Peptide Fragmentation Suitable for Solid-Phase Microsequencing

USE OF *N*-BROMOSUCCINIMIDE AND BNPS-SKATOLE {3-BROMO-3-METHYL-2-[(2-NITROPHENYL)THIO]-3*H*-INDOLE}

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BNPS-skatole {3-bromo-3-methyl-2-[(2-nitrophenyl)thio]-3*H*-indole} and *N*-bromosuccinimide have been used to specifically cleave at peptide bonds after amino acids with available C- γ =C- δ double bonds, i.e. tryptophan, tyrosine and histidine. The resulting C-terminal lactones conveniently attach to amino-glass supports for sequencing with DABITC (4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate). Also, peptides having such C-termini, i.e. from a chymotryptic digest, can be readily made to react with these reagents and thus be easily attached and sequenced by solid-phase methods.

CNBr cleavage (Gross, 1967) at methionine residues is the most widely used method available for generating large peptide fragments, which, with the progressive improvements in Edman-type degradations, are becoming easier to sequence. For those laboratories with solid-phase sequencers, this cleavage method also provides a C-terminal homoserine lactone through which the fragment can be coupled to amino-substituted resins (Horn & Laursen, 1973). Similarly, cleavages at tryptophan, tyrosine and/or histidine residues by such reagents as BNPS-skatole (Fontana, 1972) and *N*-bromosuccinimide (Ramachandran & Witkop, 1967) have been known for some time to give variable yields of cleavage and to produce C-terminal lactone derivatives at the cleavage points. It was only quite recently shown by Wachter & Werhahn (1979*a*) that fragments produced by exposure to the mixture dimethyl sulphoxide/HBr/HCl/acetic acid, as described by Savige & Fontana (1977) for tryptophan cleavage, could be coupled via their dioxindolylalanine lactones to aminopropyl-glass. The cleavage (~70%), coupling (~90%) and repetitive (~90%) yields for five peptide fragments were most acceptable. However, there are potential limitations to this approach, since oxidation of cysteine to cystine and methionine to its sulfoxide occurs (Savage & Fontana,

1977), and tyrosine residues are modified to their mono- and di-bromo derivatives (Wachter & Werhahn, 1979*a*). The use of *o*-iodosylbenzoic acid has been introduced by Mahoney & Hermodson (1979) as an alternative method for selective tryptophan cleavage. However, an extensive study by Wachter & Werhahn (1979*b*) has indicated that this reagent is not as specific as originally reported.

In the present paper we illustrate the usefulness of BNPS-skatole for tryptophan cleavages. Subsequent cleavages at tyrosine and/or histidine residues, neither of which is modified by this reaction, can then be performed by using *N*-bromosuccinimide. The coupling of peptides containing neither C-terminal lactones (CNBr cleavage) nor ϵ -amino groups of lysine (tryptic digestion) has always been unreliable. The treatment of peptides originating from chymotryptic digestions with *N*-bromosuccinimide has indicated that those possessing C-terminal histidine, tryptophan or tyrosine residues can be conveniently coupled and sequenced automatically at the low nanomolar level. A preliminary account of some of this work was recently presented at the Third International Conference on Solid-Phase Methods in Protein-Sequence Analysis, held in Heidelberg, German Federal Republic (Wilson & Hughes, 1979).

Material and Methods

Peptide/protein cleavages and attachments

BNPS-skatole cleavages (Fontana, 1972) were

Abbreviations used: BNPS-skatole, 3-bromo-3-methyl-2-[(2-nitrophenyl)thio]-3*H* indole; DABITC, 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate; DABTH, 4-*NN*-dimethylaminoazobenzene 4'-thiohydantoin; h.p.l.c., high-pressure liquid chromatography.

performed in 70% distilled acetic acid 0.1%/phenol for 48 h at room temperature by using a 10-fold excess of the reagent. After the addition of β -mercaptoethanol (10-fold excess) and a further incubation for 5 h at 37°C, the excess BNPS-skatole was extracted with ethyl acetate and the resulting aqueous phase either freeze-dried or dried by evaporation. Attachment to aminopropyl-glass via the dioxindolylalanine lactone, after a 2–12 h incubation in anhydrous trifluoroacetic acid was carried out as previously described (Horn & Laursen, 1973). *N*-Bromosuccinimide cleavages (Ramachandran & Witkop, 1967) after tryptophan, tyrosine or histidine residues, or the exposure of chymotryptic peptides possessing such C-termini, were performed in aq. 50% distilled acetic acid for 2 h at 30°C by using a 12-fold excess of the reagent. After evaporation the peptide mixtures were treated with trifluoroacetic acid, dried, and coupled as above. Coupling yields were determined by hydrolysing a portion of the peptide-resin in 6M-HCl and subsequent amino-acid analysis on a Durrum D-500 analyser.

