeptides have been deduced by molecular cloning (Schofield *et al.*, 1987). The subunits are similar in sequence not only to each other (~35% amino acid sequence identity; ~55% similarity), but to other members of the superfamily, which include the nicotinic acetylcholine receptor and the glycine receptor (Schofield *et al.*, 1987). This led to a proposed model of the GABA_A receptor with a large extracellular hydrophilic *N*-terminal domain, four transmembrane hydrophobic domains, M1–M4, with the *C*-termini of both subunits located extracellularly and the large hydrophilic loop between M3 and M4 being intracellular (Schofield *et al.*, 1987).

The locations of the various drug-binding domains within the receptor structure are not known. It is known, however, that the benzodiazepine [³H]flunitrazepam specifically photoaffinity-labels the α -subunit with M_r 53000 in brain membranes (e.g. Mohler *et al.*, 1980)

and in purified receptor preparations (e.g. Sigel *et al.*, 1983). Czajkowski & Farb (1986) showed that the benzodiazepine recognition site is orientated towards the extracellular space by carrying out the photoaffinity-labelling reaction in chick neuronal cells and showing that this reaction was competitively blocked by the positively charged benzodiazepine Ro 7-0213. Ro 7-0213 is non-membrane-permeable (Czajkowski & Farb, 1986). Also, limited tryptic-digestion studies of photoaffinity-labelled rat and chick brain membrane preparations have shown that a fragment of M_r 24000–27000 remains anchored in the membrane (Klotz *et al.*, 1984; Eichinger & Sieghart, 1985; Czajkowski & Farb, 1986; Czajkowski *et al.*, 1989). Treatment with higher concentrations of trypsin resulted in the appearance of a 6000- M_r fragment into the supernatant (Klotz *et al.*, 1984). Since the orientation of the receptor in the membranes at this time is predicted and not yet established, these experiments are of limited value for interpretation, even with the knowledge of the primary structure of the α -subunit.

In order to overcome these problems, we have adopted the approach of photoaffinity-labelling the purified receptor with [³H]flunitrazepam and subjecting this material to defined chemical cleavage with CNBr. The products of the reaction are identified with GABA_A-receptor-sequence-specific antibodies. This approach is of value for the study of rare proteins because it is difficult to purify them in sufficient amounts for amino acid microsequencing and also, particularly in the case of the GABA_A receptor peptides, isolation to homogeneity is complicated by the existence of multiple subunit isoforms (Levitan *et al.*, 1988; Pritchett *et al.*, 1988a). We report below that the benzodiazepine photoaffinity-labelling site does not lie within the amino acid sequences α 1 1–58 and α 1 149–429.

Abbreviations used: GABA, γ -aminobutyric acid; SDS/PAGE, SDS/polyacrylamide-gel electrophoresis.

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EXPERIMENTAL

Materials

[*N*-methyl-³H]Flunitrazepam (85 Ci/mmol), biotinylated anti-(rabbit Ig), streptavidin biotinylated peroxidase complex and low-*M_r* rainbow-coloured pre-stained markers were purchased from Amersham International. Biotinylated concanavalin A was from Vector Laboratories, Burlingame, CA, U.S.A. Pre-stained protein standards were from Bethesda Research Laboratories, Gaithersburg, MD, U.S.A. Flunitrazepam and Ro 7-1986/1 were kindly donated by Dr. H. Mohler, Hoffman-La Roche, Basel, Switzerland, and clorazepate was a gift from Boehringer Ingelheim, Bracknell, Berks., U.K. The anti-(GABA_A-receptor sequence)-specific antibodies used were as previously described (Duggan & Stephenson, 1989; Stephenson *et al.*, 1989), and all other materials were as noted elsewhere (Sigel *et al.*, 1983; Stephenson *et al.*, 1986a).

Preparation of purified GABA_A receptor

The GABA_A receptor was purified from adult bovine cerebral cortex by the sodium deoxycholate/Triton X-100 benzodiazepine affinity-chromatography method as described previously (Sigel *et al.*, 1983).

