

Met174 side chain is the site of photoinsertion of a substance P competitive peptide antagonist photoreactive in position 8

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Abstract Numerous photoaffinity studies of the NK-1 receptor have been carried out with peptide agonist analogues of substance P (SP). However, no information is available with regard to the domain interaction of peptide antagonists within this receptor. We describe herein the photoaffinity labelling of the SP receptor with a peptide antagonist analogue, Bapa⁰[(pBzl)-Phe⁸,DPro⁹,MePhe¹⁰,Trp(CHO)¹¹]SP. Photolabelling, enzymatic or chemical cleavage of the covalent complex, purification via streptavidin-coated beads and matrix-assisted laser desorption/ionization time of flight mass spectrometry analysis led us to show that the methyl of Met174 side chain, within the receptor's second extracellular loop, is covalently linked to the antagonist photoreactive at position 8.

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Key words: NK-1 receptor; Antagonist; Substance P; Photolabelling; Matrix-assisted laser desorption/ionization time of flight mass spectrometry

1. Introduction

Most photolabelling studies on receptors have been carried out with peptide agonists while the use of photoreactive antagonists is more rare [1]. Among receptors of the rhodopsin class I G protein-coupled receptor family, the substance P (SP) NK-1 receptor has been widely studied with photoreactive peptide agonists [2–8] whereas only one study describes photoaffinity labelling with the use of a non-peptide antagonist, derived from CP-96,345, of the quinuclidin family [9].

Many peptide antagonists have been reported for the NK-1 receptor (see [10] for review). Generally these SP analogues contain at least one D-Trp or a β turn type II' constraint such as a spirolactame, a DPro-(NMe) amino acid or DPro-alkyl-Pro, or are modified on the C-terminal methionine [10].

To understand the molecular basis of the interaction between antagonist or agonist ligands and the NK-1 receptor, numerous mutagenesis studies have been carried out (see [11] for review). Until now, site-directed mutagenesis studies and construction of deleted or chimeric NK-1 receptors led to the

conclusion that the region of the binding site for non-peptide antagonists differs from the binding site for the peptide agonists [11].

Briefly, residues of crucial importance for peptide agonist binding were initially identified in the N-terminal extension and in the first and second extracellular loops [11]. Substitutions of these residues led to dramatic impairment in SP binding without affecting non-peptide antagonist binding. In contrast, residues involved in the binding of antagonists lie in bundles between transmembrane domains 3, 4, 5 and 6 [11]. As a prerequisite these mutagenesis studies suppose that retention of the high affinity binding for antagonists (or agonists) is a meaningful criterion ascertaining that the overall structure of the receptor protein is preserved, therefore permitting the analysis of the agonist (or antagonist) binding. In this manner these studies suggest that there is either limited or no overlap in the binding sites for agonists and non-peptide antagonists [11]. However, there are few data available concerning interaction of peptide antagonists within the NK-1 receptor.

Therefore, photoaffinity labelling appeared to be a complementary approach to determine domains of interactions of agonists and peptide antagonists within the NK-1 receptor. The only antagonist used so far in photoaffinity studies is an azido derivative of the non-peptide antagonist CP-96,345 [9]. It has been shown that this ligand binds the third extracellular loop of the NK-1 receptor [9], while so far all peptide agonists photoactivatable in different positions only cross-link the second extracellular loop of the NK-1 receptor [2–4,6–8] and the N-terminal domain [2,5].

Therefore, to try to define the interaction site between peptide antagonists and the NK-1 receptor, a photoreactive analogue of SP has been synthesized that contains a biotinyl sulfone aminopentanoic acid (Bapa) at the N-terminus of the sequence and a *para*-benzoyl-L-phenylalanine as the photoreactive amino acid [12]. The analogue used herein is Bapa⁰[(pBzl)Phe⁸,DPro⁹,MePhe¹⁰,Trp(CHO)¹¹]SP. We have previously shown that this peptide has an affinity for the NK-1 receptor transfected in CHO cells and antagonist activity in both phospholipase C and adenylate cyclase second messenger pathways [13]. Although quite low (~ 350 nM), this binding affinity was still adequate for photolabelling studies [13].