DABITC degradation and DABTH identification

Micro sequence analyses (<20 nmol level) of the peptides originating from the above-mentioned cleavage methods were performed automatically by using the recently introduced DABITC methodology. LKB 4020 and Sequemat Mini-15 sequencers were operated as previously described (Hughes *et al.*, 1979b). Conversions were performed manually (1M-HCl; Edman & Begg, 1967) and automatically (aq. 35% trifluoroacetic acid; Hughes *et al.*, 1979b). Amino acid DABTH derivatives were identified by either isocratic (silica columns) or gradient (reverse-phase) h.p.l.c. with the instruments and chromatographic conditions previously published (Wilson *et al.*, 1979b). Together, these systems are capable of detecting, with the exception of lysine, all DABTH derivatives at the <100 pmol level.

Results and Discussion

The degradation of the peptide originating from BNPS-skatole cleavage at tryptophan-14 in the α -chain of human haemoglobin (67% cleavage) is shown in Fig. 1(a). After coupling to aminopropyl-glass (54% yield) the sequence was determined at the 4.5 nmol level (92.6% repetitive yield based on alanine-5, -12 and -13). DABTH identification at each cycle was clearly possible, with the exceptions being at: (i) position 1, which was not analysed, since it appears as the phenylthiohydantoin derivative (see Hughes *et al.*, 1979a). (ii) positions 7 and 11, which are lysine residues and cannot be identified with this system; and (iii)

position 14, the dioxindolylalanine lactone, which cannot be identified on this reverse-phase system. However, it should be pointed out that for the other four peptides that were sequenced after BNPS-skatole cleavages (see Table 1), where amino acid DABTH derivatives were identified by isocratic h.p.l.c. (Wilson *et al.*, 1979b), a characteristic peak was noted that chromatographed on the polar column just after the threonine DABTH derivative. The use of *N*-bromosuccinimide for the cleavage at tyrosine residues and subsequent sequencing is indicated in Fig. 1(b). The peptide, modified and attached with a yield of 50%, was easily sequenced at the 7 nmol level. The peaks marked as S⁺ and T⁺ most likely represent the dehydro-DABTH derivatives of serine and threonine (see also Wilson *et al.*, 1979a). The poor yield for asparagine-1 was unexpected, and the peaks marked as M⁺ at position-10 probably are methionine sulphone DABTH derivatives. Hydrolyses of amino-acid mixtures and numerous peptides after *N*-bromosuccinimide modification have indicated that the recoveries of some residues, as determined by amino-acid analysis, are effected: methionine is oxidized to the sulphone; tyrosine totally disappears, but a peak is seen eluting in the same area as histidine; cysteine is oxidized to cysteic acid; arginine and aminoethylcysteine are partially lost; totally destroyed are cystine, carboxymethylcysteine, pyridylethylcysteine, histidine and tryptophan.

Coupling through the lactone formed from the reaction of a C-terminal tyrosine with *N*-bromosuccinimide has proved to be of general application; typical sequence results are shown in Fig. 1(c). The significant amount of deamidation occurring at residues 4, 8 and 9 is due to the manual conversion method and can be virtually eliminated by automatic conversion (see asparagine-9 in Fig. 1a). In contrast with the DABTH lactone produced by BNPS-skatole cleavage at tryptophan, no derivative from the *N*-bromosuccinimide reaction could be detected by isocratic h.p.l.c.. Table 1 lists other peptides that have been modified at histidine and/or tyrosine residues and sequenced. To date, no unexpected cleavages have been noted, nor have any peptides, after modification, failed to couple. Although the coupling yields have varied significantly (23–89%), some 10 nmol of starting material would have been sufficient for the total sequence of any peptide here presented.

In summary, the structural analyses of proteins have been hindered by the shortage of methods for the selective cleavage of polypeptide chains at specific amino acids. The CNBr method is widely used, since the reaction is specific and the extent of cleavage is essentially complete, peptide bonds involving Met–Ser and Met–Thr being exceptions.

