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IDENTIFICATION OF THE FRAGMENT PHOTOAFFINITY-LABELED WITH AZIDODANSYL-RHIZOXIN AS Met-363–Lys-379 ON β-TUBULIN

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Abstract—The rhizoxin (RZX)-binding site on porcine brain tubulin was investigated by photoaffinity labeling with the 5-azido-1-naphthalene sulfonyl (azidodansyl) derivative of RZX, nor-rhizoxin-22-al-5′-azidonaphthalene-1′-sulfonylhydrazone (azidodansylrhizoxin: Adan–RZX). Upon ultraviolet irradiation, Adan–RZX generates a highly reactive nitrene, which irreversibly binds to an amino acid residue(s) near the RZX-binding site. The label was found to be on β-tubulin. Enzymatic digestion of the labeled tubulin generated only one major fluorescent peak on C18 reverse phase HPLC analysis. The labeled site(s) was mapped by using various combinations of highly specific peptidases in succession. That is, the labeled fragment generated by the first peptidase was purified by HPLC and exposed to a second peptidase; if the retention time in HPLC changed after the second digestion, the fragment generated in the first digestion must have contained the recognition site(s) of the second enzyme. From the results of these successive digestions and the known polypeptide sequences, we could identify the labeled fragment as Met-363–Lys-379 of β-tubulin. This peptidase combination technique should be widely applicable.

Rhizoxin (RZX†, Fig. 1) is a toxic 16-membered macrolide produced by *Rhizopus chinensis*, the pathogen of rice seedling blight [1, 2]. It is an antitumor antibiotic [3] and shows a potent antimitotic effect, through inhibition of microtubule assembly [4] in mammalian, plant and fungal cells. Microtubules are polymers of a heterodimer formed from two globular subunits, i.e. α- and β-tubulins. Both tubulins have molecular masses of about 50,000 Da, similar amino acid compositions and similar overall shapes.

Various antimitotic compounds, including colchicine (CLC), benzimidazoles, vinca alkaloids such as vinblastine (VLB) and maytansine (MAY), bind to tubulin. Vinca alkaloids bind at a site that appears to be unrelated to the CLC-binding site, but overlaps with the MAY-binding site, and the two distinct binding sites on tubulin of these antimitotic compounds have been classified as the CLC site and the VLB–MAY site [5].

We found that RZX binds to tubulin competitively with respect to ansamitocin P-3, a maytansinoid compound, but only partially competitively with respect to VLB. Therefore, the VLB–MAY site might be differentiated into two sites, a VLB site and a MAY–RZX site, or more [6–8]. Another drug, phomopsin-A, was recently shown to bind to the MAY–RZX site on porcine tubulin [9]. The study of RZX-binding site is, therefore, becoming of increasing importance in connection with the three-dimensional structure of tubulin and for the design of new antimitotic drugs.

We recently showed that RZX binding to tubulin is determined by the nature of the 100th amino acid residue of β-tubulin [10]. To obtain further information on the structure of the RZX-binding site, we designed and synthesized the photoaffinity labeling reagent Adan–RZX, which is an RZX derivative coupled to a 5-azido-1-naphthalenesulfonic acid moiety (Fig. 1). As the parent ligand, we chose nor-rhizoxin-22-al, because analysis of the structure–activity relationship of a number of RZX derivatives indicated that the side chain terminal of RZX is not essential for its tubulin-binding activity [11]. It has been established that Adan–RZX inhibits tubulin polymerization by binding to tubulin and that it shares the same binding site as RZX and ansamitocin P-3 by means of competition experiments [12]. Therefore, Adan–RZX should be an effective, RZX-binding site-specific photoaffinity labeling probe.

Several applications of the photoaffinity labeling technique to examine drug-binding sites on tubulin have been reported [13–15]. However, these studies were restricted to CLC derivatives and VLB derivatives, and only sought to identify the subunit (α or β) to which these drugs bind. In the present study, we have determined the sequence of the

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† Abbreviations: RZX, rhizoxin; CLC, colchicine; VLB, vinblastine; MAY, maytansine; Adan–RZX, azidodansylrhizoxin (nor-rhizoxin-22-al-5′-azidonaphthalene-1′-sulfonylhydrazone); MES, 2-(*N*-morpholino)-ethanesulfonic acid; EGTA, 1,2-di(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; Dan–RZX, dansylrhizoxin (nor-rhizoxin-22-al-5′-dimethylaminonaphthalene-1′-sulfonylhydrazone); SDS, sodium dodecyl sulfate.