Photoaffinity-labelling and CNBr cleavage of the purified GABA_A receptor

The purified GABA_A receptor was photoaffinity labelled with [³H]flunitrazepam as described previously (Sigel *et al.*, 1983; Stephenson *et al.*, 1986b). The receptor (4 ml, 60–80 pmol of [³H]flunitrazepam-binding sites) and [³H]flunitrazepam (400 μl, 100 nM final concn.) were incubated on ice for 60 min. Samples were irradiated at 254 or 366 nm for three 15 min periods with 5 min intervals between successive irradiations to prevent overheating, at 6 cm distance at 4 °C with shaking. Excess unlabelled flunitrazepam (10⁻⁵ M final concn.) was added and samples were further incubated in the dark for 60 min. Non-specific incorporation of radioactivity was determined by carrying out the photoaffinity-labelling reaction in the presence of non-radioactive flunitrazepam.

The photoaffinity-labelled receptor was precipitated by the addition of an equal volume of 24% (w/v) trichloroacetic acid. Samples were incubated on ice for 15 min, centrifuged (10000 g, 15 min) and the supernatant discarded. The protein pellets were further washed with 12% (w/v) trichloroacetic acid and twice with acetone. Aliquots of the photoaffinity-labelled purified GABA_A receptor were precipitated and washed and either subjected to SDS/PAGE under reducing conditions followed by fluorography to show the specificity of photoaffinity labelling of the intact receptor or they were counted for radioactivity to determine the efficiency of the photolabelling reaction. In the latter case, the protein pellets were counted for radioactivity to determine specific irreversible incorporation.

CNBr cleavage of the photoaffinity-labelled receptor was carried out according to the method of Gross (1967). The precipitated protein was dissolved in 70% (v/v) formic acid (500 μl). CNBr [25 μl of a 10% (w/v) CNBr in 70% (v/v) formic acid stock solution] was added, followed by 70% (v/v) formic acid (500 μl). The mixture was incubated for 21 h at room temperature in the dark and then the sample was freeze-dried. The CNBr cleavage products were dissolved with boiling in SDS/PAGE

sample buffer in the presence of 20 mM-dithiothreitol and analysed as described below.

SDS/PAGE and Western blotting

The purified GABA_A receptor and the CNBr cleavage products were analysed by SDS/PAGE under reducing conditions in either 12%-(w/v)-polyacrylamide slab gels or 15.5–20% polyacrylamide-gradient slab gels. Proteins were stained by the silver-stain method as described previously (Mamalaki *et al.*, 1987), and radioactivity was determined by immersion of the gel in Autofluor (National Diagnostics), followed by gel drying and exposure to pre-flashed Fuji X-ray film at -80 °C. *M_r* values of peptides in gradient gels were calculated from standard calibration curves of log *M_r* versus acrylamide monomer concentration (%). Alternatively, samples were transferred to nitrocellulose membranes and Western blotting was carried out as previously described (Stephenson *et al.*, 1986a; Duggan & Stephenson, 1989). The antibodies used were anti-[α1-(1–15)-peptide], anti-[α1-(324–341)-peptide] and anti-[α1-(413–429)-peptide] at dilutions of 1:100 as described by Duggan & Stephenson (1989) and Stephenson *et al.* (1989). Radioactivity was determined either by scanning on a Berthold t.l.c. linear analyser or by immersion in Autofluor, drying and exposure to pre-flashed Fuji X-ray film as described above.

Biotinylated concanavalin A overlay

Protein and peptides were transferred to nitrocellulose as for Western blotting, except that the transfer buffer contained 0.1% (w/v) SDS. The nitrocellulose was rinsed superficially with 50 mM-Tris/HCl, pH 7.4, containing 0.9% (w/v) NaCl for 10 min at room temperature. It was quenched by incubation with 2% (w/v) polyvinylpyrrolidone in 50 mM-Tris/HCl, pH 7.4, containing 0.9% (w/v) NaCl, 5 mM-MgCl₂ and 1.5 mM-CaCl₂ for 1 h at 37 °C. The nitrocellulose was incubated with biotinylated concanavalin A (10 μg/ml) in 50 mM-Tris/HCl, pH 7.4, containing 0.9% (w/v) NaCl and 2% (w/v) polyvinylpyrrolidone for 60 min at room temperature. Blots were washed with four changes of 50 mM-Tris/HCl, pH 7.4, containing 0.9% (w/v) NaCl, 2% (w/v) polyvinylpyrrolidone and 0.2% Tween at room temperature over a period of 20 min.

