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# Photolabeled tryptic degradation products of benzodiazepine-binding proteins are glycopeptides

## Implications for localization of cleavage sites

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Crude synaptic membranes of avian and mammalian brain tissue were photolabeled with the benzodiazepine-receptor ligand [$^3$H]flunitrazepam and subsequently treated extensively with trypsin followed by incubation with endoglycosidase F. SDS-polyacrylamide gel electrophoresis and fluorography revealed that the final tryptic degradation product of 25 kDa in both pigeon and calf brain is deglycosylated in two steps. These results were confirmed by immunoblots of similarly pretreated membranes of pig brain using the α-subunit-specific monoclonal antibody bd-24. Benzodiazepine-receptor binding and its enhancement by GABA are largely retained after trypsinization. Based on the proposed transmembrane topology for the α-subunits of the GABA/benzodiazepine receptor, we suggest that the large N-terminal domain of benzodiazepine-binding proteins is protected against tryptic cleavage.

Aminobutyric acid receptor, γ-; Benzodiazepine receptor; Deglycosylation; Tryptic degradation; Photoaffinity labeling; Transmembrane topology; Immunoblotting

## 1. INTRODUCTION

The GABA/benzodiazepine receptor ionophore complex (GABA/BZR) in mammals is presumably composed of two α- and two β-subunits [1]. The α-subunit can be photolabeled with [$^3$H]flunitrazepam (FNZ) [2]. The photolabeled benzodiazepine (BZ)-binding proteins show species variation and regional heterogeneity [3,4] of the apparent molecular masses even after deglycosylation [5] as revealed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorography. In mammals, the main photolabeled BZ-binding protein of 53 kDa is deglycosylated to 49 kDa, a value which corresponds to that of the amino acid chain of the α-subunit as deduced from its cDNA [6]. Since the BZ-binding protein is deglycosylated in two steps, both potential glycosylation sites present in the amino acid chain of the α-subunit [6] appear to be glycosylated in vivo.

After tryptic degradation of the BZ-binding proteins a photolabeled 40 kDa peptide is detectable in various species. In avians and mammals high concentrations of trypsin lead to further degradation to a product of 25 kDa [7]. Treatment of tryptically degraded membranes with endoglycosidase F (Endo F) should clarify whether the 40 and 25 kDa fragments are glycosylated. This information will help to localize approximately the tryptic cleavage sites with regard to the proposed transmembrane topology of the α-subunit [6].

Therefore, the size of the trypsinized and/or deglycosylated photolabeled BZ-binding proteins from various species was determined by SDS-PAGE followed by fluorography. In addition, immunoblots of similarly pretreated membranes of

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pig brain were performed using the $\alpha$-subunit-specific monoclonal antibody (mAb) bd-24 [8], which cross-reacts with the pig brain $\alpha$-subunit [9,10]. Thereby the molecular masses and $\alpha$-subunit specificity of the photolabeled fragments could be assessed.

## 2. MATERIALS AND METHODS

Pigeon, pig and calf were obtained locally. [$^3$H]FNZ (spec. act. 81.8 Ci/mmol) and Improved En$^3$hance were from NEN. The enzyme endoglycosidase F (EC 3.2.1.96) was purchased from Boehringer Biochemica (Mannheim).

Brains were removed immediately after death or after 2 (pig) or 4 h (calf), rapidly frozen and stored at −70°C. Cortices and cerebella were investigated separately.

Membrane preparations, photoaffinity labeling with [$^3$H]FNZ and fluorography were performed as described [3]. Briefly, crude synaptic membranes were prepared by homogenizing and washing the membranes four times in 50 mM Tris-citrate buffer (pH 7.1), containing protease inhibitors. Incubation (90 min in the dark) and irradiation (10 min at a wavelength of 366 nm and a distance of 3 cm; Camag de Luxe) were performed in an ice-water bath in 50 mM Tris-citrate buffer containing 150 mM NaCl, the protease inhibitors and 10 nM [$^3$H]FNZ in the presence and absence of 3 $\mu$M clonazepam.