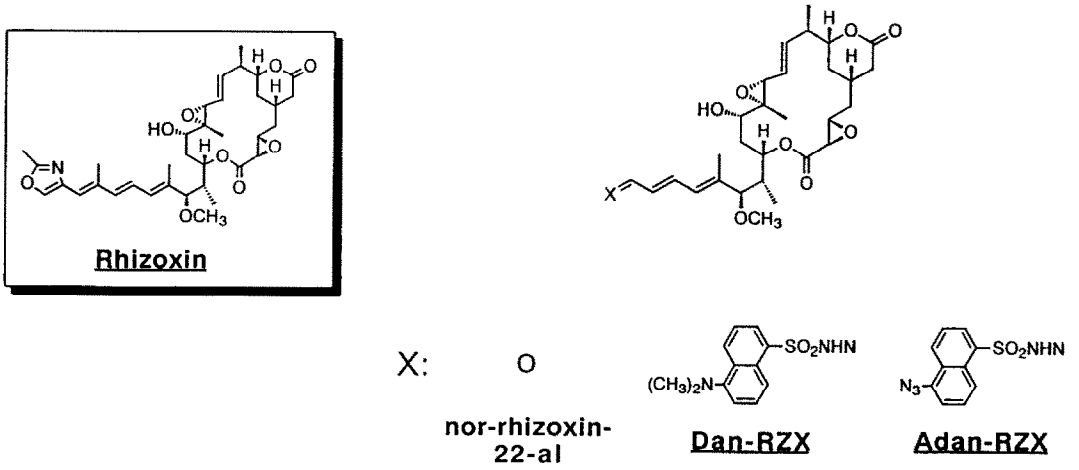

Fig. 1. Structures of RZX and Adan–RZX.

peptide to which Adan–RZX is bound on β-tubulin by the use of combinations of highly specific peptidases, in conjunction with HPLC analysis with fluorescence detection.

MATERIALS AND METHODS

Materials. RZX was obtained as described previously [1] and ansamitocin P-3 was a gift from the Central Research Institute of Takeda Chemical Industry. CLC was purchased from the Sigma Chemical Co. 5-Azidonaphthalene-1-sulfonic acid sodium salt was purchased from Molecular Probes Inc. Endoproteinase Lys-C and endoproteinase Asp-N were purchased from Boehringer Mannheim Biochemica. Clostripain (endoproteinase Arg-C) was from the Promega Co. Digestion buffers were 25 mM Tris–HCl, 1 mM EDTA (pH 8.5) for endoproteinase Lys-C; 50 mM sodium phosphate (pH 8.0) for endoproteinase Asp-N; 50 mM Tris–HCl, 2 mM dithiothreitol, 1 mM $CaCl_2$ (pH 7.5) for clostripain. MES buffer contains 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6.5), 1 mM 1,2-di(2-aminoethoxy)ethane-*N,N,N',N'*-tetraacetic acid (EGTA), 0.5 mM $MgCl_2$, and R buffer contains 20 mM Tris–HCl, 0.15 M NaCl (pH 8.0). A μBondasphere column (5 μ C18-100 Å, 3.9 mm × 15 cm; Waters Chromatography Division of Nihon Millipore Ltd) was used as a C-18 reverse-phase column.