The Vectastain ABC reagent was prepared according to the manufacturer's instructions and then incubated for 45 min at room temperature with the nitrocellulose (5 ml). The nitrocellulose was washed with 50 mM-Tris/HCl, 0.9% (w/v) NaCl, 2% (w/v) polyvinylpyrrolidone and 0.1% Tween as described above, and then developed. The developing solution was physiological buffered saline (5 ml), containing diaminobenzidine (100 μl of a 40 mg/ml stock solution), NiCl₂ (25 μl of an 80 mg/ml stock solution) and 3% (v/v) H₂O₂ (15 μl). The specificity of the reaction was controlled by carrying out the reaction with biotinylated concanavalin A in the presence of 0.2 M-α-methyl mannoside. After development, the nitrocellulose membranes were immersed in Autofluor, dried and exposed to pre-flashed X-ray film.

RESULTS

Photoaffinity labelling of the purified GABA_A receptor

In accord with previous observations, it was shown that, after u.v. irradiation, [³H]flunitrazepam was speci-

Fig. 1. Western blotting of complete and limited CNBr digestion peptides of [3 H]flunitrazepam-photoaffinity-labelled purified GABA_A receptor

The purified GABA_A receptor was photoaffinity-labelled with [3 H]flunitrazepam and subjected to complete and limited CNBr chemical cleavage. The resultant peptides were dissolved in SDS/PAGE sample buffer and subjected to SDS/PAGE under reducing conditions in 15.5–20% polyacrylamide gradient slab gels. The peptides were transferred to nitrocellulose membranes by Western blotting and probed with anti-(GABA_A receptor sequence-specific) antibodies as described in the Experimental section. Following development, some of the immunoblots were incubated in Autofluor and exposed to X-ray film at -80°C . A, M_r values for positions of pre-stained protein standard markers; lanes B–F, blotting with the anti-[α 1-(1–15)-peptide] antibodies, and samples are: B, purified GABA_A receptor; C, complete CNBr cleavage products; D, limited CNBr cleavage products; E and F, fluorographs of lane C and lane D respectively; lanes G and H, immunoblotting with anti-[α 1-(324–341)-peptide] antibodies, where G is whole receptor and H is the products of complete CNBr cleavage; lanes I and J, immunoblotting with anti-[α 1-(413–429)-peptide] antibodies, where I is whole receptor and J is after complete CNBr cleavage. $\blacktriangle$ denotes the position of the five immunoreactive peptides found for both the anti-[α 1-(324–341)-peptide] and the anti-[α 1-(413–429)-peptide] antibody immunoblots.

fically and irreversibly incorporated into the purified GABA_A receptor. It was found that $20.4 \pm 9\%$ ($n = 11$ independent receptor preparations) of the reversible benzodiazepine-binding sites were photoaffinity-labelled with [3 H]flunitrazepam. Under these photoaffinity-labelling conditions, it was shown that the radioactivity was specifically incorporated into a single band of M_r 53000 by SDS/PAGE under reducing conditions followed by fluorography. Irradiation at 254 nm and 366 nm produced the same results with respect to both the efficiency of the photoaffinity-labelling reaction and the incor-

Fig. 2. Western blotting of complete and limited CNBr digestion peptides of [3 H]flunitrazepam-photoaffinity-labelled purified GABA_A receptor on SDS/12%-PAGE

The purified GABA_A receptor was photoaffinity-labelled with [3 H]flunitrazepam and subjected to complete and limited CNBr chemical cleavage. The resultant peptides were dissolved in SDS/PAGE sample buffer and subjected to SDS/PAGE under reducing conditions in 12%-polyacrylamide slab gels. The peptides were transferred to nitrocellulose membranes and probed with anti-(GABA_A receptor sequence)-specific antibodies as described in the Experimental section. After development, some of the immunoblots were incubated in Autofluor and exposed to X-ray film at -80°C . Lanes A and B, blotting with anti-[α 1-(1–15)-peptide] antibodies with purified receptor (A) or limited CNBr cleavage products (B); lanes C and D, blotting with anti-[α 1-(413–429)-peptide] antibodies with limited CNBr cleavage products (C) and purified receptor (D); lane E, pre-stained protein standards with M_r values of 92000, 66000, 42000, 26000 and 18000 respectively; lane F, fluorograph of lane B. $\blacktriangle$ denotes the position of the five radioactive peptides which are coincident with five of the immunoreactive peptides recognized by anti-[α 1-(1–15)-peptide] antibodies.

poration of the radioactivity into the polypeptide of M_r 53000.