Fig. 1. *H.p.l.c.* analyses of amino acid DABTH derivatives arising from the degradation of peptides produced by BNPS-skatole (a) and *N*-bromosuccinimide (b and c) cleavages

The peptides, coupled to aminopropyl-glass via their lactones were sequenced in either a Sequemat (a) or LKB (b and c) instrument. After conversion (a, automatically; b and c, manually) the DABTH derivatives were analysed by gradient (a) or isocratic (b and c; apolar and polar columns are right and left respectively) h.p.l.c. systems. The amounts of peptide degraded and the percentages analysed were: (a) 5.4 nmol, 20% injected at 0.01 full-scale deflection; (b) 7.1 nmol, 10% injected at 0.05 full-scale deflection for 1–8 and 0.02 full-scale deflection for 9–13; (c) 14.0 nmol, 10% injected at 0.02 full-scale deflection. DABTH detection was at 436 nm, except for the first residues in (b) and (c), which were detected at 254 nm.

Table 1. *Peptides sequenced after modification by either BNPS-skatole or N-bromosuccinimide*

The peptides were exposed to either of the modifying reagents and attached to aminopropyl-glass. After the determination of coupling yields, sequencing was carried out with DABITC. Arrows indicate modification points; residues underlined were not identified by h.p.l.c. analyses; parentheses denote peptides or amino acids split off by the reaction. Peptide sources were *Aeromonas* aminopeptidase (AAP), mitochondrial aspartate aminotransferase (AAT), aminoethyl- α -chain of human haemoglobin (α -Chain) and *Neurospora* tyrosinase. Details of the one letter notation for amino acids are given in *Biochem. J.* (1969) **113**, 1–4.

	Peptide	Amount reacted (nmol)	Coupling yield (%)	Amount sequenced (nmol)	Source
(I) BNPS-Skatole					
1.	P R E E Q W↓	30	40	7.7	AAT
2.	P E L R K E W↓	35	42	9.7	AAT
3.	E E Q W↓ (K E L A S V V K)	50	46	17.1	AAT
4.	V L S P A D K T N V K A – A W↓ (G K ...)	108	54	4.4	α -Chain
5.	D V Y L P K P S W↓ (G N H – P I F R)	44	47	14.6	AAT
(II) N-Bromosuccinimide					
(a) Histidine modifications					
1.	L A H↓ A I H↓	18	32	3.6	AAT
2.	A S V K Q V S H↓	25	65	11.0	AAP
3.	N D Y↓ N P R I H↓	41	23	8.1	AAP
4.	A N S D P T G S H↓	28	74	17.5	AAP
(b) Tyrosine modifications					
1.	G G Y↓	100	67	15.8	Tyrosinase
2.	L A L Y↓	36	41	7.8	Tyrosinase
3.	G F T C G Y↓	28	84	12.0	AAP
4.	R D D D G K P Y↓	20	89	11.7	AAT
5.	G S Q D L A N Q Y↓ (K)	26	83	4.4	AAP
6.	R G S Q D L A N Q Y↓	24	50	14.0	AAP
7.	D A G L E L Q A Y↓ (R)	32	60	13.1	AAT
8.	D T N S K K M N L G V G – A Y↓ (R)	20	31	4.8	AAT
9.	N V V S A L Q D M T N – Y↓ (K)	49	63	7.1	AAP
10.	H↓ F I E Q G I D V V L S Q S – Y↓ A K A M G L Y↓ (G E R)	21	54	4.4	AAT

Methods for cleavage at other amino acid residues that occur with approximately the same relative frequency (Dayhoff, 1978), i.e. tryptophan and histidine, would be advantageous, provided that the reaction did not: (i) modify other amino acids and thereby render them either undetectable or non-reactive to subsequent modification; or (ii) result in extensive cleavages at more than a single specific amino acid. With the advent of suitable h.p.l.c. methodology for peptide separations at the micro-level (Hughes *et al.*, 1979a), such specific cleavage methods can be exploited to provide large peptide fragments. The present paper has demonstrated the uses of BNPS-skatole and N-bromosuccinimide as reagents for specific cleavages and the application of the resulting lactones as attachment points for solid-phase microsequencing. Additionally, making chymotryptic peptides react with N-bromosuccinimide, thereby converting C-terminal histidine, tryptophan or tyrosine residues to their lactones provides a convenient method of coupling and thus the sequencing of such fragments.

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