In this study, we have used this antagonist Bapa⁰[(pBzl)-Phe⁸,DPro⁹,MePhe¹⁰,Trp(CHO)¹¹]SP in photolabelling experiments and determined its site of photoinsertion within the human NK-1 (hNK-1) receptor expressed in CHO cells, using the same strategy of purification via streptavidin-coated beads and analysis by matrix-assisted laser desorption/ionization

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Abbreviations: SP, substance P; H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂; Bapa, biotinyl sulfone aminopentanoic acid; pBzl, *para*-benzoyl; hNK-1, human NK-1

time of flight (MALDI-TOF) mass spectrometry as previously [4,7,8].

2. Materials and methods

2.1. Materials

Bapa⁰[(pBzl)Phe⁸,dPro⁹,MePhe¹⁰,Trp(CHO)¹¹]SP has been synthesized and characterized previously [13]. The MALDI-TOF mass spectrum of the peptide gave the expected *m/z* of the protonated molecule (first isotope): 1979.94 (with formylation on Trp¹¹) or 1951.94 (without formylation). [11-³H][Pro⁹]SP (60 Ci/mmol) was synthesized at CEA (Saclay, France) according to Chassaing et al. [14]. The CHO cell clone expressing the hNK-1 receptor (6 pmol/mg) has already been described [15]. TPCK-treated trypsin was from Sigma, endo-GluC from Boehringer, CNBr from Calbiochem. Streptavidin-coated magnetic beads were obtained from Dynal.

2.2. Cell culture and membrane preparation

CHO cells expressing the hNK-1 receptor were cultured in Ham's F12 medium supplemented with 100 IU/ml penicillin, 100 IU/ml streptomycin and 10% fetal calf serum. Cultures were kept at 37°C in a humidified atmosphere of 5% CO₂. Stable transfection was maintained by geneticin (400 mg/l). Membranes were prepared as described [15] and stored in sterile buffer containing 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 10 mM MgCl₂, 5 µg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride and 10% glycerol at -80°C. Proteins in membranes were quantified using the Bradford method with bovine serum albumin as reference.

2.3. Determination of the yield of photoaffinity labelling

Determination of the yield of photolabelling Bapa⁰[(pBzl)Phe⁸,dPro⁹,MePhe¹⁰,Trp(CHO)¹¹]SP used at a concentration equal to its affinity (*K_i* = 360 ± 50 nM) was carried out as reported [8].

2.4. Photoaffinity labelling of membrane preparations

All buffers used were prepared with sterile stock solutions to prevent any external contamination of the samples. Membranes (1 mg protein) were incubated for 10 min at room temperature with 500 nM of the photoaffinity ligand, then irradiated on ice for 40 min at 365 nm (HPR 125-W lamp) at a distance of 6–10 cm. After irradiation, 10 µM [Pro⁹]SP was added for 10 min prior to centrifugation at 13000 rpm (MSE Micro Centaur) for 2 min. The pellet was then washed with 1 ml Tris buffer and centrifuged again. Photolabelled membranes were incubated for 2 h at room temperature in 30 µl denaturation buffer consisting of 17 mM dithiothreitol, 0.1% Triton X-100, 3% sodium dodecyl sulfate and 50 mM Tris-HCl pH 7.4.

2.5. Enzymatic and chemical cleavage before purification of the photolabelled receptor fragments

All enzymatic digestions were performed in sterile buffers. After denaturation, photolabelled membranes were digested at room temperature with 100 µg trypsin for 15 h as described [4,7,8]. The trypsin-digested covalent complexes were then purified with 100 µg streptavidin-coated magnetic beads and subjected or not to further digestion with endo-GluC or to CNBr cleavage in 70% formic acid as previously described [4,7,8], the various peptide fragments being analyzed by MALDI-TOF mass spectrometry. After endo-GluC or CNBr cleavage, samples were again purified with the magnetic beads before MALDI-TOF mass spectrometry analysis. CNBr cleavage was also performed directly after denaturation of the photolabelled membranes as described previously [4].