Synthesis of Adan–RZX. Adan–RZX (Fig. 1) was prepared as described previously [12]. Briefly, 5-azidonaphthalene-1-sulfonic acid sodium salt (50 mg) in dry dimethylformamide (2 mL) was added dropwise to thionyl chloride (100 μL) in dry dimethylformamide (0.5 mL) under ice cooling, and the mixture was stirred for 1 hr at room temperature. The reaction mixture was added to CH_2Cl_2, and washed with water. The organic layer was evaporated to give a residual mixture which was redissolved in dioxane (2 mL). The solution was added dropwise to 80% hydrazine hydrate (15 mg) in dioxane (1 mL), in the presence of triethylamine. After evaporation, silica gel chromatography of the residual mixture eluted with CH_2Cl_2-ethyl acetate (4:1) gave 5-azidonaphthalene-1-sulfonyl hydrazine (21 mg). This was dissolved in dioxane (2 mL) and added to nor-rhizoxin-22-al [11] (50 mg). After the evaporation of the mixture, the residue was separated by silica gel chromatography (benzene-acetone, 7:2) to give Adan–RZX (50 mg). Adan–RZX: FAB-MS 778 $(M + H)^+$; HRMS calculated for $C_{39}H_{48}N_5O_{10}S$, *m/z* 778.3122, found 778.3183. ^{1}H-NMR ($CDCl_3$) 0.79 (1H, m, 4-H), 0.94 (1H, m, 6-H), 0.96 (3H, d, 16a-H), 1.20 (3H, d, 8a-H), 1.39 (3H, s, 12a-H), 1.60 (3H, s, 18a-H), 1.8–3.3 (19H, m), 3.10 (3H, s, 1'7-OCH_3), 3.87 (1H, m, 7-H), 4.51 (1H, dd, 15-H), 5.30 (1H, s, -SO_2NH-), 5.34 (1H, dd, 10-H), 5.59 (1H, dd, 9-H), 5.99 (1H, d, 19-H), 6.17 (1H, dd, 21-H), 6.65 (1H, dd, 20-H), 7.40 (1H, dd, 2', 4', 6' or 8'-H), 7.45 (1H, d, 22-H), 7.60 (1H, dd, 3' or 7'-H), 7.70 (1H, dd, 3' or 7'-H), 8.40 (1H, dd, 2', 4', 6' or 8'-H), 8.45 (1H, dd, 2', 4', 6' or 8'-H), 8.49 (1H, dd, 2', 4', 6' or 8'-H). i.r. (KBr): 2140, 1770 cm^{-1}.

Preparation of tubulin and microtubule assembly assay. Microtubule proteins were prepared from porcine brain by the assembly–disassembly method as described previously [4]. Tubulins free of microtubule-associated proteins were isolated from the microtubule proteins by the use of phosphocellulose chromatography as described previously [16]. A tubulin polymerization assay was carried out using the turbidity measuring method by use of microtubule protein as described previously [4].

Binding of Adan–RZX to tubulin. Binding of Adan–RZX to tubulin was measured indirectly in terms of competition with nor-rhizoxin-22-al-5'-dimethylaminonaphthalene - 1' - sulfonylhydrazone (Dan–RZX dansylrhizoxin, a fluorescent probe) for the RZX-binding site [11]. Tubulin preparation (0.2 mg/0.25 mL in MES buffer) was incubated at 37° for 1 hr with Dan–RZX in the presence or absence of Adan–RZX. The mixture (0.25 mL) was subjected to Sephadex G-25 gel column

Table 1. Binding of Adan–RZX to tubulin*

Drug concentration (μM)	Amount of Dan–RZX (%)	Inhibition of Dan–RZX binding (%)
Dan–RZX (30)	100	—
+CLC (50)	96.6	0.4
+Ansamitocin P-3 (10)	38.2	61.8
+RZX (10)	52.8	47.2
+RZX (30)	38.3	61.6
+Adan–RZX (30)	89.3	10.7
+Adan–RZX (150)	38.3	61.7

* Dan–RZX was incubated with tubulin in the absence or presence of other drugs and the amounts of Dan–RZX bound to tubulin were determined by measuring the fluorescence intensity. The amount of Dan–RZX bound in the absence of any other drug was defined as 100% binding.

chromatography (bed volume; 1 mL), and eluted with MES buffer. The first 0.45 mL of eluent was rejected, and the next 0.4 mL was collected. Fluorescence intensity of the latter fraction was measured (excitation at 350 nm, emission at 520 nm) [12].