Tryptic degradation was performed as in [7]. After photolabeling membrane pellets were resuspended in 50 mM sodium phosphate buffer (pH 7.4) (0.2–0.5 mg/ml protein) containing different concentrations of trypsin (0–0.5 mg/ml). The suspensions were incubated for 30 min at 37°C in a water bath under shaking. Tryptic degradation was terminated by centrifugation at 48000 × $g$ for 20 min at 4°C followed by washing the suspensions twice with buffer containing soybean trypsin inhibitor (1 mg/ml).

For deglycosylation experiments, membranes (some of them after tryptic degradation) were resuspended in 25 mM Tris-HCl (pH 7.4), containing 0.2% SDS (0.5–1 mg/ml protein) and incubated with various concentrations of Endo F (0.025–1.25 U/ml) for 18 h at 37°C according to Schmitz et al. [5].

SDS-PAGE of the photolabeled membrane proteins was performed on 10% polyacrylamide gels. Gels were fixed, impregnated with Improved En$^3$hance, dried and exposed to Kodak X-Omat AR films for 2–4 weeks at −70°C.

For immunoblotting, both pretreated (trypsin, Endo F or combination thereof) and untreated membrane proteins of pig brain were separated by SDS-PAGE, blotted on 0.45 $\mu$m nitrocellulose sheets according to Towbin et al. [11] and incubated with the $\alpha$-subunit-specific mAb bd-24 for 12 h at 4°C. Non-specific staining was determined with antibody-free culture medium. Antibody binding was visualized using the streptavidin-biotinylated peroxidase complex (Amersham) and 3-amino-9-ethylcarbazole as substrate.

Reversible binding studies on pretreated (trypsin and/or Endo F) or untreated membranes of calf cortex and pigeon telencephalon were performed with [$^3$H]FNZ in 50 mM Tris-citrate buffer (pH 7.1), containing 150 mM NaCl using 6 concentrations of [$^3$H]FNZ (0.2–10 nM) in the absence or presence of 3 $\mu$M clonazepam. Specific binding was defined as the difference in amount of [$^3$H]FNZ bound in the absence and presence of 3 $\mu$M clonazepam. Additional binding studies were performed in the presence of 100 $\mu$M GABA. To ensure that tryptic degradation or deglycosylation of the BZ-binding proteins in calf cortex was complete prior to the reversible [$^3$H]FNZ binding studies, identically pretreated membranes were photolabeled in parallel and visualized fluorographically. Protein concentrations were determined with a modified method of Lowry et al. [12].

## 3. RESULTS

In accordance with previous results [7,13], trypsinization of pig cortex membranes converted the main photolabeled BZ-binding proteins of 53 kDa and the weakly labeled proteins of 56 and 59 kDa led to labeled proteolytic products of 45, 40 and 25 kDa (fig.1). The 53 kDa protein is tryptically degraded to 25 kDa via the intermediate product of 40 kDa [7]. The origin of the 45 kDa product, which under our labeling conditions is detectable only in cortex, is unclear. It is apparently not further degraded to 25 kDa.

When trypsinized membranes were subsequently treated with low Endo F concentrations (0.25 U/ml) the molecular mass of the 25 kDa product was reduced by approx. 2 kDa; incubation with a higher Endo F concentration (1.25 U/ml) led to further degradation to 21 kDa. Thus, the proteolytic product of 25 kDa is a glycopeptide, which is deglycosylated in two steps. We had previously shown that deglycosylation of all strongly labeled BZ-binding proteins in different vertebrates also reduces the molecular mass in two steps of approx. 2 kDa each [5]. The deglycosylation pattern of weakly and/or diffusely labeled bands, such as those of 56 and 59 kDa in pig cortex (fig.1), cannot be evaluated properly. The deglycosylated proteolytic products of 45 and 40 kDa are not discernible in fig.1. In pig cerebellum, the 25 kDa peptide is also deglycosylated to 21 kDa (not shown).