When immunoblotting was carried out on [3 H]flunitrazepam-photoaffinity-labelled receptor, it was found that the radioactive band was coincident with that which was recognized by anti-[α 1-(1–15)-peptide], anti-[α 1-(324–341)-peptide] and anti-[α 1-(413–429)-peptide] antibodies (Fig. 1). In some preparations, a minor component of M_r 46000 was seen in silver-stained gels. When present, this band was radioactive and was recognized by the anti-[α 1-(1–15)-peptide] and anti-[α 1-(324–341)-peptide] anti-

Fig. 3. SDS/PAGE of [^3H]flunitrazepam-photoaffinity-labelled purified GABA_A receptor after CNBr cleavage

The purified GABA_A receptor was photoaffinity-labelled with [^3H]flunitrazepam and subjected to CNBr chemical cleavage as described in the Experimental section. The resultant peptides were dissolved in SDS/PAGE sample buffer and subjected to SDS/PAGE in 15.5–20% polyacrylamide gradient slab gels under reducing conditions. A, M_r values of standards; lane B, silver stain of the CNBr cleavage products; lane C, purified GABA_A receptor; and D, scan of radioactivity of the CNBr bromide peptides with a Berthold t.l.c. linear analyser.

bodies, but not the anti-[$\alpha 1$ -(413–429)-peptide] antibodies (Fig. 2).

CNBr cleavage of the purified photoaffinity-labelled GABA_A receptor

Silver-stain SDS/PAGE in gradient gels of the photoaffinity-labelled purified GABA_A receptor after CNBr cleavage gave a characteristic pattern (Fig. 3). Notably, the α - and β -subunits of the receptor were completely cleaved to lower- M_r species; there was a CNBr cleavage product at M_r 33 000 and several peptides with M_r values in the range 26 000–3000. Radioactive scanning or fluorography of the CNBr cleavage products showed that the radioactivity was incorporated into a broad peptide band of M_r $11\,711 \pm 1334$ ($n = 6$), where n refers to the variation between gels for one receptor preparation (Figs. 1 and 3). The sizes of CNBr radioactive fragments from independent receptor preparations were in the range M_r 8000–12 000. Control samples were either purified receptor photolabelled with [^3H]flunitrazepam in the presence of 10^{-5} M-flunitrazepam for the determination of

non-specific incorporation of radioactivity or purified native receptor. For the former, there was no incorporation of radioactivity into the α -subunit and no radioactivity present in the CNBr cleavage products (results not shown). When the purified non-photolabelled receptor was subjected to CNBr cleavage followed by SDS/PAGE in gradient gels, the pattern of silver staining was found to be identical with that for [^3H]flunitrazepam-labelled material (results not shown).

Immunoreactivity of CNBr cleavage products of purified GABA_A receptor

The CNBr cleavage products were also tested for their reactivity with anti-(GABA_A -receptor sequence-specific) antibodies. It was found that anti-[$\alpha 1$ -(1–15)-peptide] antibodies recognized two peptide fragments of M_r $15\,293 \pm 1372$ ($n = 7$) and $14\,166 \pm 1894$ ($n = 7$) (Fig. 1). The anti-[$\alpha 1$ -(324–341)-peptide] antibodies recognized five peptide fragments of M_r 32 000, 30 000, 22 000, 18 000 and 12 000 (Fig. 1). Anti-[$\alpha 1$ -(413–429)-peptide] antibodies also recognized five peptide fragments of M_r 32 000, 29 000, 20 000, 17 000 and 11 000 (Fig. 1). In all experiments the immunoblotting was carried out by using photoaffinity-labelled GABA_A receptor and, after development of the blot, it was exposed to X-ray film. In each case, the radioactivity was clearly separated from the immunoreactive bands (Fig. 1).

In some experiments it was evident that the CNBr cleavage reaction had not gone to completion, because both silver-staining SDS/PAGE and fluorography revealed the presence of α -subunit together with cleavage products with M_r values higher than those observed previously. These partial CNBr digests were analysed for radioactivity and immunoreactivity with the sequence-specific antibodies in both SDS/PAGE gradient gels and 12% polyacrylamide gels for higher resolution. Firstly, on SDS/12%-PAGE, it appeared that the non-digested $\alpha 1$ -subunit migrated as a doublet, with the heavier staining band having an apparently higher M_r than the non-digested control $\alpha 1$ -subunit. This was the case for all three specific anti- $\alpha 1$ antibodies. The anti-[$\alpha 1$ -(1–15)-peptide] recognized seven bands with corresponding M_r 47 000, 44 000, 42 000, 40 000, 34 000, 27 000 and 25 000 as calibrated on SDS/12%-PAGE gels (Figs. 1 and 2). Preincubation of the anti-[$\alpha 1$ -(1–15)-peptide] antibody with $\alpha 1$ -(1–15)-peptide markedly decreased the immunoreactivity in the blots, demonstrating the specificity of the reaction. When these immunoblots probed with anti-[$\alpha 1$ -(1–15)-peptide] antibodies were exposed to X-ray film, five radioactive bands were found which, by overlaying, were shown to correspond to M_r values of 47 000, 44 000, 42 000, 40 000 and 34 000.