Photoaffinity labeling of tubulin with Adan–RZX. Tubulin preparation (0.8 mg/1 mL in MES buffer) was incubated with Adan–RZX (30 μM) in the absence or presence of RZX at 37° for 1 hr. The mixture was irradiated externally for 10 min with a high-pressure mercury lamp (450 W) under ice-cooling. To measure the amount of covalently bound Adan–RZX, 0.5 mL of the mixture was treated with 0.1% sodium dodecyl sulfate (SDS) at 100° for 1 min to remove non-covalently associated Adan–RZX. Then unreacted Adan–RZX and other unbound fluorescent species were eliminated by adding 0.5 mL of dextran-coated charcoal suspension, followed by centrifugation at 10,000 *g* for 10 min at 4° [17]. The supernatant was mixed with 1.3 mL of R buffer and the fluorescence intensity of the mixture was measured (Table 2).

Reduction and carboxymethylation of tubulin and enzymatic digestion of Adan–RZX-labeled tubulin. Adan–RZX-labeled tubulin was dialysed for 20 hr at room temperature against 500 mL of 0.12 M 2-mercaptoethanol, 8 M urea, 0.1% EDTA-2Na, 0.35 M Tris–HCl (pH 8.8). To the protein solution, 1.1 M ICH_2COONa-8 M urea was added to make 10% (v/v). After 1 hr in the dark at room temperature, the reaction mixture was dialysed for 1 hr against water, then for 20 hr against fresh water. The dialysate was lyophilized to give Adan–RZX-labeled, carboxymethylated tubulin. Aliquots of the resulting sample (500 μg) were dissolved in 0.25 mL of appropriate digestion buffer. Enzymatic digestions of endoproteinase Lys-C, endoproteinase Asp-N and clostripain (endoproteinase Arg-C) were carried out at an enzyme/substrate ratio of 1:100 for 18 hr at 37° according to the supplier's recommendation.

HPLC analysis. HPLC with a C-18 reverse-phase column was used for separation of enzymatic digests. The samples (0.5–1 mg) were eluted with a linear gradient of increasing 0–60% acetonitrile in 0.05% aqueous trifluoroacetic acid. Absorbance at 215 nm and fluorescence intensity (excitation at 350 nm, emission at 520 nm) were monitored.

The technique of successive digestions with different proteases. Tubulins were photoaffinity labeled and digested with a peptidase as described above. This first digest was separated by HPLC as described above. Fraction(s) exhibiting fluorescence were collected, and evaporated *in vacuo*. The residue was redissolved in a digestion buffer for a second enzymatic digestion. The second digestion and HPLC analysis were performed in the same manner as above. The retention time of the fluorescent peak in the second HPLC analysis was compared with that of the first analysis.

RESULTS

Irradiation-induced covalent binding of Adan–RZX to tubulin

Adan–RZX and RZX were found to have similar inhibitory activities on microtubule polymerization. At a concentration of microtubules of 4 μM, 50%

Table 2. Photoaffinity labeling of the RZX-site with Adan–RZX*

Drug concentration (μM)	Fluorescence intensity of covalently bound Adan–RZX (%)
Adan–RZX (30)	100
+RZX (50)	40.5

* The amounts of covalently bound Adan–RZX were measured as described in Materials and Methods.