In order to determine the subunit of origin giving rise to the various degradation products, immunoblots were performed with the $\alpha$-subunit-specific mAb bd-24. In addition to an original 53 kDa protein the two tryptic degradation products of 40 and 25 kDa were specifically immunostained in both pig cortex (fig.2) and cerebellum (not shown). When Endo F was added subsequent to trypsinization, the 53 kDa protein and its pro-

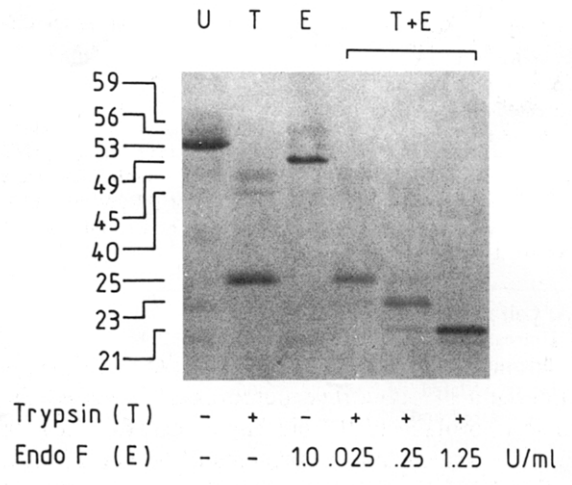


Fig.1. SDS-PAGE and fluorography of pig cortex membranes. Membranes were photolabeled with [$^3$H]FNZ, left untreated (U) or subsequently treated with trypsin (0.5 mg/ml) and/or different concentrations of Endo F, respectively. All bands are labeled specifically (nonspecific binding not shown). Here and in figs 2,3, numbers (in kDa) indicate apparent molecular masses which are calibrated with standard proteins.

teolytic products of 40 and 25 kDa were deglycosylated in two steps as shown by immunostaining (fig.2). The respective molecular masses were indistinguishable from those determined fluorographically. These results show that the protein bands of 40 and 25 kDa contain tryptic fragments of the $\alpha$-subunit and that the deglycosylation pattern observed is that of the $\alpha$-subunit. The weakly photolabeled bands of 56 and 59 kDa as well as the photolabeled tryptic degradation product of 45 kDa were not stained by bd-24.

To compare the degradation products obtained in mammalian brain with those of avian brain, the receptor in pigeon telencephalon was investigated. We had previously shown that the tryptic degradation products in avian telencephalon (45, 40, 25 kDa) and cerebellum (40, 25 kDa) have a molecular mass similar to those obtained in mammalian cortex and cerebellum, respectively [7]. We now show that in pigeon telencephalon the molecular masses of all photolabeled proteolytic products (45, 40 and 25 kDa) were reduced in two steps by deglycosylation (fig.3). Similarly, in pigeon cerebellum the proteolytic product of 25 kDa was also deglycosylated to 21 kDa (not shown).

In order to investigate the effect of trypsinization and/or deglycosylation of BZ-binding proteins on binding parameters, reversible binding studies were performed with [$^3$H]FNZ (table 1). Trypsinization slightly reduced the $B_{max}$ value (expressed as fmol/mg protein) in membranes of calf cortex whereas that of pigeon telencephalon remained virtually unchanged. Deglycosylation with Endo F resulted in significant reduction of the $B_{max}$ values in calf membranes, a decrease being also observed in pigeon telencephalon. The combined treatment with trypsin and Endo F did not significantly lower the $B_{max}$ values beyond the values obtained in membranes pretreated with En-

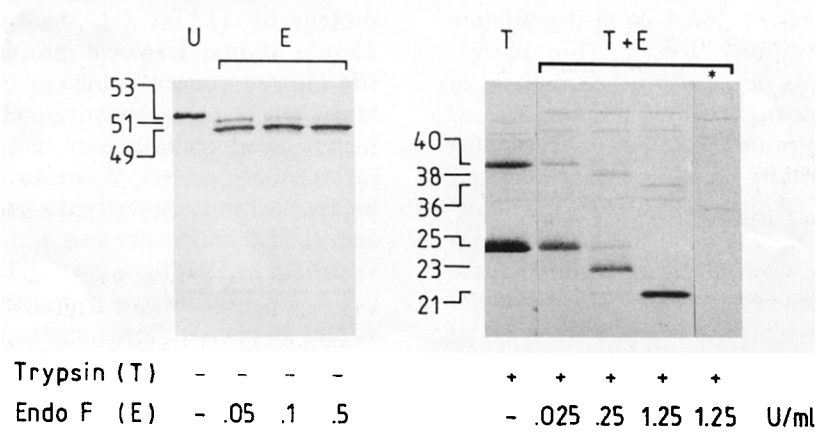


Fig.2. Immunological detection of proteins recognized by the $\alpha$-subunit-specific mAb bd-24 in pig cortex. Membranes were left untreated (U) or treated with trypsin (0.5 mg/ml) and/or different concentrations of Endo F, respectively, and subsequently subjected to SDS-PAGE and immunoblotting. Nonspecific staining (*) was determined using the antibody-free culture medium for incubation.