The pattern of distribution of immunoreactivity for the incomplete CNBr cleavage with the anti-[$\alpha 1$ -(413–429)-peptide] antibodies was distinct to that found for the N -terminal antibody. Thus the anti-[$\alpha 1$ -(413–429)-peptide] antibodies recognized eight bands of M_r 45 000, 36 000, 32 000, 29 000, 24 000, 23 000, 21 000 and 17 000. Overlaying of the immunoblot and the fluorograph obtained from the immunoblot showed that those bands of M_r 45 000 and 36 000 could not be distinguished from those of M_r 45 000 and 34 000 that were coincident with those recognized by the anti-[$\alpha 1$ -(1–15)-peptide] antibodies and thus may be radioactive. Even after 3 months exposure time, no radioactivity was detected with the M_r -32 000 peptide.

Fig. 4. Concanavalin A lectin overlay of complete and limited CNBr digestion peptides of [3 H]flunitrazepam-photoaffinity-labelled purified GABA_A receptor in SDS/12%-PAGE

The purified GABA_A receptor was photoaffinity-labelled with [3 H]flunitrazepam and subjected to complete and limited CNBr chemical cleavage. The resultant peptides were dissolved in SDS/PAGE sample buffer and subjected to SDS/PAGE under reducing conditions in 12%-polyacrylamide slab gels. The peptides were transferred to nitrocellulose membranes and probed with biotinylated concanavalin A as described in the Experimental section. After development, the membranes were incubated in Autofluor and exposed to X-ray film at -80°C . Lane A, ovalbumin (M_r 42 000 of the prestained protein standards); lane B, purified receptor; lane C, complete CNBr cleavage products; lane D, limited CNBr cleavage products; E, F and G are equivalent to lanes B, C, and D, except that they were pre-incubated with 0.2 M- α -methyl mannoside; lane H is a fluorograph of lane C, and lane I is a fluorograph of lane D. $\blacktriangle$ denotes the position of the five radioactive bands also shown in Fig. 2.

Reactivity of CNBr cleavage products of purified GABA_A receptor with biotinylated concanavalin A

Further identification of the CNBr cleavage fragments was obtained by studying their reactivity with biotinylated concanavalin A to ascertain which of the peptides are glycosylated. Both the α - and β -polypeptides reacted with biotinylated concanavalin A, and the reactivity was blocked by α -methyl mannoside, which showed that both the α - and β -polypeptides contain glucose and/or mannose residues (Figs. 4 and 5). The pattern of the peptides reactive with biotinylated concanavalin A after complete and limited CNBr cleavage in both SDS/12%- and SDS/gradient-PAGE under reducing conditions are

Fig. 5. Concanavalin A lectin overlay of complete and limited CNBr digestion peptides of [3 H]flunitrazepam-photoaffinity-labelled purified GABA_A receptor on 15.5–20%-polyacrylamide gels

The purified GABA_A receptor was photoaffinity-labelled with [3 H]flunitrazepam and subjected to complete and limited CNBr chemical cleavage. The resultant peptides were dissolved in SDS/PAGE sample buffer and subjected to SDS/PAGE under reducing conditions in 15.5–20%-polyacrylamide gels. The peptides were transferred to nitrocellulose membranes and probed with biotinylated concanavalin A as described in the Experimental section. After development, membranes were scanned on a Berthold t.l.c. linear analyser or soaked in Autofluor and exposed to X-ray film at -80°C . Lane A, pre-stained protein standards; lane B, purified receptor; lane C, limited CNBr cleavage products; D, complete CNBr cleavage products; E and F are fluorographs of lanes C and D respectively, and G is the scan of the radioactivity of lane F obtained by using a Berthold t.l.c. linear analyser, before exposure to Autofluor and X-ray film.

shown in Figs. 4 and 5. It can be seen that, for complete CNBr digestion, the strongest specific labelling was found for a doublet with M_r 15 000 and 14 000, and this corresponded to those peptides which were immunoreactive with anti-[α 1-(1–15)-peptide] antibodies. A weaker reaction was seen with several higher- M_r species and weakly with one of lower M_r , which was not coincident with the peak of radioactivity from the [3 H]flunitrazepam. For the incomplete digestion, the reactivity with biotinylated concanavalin A followed a distribution pattern similar to that for the immunoreactivity with anti-[α 1-(1–15)-peptide] antibodies; however, the multiplicity of the bands

precluded their unambiguous assignment with the radioactive peptides seen after fluorography.