2.6. Identification of the labelled receptor fragments by MALDI-TOF MS analysis

Two different sample preparations were studied. The first one corresponded to the elution of covalently bound peptides from the magnetic beads with 3 µl of the MALDI matrix α -cyano-4-hydroxycinnamic acid (HCCA) in 4:1 (v/v) CH₃CN/H₂O (0.1% trifluoroacetic acid) as described previously [4,7,8]. After 10 min incubation, 1 µl bead-free supernatant was deposited on the sample holder for MALDI-TOF MS analysis. The second one was a direct deposit of 1 µl of matrix containing beads. In both types of deposit, spreading out the drop by touching the metal surface with the pipette tip promoted formation of a 'seeding layer' that generated a homogeneous film of microcrystal. Generally, this second type of deposit gave more intense and durable signal during mass spectrum acquisition. Positive ion mass spectra (averaged over 256 laser shots) were obtained in positive mode on a Voyager Elite (PerSeptive Biosystems) mass spectrometer in the reflector and delayed extraction mode. Peptides for external calibration were deposited on the target close to the photolabelled samples. This deposit contains a few pmol of neurotensin and the accuracy in the mass range (800–4000 U) was estimated to be 30 ppm. Alternatively, internal calibration using the protonated molecule of the ligand (one point calibration) was also applied that gave a similar accuracy. For samples giving a very low ion signal (resulting from a loss in peptide quantities due to the combined and repeated digestions and purification steps), the final mass spectrum was obtained from the average of 10–20 mass spectra (256 laser shots each). Typically, the amount of peptide on these targets was estimated to be in the low fmol range. Peptide receptor domains corresponding to the mass peaks obtained were identified by using the Protein Analysis WorkSheet freeware edition (Proteomics, <http://www.proteomics.com>) and applied to the hNK-1 receptor using the different cleavages.

3. Results

The synthesis (Boc strategy) and biological activity of the photoreactive peptide antagonist Bapa⁰[(pBzl)Phe⁸,dPro⁹,MePhe¹⁰,Trp(CHO)¹¹]SP have already been described [13]. Briefly, this analogue competes with [11-³H][Pro⁹]SP specific binding (*K_i* value was 360 ± 50 nM) to the NK-1 receptor and has antagonist activity in the adenylate cyclase and phospholipase C pathways [13]. Deformylation of the C-terminal tryptophan did not improve the binding potency of the peptide analogue.

We first examined the efficiency of photolabelling with this photoreactive peptide. The yield of covalent photoaffinity labelling was determined to be 24 ± 2%. Despite this apparent low yield of photolabelling, further analysis to identify the site of photoinsertion within the receptor was carried out.

After irradiation, the covalent complex antagonist ligand/NK-1 receptor was digested for 15 h with trypsin at room temperature. We observed by MALDI-TOF MS analysis of the tryptic or combined trypsin/endo-GluC digests that Bapa⁰[(pBzl)Phe⁸,dPro⁹,MePhe¹⁰,Trp(CHO)¹¹]SP is cleaved by the chymotrypsin-like activity derived from trypsin (from Sigma) as previously described [4,7,8]. Protonated molecules at

Table 1
Monoisotopic masses (MH⁺) expected for the first isotope of the different forms of the SP peptide antagonist analogue photoreactive in position 8

Peptide	MH ⁺ expected
Bapa ⁰ [(pBzl)Phe ⁸ ,dPro ⁹ ,MePhe ¹⁰ ,Trp(CHO) ¹¹]SP	1979.96
Bapa ⁰ [(pBzl)Phe ⁸ ,dPro ⁹ ,MePhe ¹⁰ ,Trp ¹¹]SP	1951.96
Bapa ⁰ [(pBzl)Phe ⁸ ,dPro ⁹ ,MePhe ¹⁰ ,Trp(CHO) ¹¹]SP monooxidized ^a	1995.95
Bapa ⁰ [(pBzl)Phe ⁸ ,dPro ⁹ ,MePhe ¹⁰ ,Trp ¹¹]SP monooxidized ^a	1967.95
Bapa ⁰ [(pBzl)Phe ⁸ ,dPro ⁹ ,MePhe ¹⁰ ,Trp(CHO) ¹¹]SP doubly oxidized ^a	2011.95

^aCNBr chemical treatment of the peptide, in acid conditions (HCOOH 70%) led to mono- and dioxidation of the tryptophan residue as described [16].