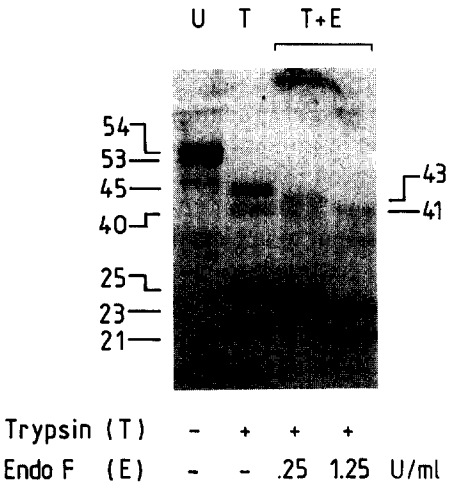


Fig.3. SDS-PAGE and fluorography of telencephalic membranes of pigeon brain. Membranes were photolabeled with [$^3$H]FNZ, left untreated (U) or subsequently treated with trypsin (0.5 mg/ml) and/or different concentrations of Endo F, respectively.

Table 1

Comparison of binding parameters of reversible [$^3$H]flunitrazepam binding and its enhancement by 100 $\mu$M GABA in untreated or pretreated (trypsin and/or endoglycosidase F) brain membranes (results are of single determinations)

| Membranes | $B_{max}$ (fmol/mg protein) | $K_d$ (nM) | |
|---|---|---|---|
| | | − GABA | + GABA (100 $\mu$M) |
| (A) Calf cortex | | | |
| Untreated | 1433 | 2.9 | 1.6 |
| Trypsinized | 1090 | 4.6 | 2.8 |
| Deglycosylated | 466 | 3.2 | 1.7 |
| Trypsinized and deglycosylated | 461 | 2.8 | 0.9 |
| (B) Pigeon telencephalon | | | |
| Untreated | 1127 | 2.1 | |
| Trypsinized | 1136 | 2.1 | |
| Deglycosylated | 892 | 3.8 | |
| Trypsinized and deglycosylated | 622 | 3.4 | |

do F alone. The $K_d$ values of the untreated and pretreated membranes were similar. To ascertain that tryptic digestion had occurred prior to the ligand-binding experiments calf cortex membranes, which were pretreated identically to those used for the reversible binding studies with trypsin or Endo F, were subsequently photolabeled and subjected to SDS-PAGE. Fluorography of the trypsinized membranes revealed labeling of mainly the proteolytic product of 25 kDa and to a lesser extent those of 45 and 40 kDa. The pattern was compatible with that observed upon degradation with a high concentration of trypsin. Thus, reversible binding primarily occurred to the fragment of 25 kDa. When calf cortex membranes were treated with Endo F alone prior to the reversible binding study, the carbohydrate moieties were completely removed as shown fluorographically by the reduction in size of the 53 kDa band to one of 49 kDa (not shown). Thus, we were able to ensure that the reversible binding studies had been performed with either the fully deglycosylated tryptic fragment of the receptor or deglycosylated receptor. Our results confirm that the proteolytic products can bind [$^3$H]FNZ reversibly and irreversibly [13]. The same applies to the deglycosylated BZ-binding proteins. Furthermore, reversible [$^3$H]FNZ binding was enhanced by GABA to a similar extent in enzymatically pretreated and untreated membranes of calf cortex (table 1).