DISCUSSION

In the present paper we have described studies in which we have begun to map the site of benzodiazepine photoaffinity labelling within the GABA_A-receptor structure. The strategy that we have adopted is the use of CNBr chemical cleavage of the purified [³H]flunitrazepam-photoaffinity-labelled receptor. The reasons for this were twofold. First, CNBr cleaves specifically at the C-terminal end of methionine residues, and the number of methionine residues in the $\alpha 1$ subunit is 12; thus both the number of peptide fragments is low and their predicted sizes (Fig. 6) make them amenable to study by SDS/PAGE. Secondly, the cleavage method is a chemical one, and thus it circumvents any problems that may be incurred in the further purification of these peptides, as would be the case with proteolytic methods.

We have shown that, for the intact receptor, after [³H]flunitrazepam photoaffinity-labelling, the radioactivity is coincident in two SDS/PAGE systems with the subunit labelled with three GABA_A-receptor $\alpha 1$ -subunit-specific antibodies. Complete CNBr cleavage, defined by the complete disappearance of α -subunit, gave a radioactive peptide of M_r 12000, a doublet with M_r 15000 and 14000, which was recognized by anti-[$\alpha 1$ -(1-15)-peptide] antibodies and five peptide fragments of M_r 32000, 30000, 22000, 18000 and 12000, which were recognized by both anti-[$\alpha 1$ -(324-341)-peptide] and anti-[$\alpha 1$ -(413-429)-peptide] antibodies. Since there is no methionine between the sequences $\alpha 1$ 324-341 and $\alpha 1$ 413-429, antibodies against these peptides recognize the same chemical cleavage products. Thus the peptide of M_r 14000 must correspond to the $\alpha 1$ protein sequence 1-58 and the C-terminal CNBr cleavage products to $\alpha 1$ 287-429 (12000), $\alpha 1$ 267-429 (18000), $\alpha 1$ 237-429 (22000), $\alpha 1$ 214-429 (30000) and $\alpha 1$ 49-429 (Table 1 and Fig. 6). The M_r -15000 peptide may correspond to $\alpha 1$ 1-81, although it is possible that the peptide recognized by the anti-[$\alpha 1$ -(1-15)-peptide] antibody is the $\alpha 2$ 1-58 subunit sequence, since it has been pointed out that there are several amino acid identities in the sequences $\alpha 1$ 1-15 and $\alpha 2$ 1-15 which may be sufficient to cause immunocross-reactivity between subunits (Duggan & Stephenson, 1989). The α -subunit is known to be N-glycosylated, and it has been shown that treatment of the purified GABA_A receptor with endoglycosidase F results in a decrease in the M_r of the α -subunit of 9000 (Mamalaki *et al.*, 1987).

Table 1. Summary of M_r values for the predicted CNBr cleavage products for sequential cleavage from the N- and C-termini of the $\alpha 1$ -subunit of the bovine GABA_A receptor

M_r values for the predicted CNBr cleavage products were calculated from the deduced primary structure of the $\alpha 1$ -subunit and are given to two significant figures. In the calculation it was assumed that the 9000 M_r contribution of the carbohydrate of the $\alpha 1$ -subunit (Mamalaki *et al.*, 1987) is attached at threonine-13.

Predicted peptide sequence	$10^{-3} \times M_r$	Predicted peptide sequence	$10^{-3} \times M_r$
1-429	53	1-429	53
1-286	46	59-429	36
1-266	44	82-429	33
1-236	40	91-429	32
1-213	37	113-429	30
1-148	29	115-429	30
1-141	28	132-429	29
1-131	27	142-429	28
1-114	24	149-429	28
1-112	23	214-429	24
1-90	21	237-429	21
1-81	20	267-429	18
1-58	16	287-429	16

When account is taken of the contribution by weight of the carbohydrate moieties to the peptides, there is good agreement between the sizes of the CNBr fragments predicted from the primary structure of the $\alpha 1$ -subunit and the experimental results (Table 1 and Fig. 6). (It was assumed in these calculations that most of the carbohydrate was attached at threonine-13; see below.) Since the peak of radioactivity did not correspond to any of the immunoreactive peptides, it must lie within the amino acid sequence $\alpha 1$ 58-148 or be associated with the $\gamma 2$ -subunit (Pritchett *et al.*, 1988a, 1989).