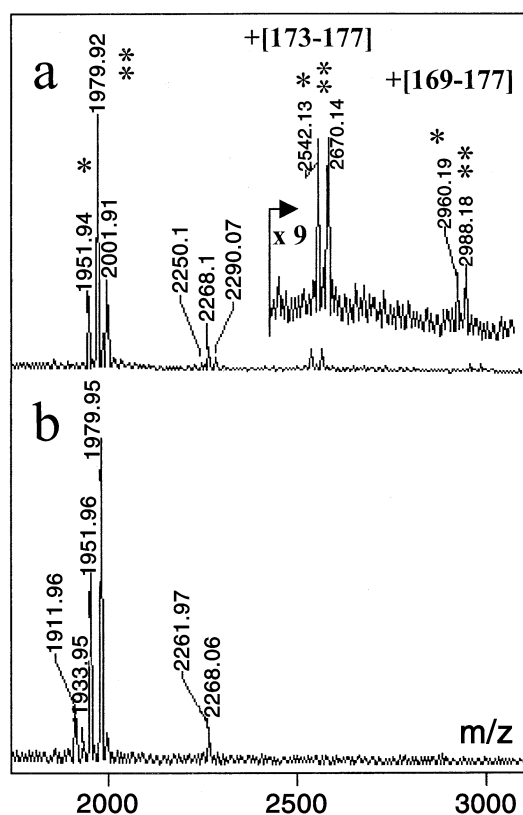


Fig. 1. Typical MALDI-TOF mass spectra obtained with Bapa⁰[(pBzl)Phe⁸,dPro⁹,MePhe¹⁰,Trp(CHO)¹¹]SP and Bapa⁰[(pBzl)Phe⁸,dPro⁹,MePhe¹⁰,Trp¹¹]SP. Asterisks * and ** refer to species containing the deformylated or formylated photoreactive ligand, the square brackets indicating the receptor domain covalently linked (see text). a: After photolabelling and combined trypsin and endo-GluC digestions. b: Control experiment without photolabelling, with combined trypsin and endo-GluC digestions.

m/z 1110.54 and 982.45 (first isotope) were observed. They correspond to the N-terminal fragments 1–6 and 1–5 of this peptide (not shown). Smaller fragments of the peptide, arising from cleavage of Arg–Pro or Lys–Pro bonds, could not be observed because mass spectrum acquisition was done with a low mass gate of 800 or 1000, in order to increase the signal to noise ratio in higher mass range. However, even with a lower mass gate, possible interference with the matrix signals might have hampered the reliable detection of these peptide fragments.

In all mass spectra, we could also observe the protonated molecule of two forms of the peptide, containing or not a formyl on the tryptophan residue in position 11 (m/z expected at 1979.96 and 1951.96 respectively, see Table 1). The major