## 4. DISCUSSION

Tryptic degradation products of the photolabeled BZ-binding proteins, which can be detected fluorographically, are glycopeptides. In pig cortex (fig.1) and cerebellum the molecular mass of the 25 kDa fragment is reduced by approx. 4 kDa by deglycosylation. Similar to the main BZ-binding protein of 53 kDa [5], deglycosylation of the 25 kDa tryptic fragment proceeds in two steps (fig.1). Low concentrations of Endo F apparently may cleave one carbohydrate chain of 2–3 kDa; higher concentrations may remove an additional carbohydrate moiety of similar size. Similarly, in avian telencephalon deglycosylation of both the 45 and 25 kDa fragments also occurs in two steps as visualized by fluorography (fig.2). Deglycosylation in two steps of the tryptic degradation products of 40 and 25 kDa was also demonstrated by immunoblotting using the $\alpha$-subunit-specific mAb bd-24 (fig.2). The results indicate that the 40 and 25 kDa fragments are derived from the $\alpha$-subunit of GABA/BZR. In accordance with previous results [13] the tryptic degradation products can be labeled reversibly and irreversibly, indicating that they

encompass both the reversible BZ-binding site and the irreversible binding site for [$^3$H]FNZ (table 1).

Not all photolabeled proteins were immunoreactive with the mAb bd-24. The photolabeled 56 and 59 kDa BZ-binding proteins in pig cortex could represent additional $\alpha$-subunits, which escape immunodetection with the mAb bd-24 [14]. In accordance with this assumption, it was recently found that these proteins can be immunostained with the $\alpha$-subunit-specific mAb bd-28 [8,15]. The fragment of 45 kDa labeled in pig cortex possibly results from tryptic degradation of the 56 and 59 kDa proteins, thus explaining why it also escapes immunodetection.

The $\alpha$-subunit of the GABA/BZR from bovine [6] and rat brain [16,17] is believed to consist of a large extracellular N-terminus followed by four transmembrane regions (M1–M4) and a short extracellular C-terminus region. The main BZ-binding protein (53 kDa) in pig brain seems to share homologies with the bovine and rat $\alpha$-subunits as shown by its size. Additionally, both the bovine and pig $\alpha$-subunits immunoreact with bd-24. Assuming that the pig $\alpha$-subunit also contains two potential glycosylation sites, our results suggest that the large extracellular N-terminal domain containing the glycosylation sites is protected against tryptic proteolysis. This 25 kDa peptide fragment may encompass most, if not all, of the extracellular domain. The 40 kDa glycopeptide fragment might additionally encompass M1–M3 and part of the intracellular loop between M3 and M4. Accordingly, the tryptic cleavage site leading to the 40 kDa glycopeptide may be localized intracellularly between M3 and M4.

Since low trypsin concentrations lead to the 40 kDa peptide in several vertebrates despite variation in the original molecular masses of the BZ-binding proteins [7], we propose that the bulk of the photolabeled BZ-binding proteins is structurally homologous between species. Accordingly, the variation in molecular masses of the original BZ-binding proteins should be due to structural changes in the intracellular loop between M3 and M4, or at the C-terminus.

It seems surprising that the large extracellular N-terminal domain is protected against cleavage even at high trypsin concentrations. However, the preservation of the BZ-binding site which is thought to be located in the extracellular domain supports this conclusion (table 1): (i) the 25 kDa peptide encompasses most of the reversible and irreversible binding sites for [$^3$H]FNZ; (ii) reversible binding of [$^3$H]FNZ could be enhanced by GABA in membranes pretreated with high trypsin concentrations to an extent comparable with fresh membranes (table 1). Radioligand binding occurs mainly to the 25 kDa peptide, since photolabeling and SDS-PAGE of trypsinized membranes revealed the fragment as the main degradation product. The fact that reversible binding of [$^3$H]FNZ to the 25 kDa fragment can be enhanced by GABA suggests that a low-affinity GABA-binding site may also be located on the 25 kDa fragment.

Recently, a structural basis for the heterogeneity of the $GABA_A$ receptor has become apparent through the isolation of additional cDNA clones encoding proteins closely related to the $\alpha$-subunit [16,18]. The deduced amino acid sequences of the additional cDNAs [18] encompass more than two potential sites for *N*-linked glycosylation. However, it is unclear as to what extent the corresponding proteins are expressed in brain and whether they are susceptible to photolabeling with [$^3$H]FNZ. Our results suggest that in vertebrates most, if not all, subunits which are strongly photolabeled with [$^3$H]FNZ may encompass two glycosylation sites in vivo [5]. Since the size of the main tryptic degradation products of the BZ-binding proteins is phylogenetically conserved the photolabeled proteins appear to be structurally homologous. Intraspecies and interspecies variation in molecular masses of BZ-binding proteins may result from structural differences in the intracellular loop between M3 and M4 or at the C-terminus.

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