Additional information was obtained from the limited CNBr cleavage experiments. Thus anti-[$\alpha 1$ -(1-15)-peptide] antibodies were reactive with peptide fragments which correspond to $\alpha 1$ 1-286 (47000), $\alpha 1$ 1-266 (44000), $\alpha 1$ 1-236 (42000), $\alpha 1$ 1-213 (40000), $\alpha 1$ 1-148 (34000), $\alpha 1$ 1-141 (27000) and $\alpha 1$ 1-131 (25000). Again, there is good agreement between the predicted and the experimental sizes of the CNBr fragments (Table 1). The radioactivity was coincident for the exposure time of

Fig. 6. Diagrammatic representation of the primary structure of the $\alpha 1$ subunit of the bovine GABA_A receptor

The Figure shows the positions of the twelve methionine residues present in the linear sequence of the $\alpha 1$ -subunit. The dark areas show the putative transmembrane domains M1, M2, M3 and M4, and $\blacktriangle$ denotes the positions of the putative extracellular N-glycosylation sites. The protein sequences shown in single letter code are the synthetic peptides which were used for antibody production in their relative positions within the $\alpha 1$ -subunit primary structure.

3 months with the M_r -47000, -44000, -42000, -40000 and -34000 peptides, which indicates that it may be associated with the $\alpha 1$ 1-148 sequence, although the possibility that the radioactivity is associated with peptides derived from the $\gamma 2$ -subunit cannot be ruled out (see below). For the reactivity of the anti-[$\alpha 1$ -(413-429)-peptide] antibodies with the partial CNBr digestion, radioactivity was associated with the $\alpha 1$ -(59-429)-peptide, but beyond that the resolution was lost.

Further substantiation of the identification of the peptides was obtained by their ability to react specifically with biotinylated concanavalin A. In all experiments, for the complete CNBr digestion, there were two strongly reactive peptides with M_r values of 15000 and 14000. These were identical with the pair of peptides recognized by the anti-[$\alpha 1$ -(1-15)-peptide] antibodies and thus must correspond to the $\alpha 1$ 1-58 amino acid sequence. This contains one consensus glycosylation sequence at threonine-13 and thus confirms the identity of this peptide and, because the signal was strong, we suggest that it contains most of the carbohydrate, as predicted from the endoglycosidase experiments (Mamalaki *et al.*, 1987). There are two other consensus glycosylation sequences within the $\alpha 1$ -subunit. Threonine-340, with the current receptor model, is in the cytoplasmic loop domain, and it is assumed that it is not glycosylated; threonine-113 is extracellular, but under the conditions of complete chemical cleavage, it will be too small for the SDS/PAGE system employed here, i.e. two amino acids. For the partial cleavage, a multiplicity of reactive products were seen. It should be noted that, in this case, reactivity with all subunit isoforms present will be found, and the results are difficult to interpret. Nevertheless, the pattern of distribution of glycopeptides followed most closely the anti-[$\alpha 1$ -(1-15)-peptide] immunoreactive peptides. The $\alpha 1$ -subunit is the most prevalent of the GABA_A receptor polypeptides (Wisden *et al.*, 1988).

The M_r of the radioactive CNBr-cleaved material is in the range 8000-12000, which would correspond to a sequence of approx. 80-100 amino acid residues. Within the sequence $\alpha 1$ 59-148 there are six methionine residues, which, if cleavage occurred at all of these, would give rise to peptides with M_r values less than 10000. It is possible that, in the purified receptor, these methionine residues are oxidized and therefore not reactive with CNBr. Alternatively, it may be that the M_r of the photoaffinity-labelled peptide is overestimated in the SDS/PAGE system employed. It is proven, however, that the site is not contained in the sequences of the $\alpha 1$ -subunit 1-58 and 148-429.