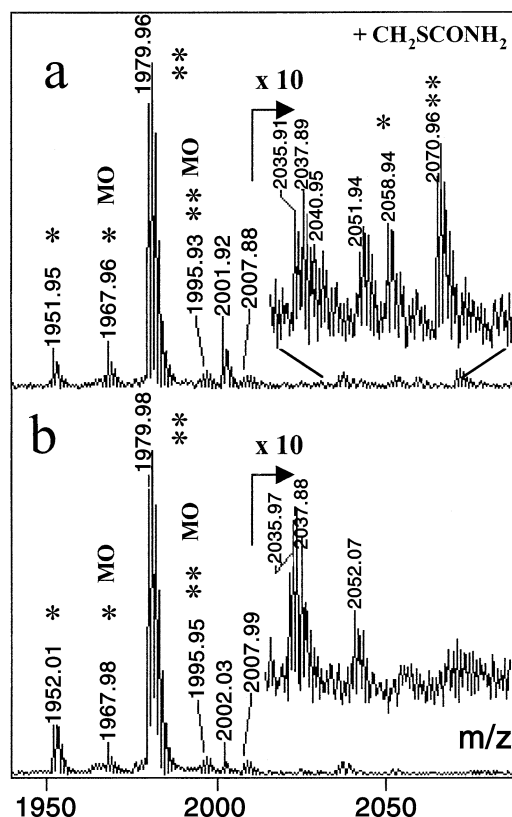


Fig. 2. Typical MALDI-TOF mass spectra obtained with Bapa⁰[(pBzl)Phe⁸,dPro⁹,MePhe¹⁰,Trp(CHO)¹¹]SP and Bapa⁰[(pBzl)Phe⁸,dPro⁹,MePhe¹⁰,Trp¹¹]SP. Asterisks * and ** refer to species containing the deformylated or formylated photoreactive ligand, and MO to monooxidized species (see text). a: After photolabelling and combined trypsin and CNBr cleavages. b: Control experiment without photolabelling, with combined trypsin digestion and CNBr cleavage.

peak always corresponded to the formylated form of the peptide, the minor peak corresponding to the deformylated one could arise from long-lasting or short-term incubation respectively at basic (trypsin digestion) or acid pH (during mixing with HCCA, the matrix for MALDI-TOF analysis). This partial deformylation of the peptide turned out to be advantageous for the mass spectrum analysis since two peaks with a mass difference of 28 u were proved to be the peptide antagonist signature.

After trypsin treatment, reproducible MALDI mass spectra (over seven independent experiments) showed two protonated molecules at m/z 2960.33 $\pm$ 0.08 and 2988.30 $\pm$ 0.1 (Table 2). Blank experiments (without photolabelling) confirmed that these ions are the main feature of the photolabelling. Signal

Table 2
Major feature of MALDI-MS analysis

Treatment	MH ⁺ measured ^a	Covalent complex (receptor)–(photoligand)	MH ⁺ expected
Trypsin	2960.33 $\pm$ 0.08 2988.30 $\pm$ 0.1	(S ¹⁶⁹ -R ¹⁷⁷)–(1–11) (S ¹⁶⁹ -R ¹⁷⁷)–(1–11(CHO))	2960.39 2988.39
Trypsin+Endo-Glu C	2542.24 $\pm$ 0.1 2570.24 $\pm$ 0.1	(T ¹⁷³ -R ¹⁷⁷)–(1–11) (T ¹⁷³ -R ¹⁷⁷)–(1–11(CHO))	2542.22 2570.22
Trypsin+CNBr	2058.97 $\pm$ 0.05 2070.97 $\pm$ 0.05	See text See text	2058.94 2070.94
CNBr	2052.95 $\pm$ 0.05 2070.95 $\pm$ 0.05	See text See text	2052.94 2070.94

^a Average values and uncertainties were obtained from various experiments with different samples and calibrations (see text).

at m/z 2960.33 can only correspond to the covalent attachment of the entire SP analogue, Bapa⁰[(*p*Bzl)Phe⁸, DPro⁹, MePhe¹⁰, Trp¹¹]SP, to the receptor domain S¹⁶⁹TTET-MPSR¹⁷⁷ (average measured 1008.37, 1008.43 expected). The signal at m/z 2988.30 corresponds to the covalent attachment of the formylated form of the photoreactive analogue to the same receptor domain (average measured 1008.37, 1008.43 u expected) (Fig. 1a). Subsequent cleavage of the tryptic fragment on magnetic beads with endo-GluC led to the identification in MALDI of new peptides (Fig. 1a, Table 2). Peaks at m/z 2542.24 ± 0.1 (average measured 590.28 u, 590.26 u expected) and 2570.24 ± 0.1 (average measured 590.28 u, 590.26 u expected) correspond to a unique receptor fragment T¹⁷³MPSR¹⁷⁷ linked to respectively the deformylated or formylated peptide. A blank experiment without UV irradiation only showed signals corresponding to the entire photoreactive peptide, formylated and non-formylated forms, and the corresponding trypsin/chymotrypsin digestion-derived peptide fragments (m/z 1110.55 measured, not shown) (Fig. 1b).