It has been reported that a histidine residue is important for agonist benzodiazepine binding. Treatment of both membrane-bound receptor (Burch & Ticku, 1981; Lambolez & Rossier, 1987) and purified GABA_A receptor (Duggan & Stephenson, 1987) with the histidine modifying reagent, diethyl pyrocarbonate, resulted in the specific loss of benzodiazepine-binding sites. This inactivation was prevented by preincubation with unlabelled benzodiazepine agonists and reversed by treatment with hydroxylamine. Although it is not requisite that this histidine residue should lie within the sequence of the $\alpha 1$ -subunit, i.e. $\alpha 1$ 59-148, which has not been excluded from containing the site of photoaffinity labelling, it is notable that there are three histidine residues in this region at residues 102, 110 and 142. Each of these histidine residues is conserved in the $\alpha 2$ - and $\alpha 3$ -subunit

isoforms of the receptor. The $\alpha 1$ - and the $\alpha 3$ -subunits have been shown to be photoaffinity-labelled with benzodiazepines (Kirkness & Turner, 1988).

It was reported that when the GABA_A-receptor bovine α - and β -subunits were expressed in *Xenopus* oocytes (Levitan *et al.*, 1988) or the corresponding human subunits in transfected cells (Pritchett *et al.*, 1988a) GABA-gated chloride-ion fluxes, which were potentiated by barbiturates but not by benzodiazepines, were formed. Recently, however, a novel subunit ($\gamma 2$) has been identified (by homology hybridization) from human foetal brain that confers both benzodiazepine-sensitivity on GABA_A-gated channels and the detection of specific benzodiazepine-binding activity to receptors in cells transiently transfected with α -, β - and $\gamma 2$ -subunit cDNAs (Pritchett *et al.*, 1988b, 1989). The $\gamma 2$ -subunit is related in sequence to the α - and β -subunits to the extent of 30-40% amino acid identity; it has a predicted M_r of 49800 and three potential sites for N-glycosylation. If the $\gamma 2$ -subunit is present in the bovine GABA_A receptor and it is photoaffinity-labelled with [³H]flunitrazepam, it would be predicted to be indistinguishable from the labelled $\alpha 1$ -subunit on one-dimensional SDS/PAGE. There are several points to be made with regard to the role of the $\gamma 2$ subunit in benzodiazepine binding. First, the $\gamma 2$ -subunit cDNAs can form GABA_A-gated channels which are potentiated by barbiturates when expressed singly in cells, but benzodiazepine potentiation and binding was found only with the co-expression of $\alpha 1$ -, β - and $\gamma 2$ -subunit cDNAs (Pritchett *et al.*, 1988a, 1989). Secondly, Kirkness & Turner (1988) have shown that anti-[bovine $\alpha 1$ -(101-109)-peptide] antibodies recognize two polypeptides in porcine cerebral cortex which are coincident with [³H]flunitrazepam-photoaffinity-labelled subunits. These subunits correspond in M_r to the $\alpha 1$ - and the $\alpha 3$ -polypeptides as identified with isoform-specific antibodies (Stephenson *et al.*, 1989). Thirdly, we have shown that anti-[$\alpha 1$ -(324-341)-peptide] antibodies specifically immunoprecipitate radioactivity from denatured benzodiazepine-photoaffinity-labelled purified preparations (F. A. Stephenson & M. J. Duggan, unpublished work). Thus these observations all support an integral role of the $\alpha 1$ - and the $\gamma 2$ -subunits in benzodiazepine binding. In the studies that we have described here, we have used antibodies to amino acid sequences which are not conserved between the $\alpha 1$ - and the $\gamma 2$ -polypeptides, and thus the finding that, after complete CNBr cleavage, the agonist benzodiazepine-photoaffinity-labelling site does not lie within the sequences $\alpha 1$ 1-58 and $\alpha 1$ 146-429 is valid. For the partial-CNBr-cleavage experiments, however, although the radioactivity in the peptide fragments is coincident with the peptides recognized by the anti-($\alpha 1$ -subunit) antibody, we cannot exclude the possibility that the radioactivity is associated with $\gamma 2$ -subunit CNBr products, since the distribution of methionine residues in the $\gamma 2$ -subunit would predict a limited cleavage pattern similar to that obtained for the $\alpha 1$ -subunit with respect to M_r (Pritchett *et al.*, 1989). Further studies using anti-($\gamma 2$ -subunit) antibodies are required to answer the questions concerning the roles of the $\alpha 1$ - and the $\gamma 2$ -polypeptides in the formation of the benzodiazepine-binding site.

This work was supported by the Medical Research Council. We thank Dr. M. R. Hanley for the use of the Berthold t.l.c. linear analyser and Gill Patterson for word processing. F. A. S. is a Royal Society University Research Fellow.

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Received 21 April 1989/26 June 1989; accepted 17 July 1989