Subsequent cleavage of the tryptic fragment on magnetic beads with CNBr led to the identification of two new peptides (Fig. 2a, Table 2), at m/z 2058.97 ± 0.05 and 2070.97 ± 0.05. They correspond to respectively the deformylated/monooxidized and formylated peptides with an increase in mass of 91 u. This mass increment corresponds to the insertion of –CH₂SCONH₂ into these photoreactive peptides. These peaks were never observed with membranes incubated with the pho-

toactive ligand without irradiation (Fig. 2b). Thus, the triplet radical of the benzophenone moiety of the antagonist peptide reacted with the methyl group of the Met174 side chain in the hNK-1 receptor, which was subsequently cleaved by CNBr [4]. Then, the resulting –CH₂SCN modified peptides were hydrolyzed into –CH₂SCONH₂ in the acid conditions used for CNBr cleavage or, to a lesser extent, for MALDI-TOF analysis [4]. The same results were obtained when CNBr cleavage was performed directly on photolabelled membranes, after denaturation. In that case, both the hydrolyzed form (Δm = +91 corresponding to –CH₂SCONH₂) and the intact form (Δm = +73 corresponding to –CH₂SCN) added to the formylated peptide were observed at m/z 2070.95 ± 0.05 and 2052.90 ± 0.05 respectively (Fig. 3a). In contrast, the peak of the deformylated monooxidized peptide expected at m/z 2059.06 was absent. Blank experiments confirmed that these ions are the main feature of the photolabelling (Fig. 3b).

Chemical CNBr treatment of the ligands alone was also studied and led to the appearance of monooxidized forms of the deformylated and formylated peptides at m/z 1967.98 ± 0.03 and 1995.98 ± 0.03, respectively. In addition, the doubly oxidized form of the formylated peptide could be detected at m/z ~2011.90 (not shown). Although this peptide antagonist does not contain a methionine residue, mono- or dioxidation of the peptide could still occur, probably on the tryptophan residue as previously shown [16].

4. Discussion

Cross-linking studies with photoreactive analogues of SP are mainly reported for peptide agonists [2–8]. All these studies have used *para*-benzoyl phenylalanine as the photoreactive probe to be inserted in the peptide sequence. It has been shown that this photoreactive amino acid in position 3 of the sequence of SP contacts the N-terminal domain and the second extracellular loop of the receptor [2,5], while in positions 4, 5 and 8, benzophenone only links the second extracellular loop [2–4,6–8].

Besides these studies with peptide agonists, only one report concerns photolabelling of the NK-1 receptor with an antagonist [9]. An azido derivative of (2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-((2-methoxyphenyl)methyl)-1-azabicyclo[2.2.2]octan-3-amine or CP-96,345, a potent non-peptide antagonist, led McDonald et al. to identify the third extracellular loop of the NK-1 receptor as the site of photoinsertion [9].

Here we show with the use of a peptide antagonist photoreactive in position 8 that the site of insertion is Met174 and more precisely the methyl group of the side chain. This methyl of the Met174 side chain is also the site we identified for the peptide agonist, photoactivatable at the same position [4,7]. Therefore, unlike agonists and non-peptide antagonists there should be at least a partial overlap in the binding site of agonists and peptide antagonists.

All the experiments in MALDI-TOF were done in the reflector mode and the delayed extraction was optimized. Taking into account the low peptide signal, an average of 5–10 (or more) MALDI-TOF mass spectra (256 laser shots each) was required to get a good signal to noise ratio and reliable statistics on the isotope pattern. Interestingly, this efficient average procedure gives narrower peaks and likely a good precision on m/z measurements. In addition, the external calibration was done with a deposit (same matrix+known pep-

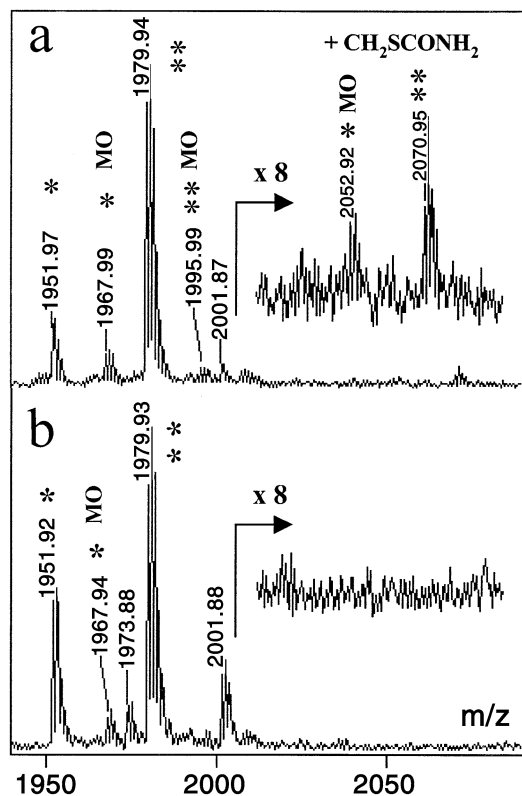


Fig. 3. Typical MALDI-TOF mass spectra obtained with Bapa⁰[(*p*Bzl)Phe⁸, DPro⁹, MePhe¹⁰, Trp(CHO)¹¹]SP and Bapa⁰[(*p*Bzl)Phe⁸, DPro⁹, MePhe¹⁰, Trp¹¹]SP. Asterisks * and ** refer to species containing the deformylated or formylated photoreactive ligand, and MO to monooxidized species (see text). a: After photolabelling and CNBr cleavage. b: Control experiment without photolabelling, with CNBr cleavage.

tide) close to the sample studied (1–2 mm). All these experimental conditions converge towards a precision of m/z measurement typically better than the 50 ppm expected from the qualification record of our apparatus (30 ppm).

Photolabelling was done with 1 mg of membrane proteins containing about 5000 fmol of hNK-1 receptor. We used a concentration of photolabelled antagonist that is close to its binding affinity, meaning that no more than 50% of receptors can be bound. The yield of photolabelling was about 20% for that analogue. Thus 500 fmol of receptors could be covalently bound. We estimated that about 50% of this covalent complex is then lost during enzymatic digestion because the antagonist peptide can be cleaved between biotin, used for purification, and the photoreactive amino acid; 250 fmol of covalent complex remained. We previously measured that the yield of purification using streptavidin-coated magnetic beads is about 80–90% [17], leading to 200 fmol of purified complex. One-third is then deposited on the target for MALDI analysis, i.e. about 60 fmol. Finally, the sample is never exhausted by the laser shots. Moreover, this estimation of fmol amount is in good agreement with the ion signal intensity studied with known quantities of the photoreactive ligand alone, in similar MALDI conditions. For all these experimental reasons we estimated that the quantity of covalent complex being analyzed is in the low fmol range.

Insertion of benzophenone-containing SP analogues on the side chain of methionine is now frequently observed [3,4,8]. In fact, it was recently suggested that methionine is the amino acid preferred by benzophenone [18].

Benzophenone is widely used in photolabelling of non-purified proteins because it produces highly efficient covalent modifications of macromolecules with remarkable site specificity [19], as exemplified herein where non-specific photolabelling was not observed. This particular reactivity of benzophenone for methionine is now elegantly used as a general methodology to analyze the site of photoinsertion of peptide analogues within their specific receptor [20,21]. Described as the methionine proximity assay, this method uses *p,p'*-nitrobenzoylphenylalanine (NO₂Bpa), which displays more selectivity than benzophenone for Met residues. Therefore a photoreactive analogue “containing such modified benzophenone moieties does not label a receptor protein unless a Met residue is in the immediate vicinity” [20,21]. It should be kept in mind that at the atom level, the photoinsertion does not always occur on the methyl but also on the C_γH₂ of the methionine side chain and in that case, CNBr cleavage is atypical as we recently demonstrated [8,22]. Insertion of the photoprobe on CH₃ gives a mass increase of 73 (–CH₂SCN) or 91 (–CH₂SCONH₂) after CNBr cleavage. However, photoinsertion

in C_γH₂ leads to a loss in mass of 48 or 64 corresponding to the formation after CNBr treatment of the ethylenic and epoxide or ketone derivatives of the photoprobe as described [8,22].

References

- [1] Dorman, G. and Prestwich, G.D. (2000) Trends Biotechnol. 18, 64–77.
- [2] Li, Y.-M., Marnerakis, M., Stimson, E.R. and Maggio, J.E. (1995) J. Biol. Chem. 270, 1213–1220.
- [3] Kage, R., Leeman, S.E., Krause, J.E., Costello, C.E. and Boyd, N.D. (1996) J. Biol. Chem. 271, 25797–25800.
- [4] Girault, S., Sagan, S., Bolbach, G., Lavielle, S. and Chassaing, G. (1996) Eur. J. Biochem. 240, 215–222.
- [5] Bremer, A.A., Leeman, S.E. and Boyd, N.D. (2001) J. Biol. Chem. 276, 22857–22861.
- [6] Li, H., McDonald, D.M., Hronowski, X., Costello, C.E., Leeman, S.E. and Boyd, N.D. (2001) J. Biol. Chem. 276, 10589–10593.
- [7] Lequin, O., Bolbach, G., Frank, F., Convert, O., Chassaing, G., Lavielle, S. and Sagan, S. (2002) J. Biol. Chem. 277, 22386–22394.
- [8] Sachon, E., Bolbach, G., Chassaing, G., Lavielle, S. and Sagan, S. (2002) J. Biol. Chem. 277, 50409–50414.
- [9] McDonald, D., Silberman, S.C., Lowe III, J.A., Drozda, S.E., Leeman, S.E. and Boyd, N.D. (1996) Mol. Pharmacol. 49, 808–813.
- [10] Quartara, L. and Maggi, C.A. (1997) Neuropeptides 31, 537–563.
- [11] Gether, U. (2000) Endocr. Rev. 21, 90–113.
- [12] Hatanaka, Y. and Sadakane, Y. (2002) Curr. Top. Med. Chem. 2, 272–288.
- [13] Sachon, E., Girault-Lagrange, S., Chassaing, G., Lavielle, S. and Sagan, S. (2002) J. Peptide Res. 59, 232–240.
- [14] Chassaing, G., Lavielle, S., Julien, S. and Marquet, A. (1985) Tetrahedron Lett. 26, 623–626.
- [15] Sagan, S., Beaujouan, J.-C., Torrens, Y., Saffroy, M., Chassaing, G., Glowinski, J. and Lavielle, S. (1997) Mol. Pharmacol. 52, 120–127.
- [16] Bienvenut, W.V., Déon, C., Pasquarello, C., Campbell, J.M., Sanchez, J.-C., Vestal, M.L. and Hochstrasser, D.F. (2002) Proteomics 2, 868–876.
- [17] Girault, S., Chassaing, G., Bolbach, G., Blais, J.-C. and Brunot, A. (1996) Anal. Chem. 68, 2122–2126.
- [18] Deseke, E., Nakatani, Y. and Ourisson, G. (1998) Eur. J. Org. Chem. 2, 243–251.
- [19] Dorman, G. and Prestwich, G.D. (1994) Biochemistry 33, 5661–5673.
- [20] Rihakova, L., Deraët, M., Auger-Messier, M., Pérodin, J., Boucard, A.A., Guillemette, G., Leduc, R., Lavigne, P. and Escher, E. (2002) J. Recept. Signal Transduct. Res. 22, 297–313.
- [21] Pérodin, J., Deraët, M., Auger-Messier, M., Boucard, A.A., Rihakova, L., Beaulieu, M.-E., Lavigne, P., Parent, J.-L., Guillemette, G., Leduc, R. and Escher, E. (2002) Biochemistry 41, 14348–14356.
- [22] Sachon, E., Milcent, T., Sagan, S., Convert, O., Chassaing, G. and Lavielle, S. (2002) Tetrahedron Lett. 43, 7485–7489.