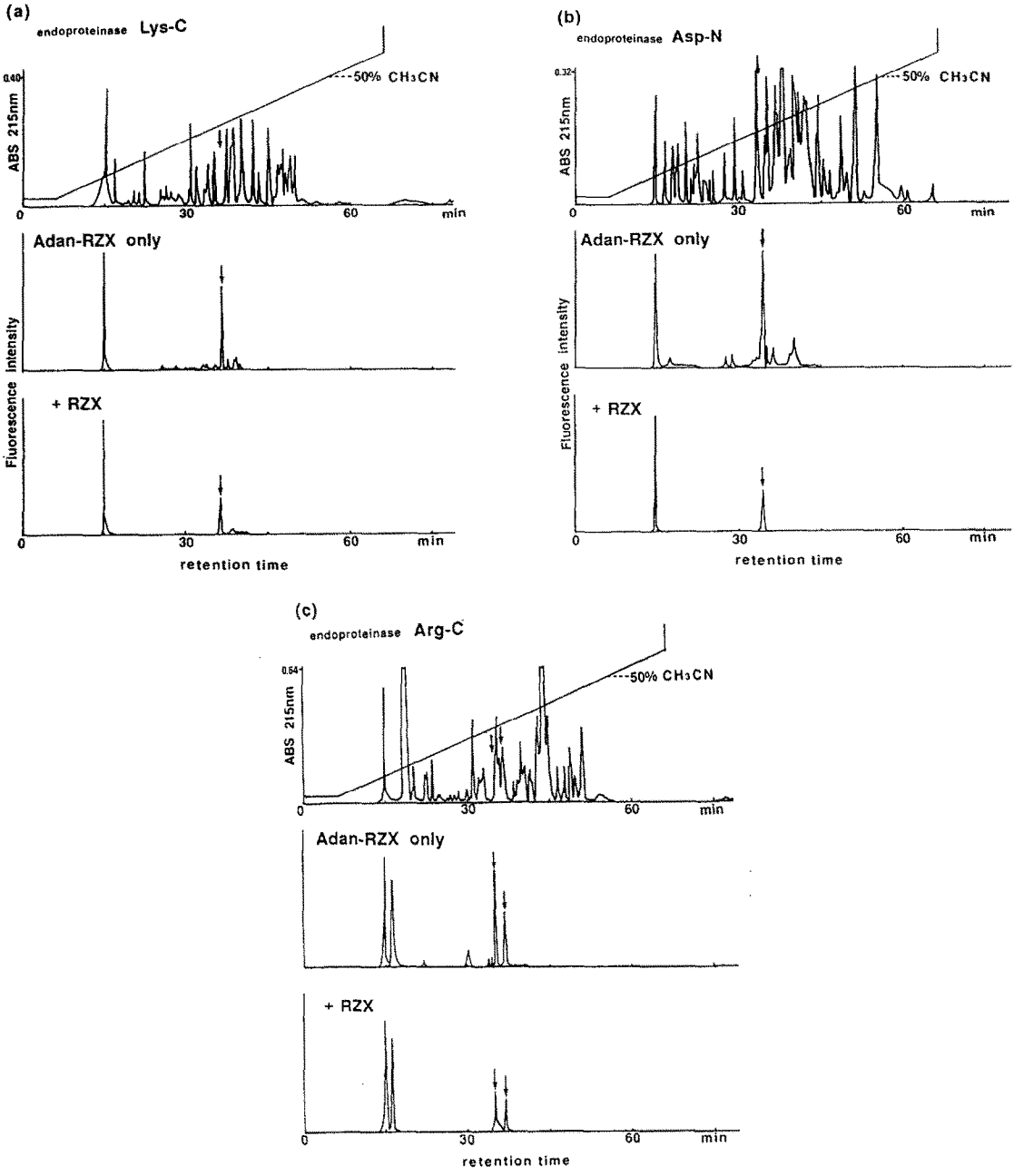

Fig. 2. HPLC analysis of enzymatic digests of photolabeled tubulin. Monitored by measuring absorbance at 215 nm and fluorescence intensity (excitation at 350 nm and emission at 520 nm). (a) Digests with endoproteinase Lys-C; (b) Digests with endoproteinase Asp-N; (c) Digests with clostripain (endoproteinase Arg-C). The top HPLC profiles were monitored in terms of absorbance, and the middle and bottom profiles in terms of fluorescence intensity. The digests of tubulin photolabeled with Adan–RZX are shown in the middle profiles; the digests of tubulin photolabeled in the presence of RZX are shown in the bottom profiles.

inhibition was attained with 7 μM Adan–RZX (IC_{50} value of RZX is 3 μM) [12]. The binding site of Adan–RZX was analysed by a binding competition assay with Dan–RZX, a fluorescent derivative of RZX, which binds specifically to the RZX-binding site on tubulin. Adan–RZX competed with bound Dan–RZX for the binding site, as shown in Table 1. From the results, the affinity of Adan–RZX to tubulin was estimated to be approximately one fifth of that of RZX. Ultraviolet irradiation of the incubation mixture containing Adan–RZX and tubulin led to the irreversible binding of Adan–RZX

to tubulin as described previously [12]. Adan–RZX bound without irradiation could be removed by SDS-treatment, but this was not the case with the irradiated sample. The efficiency of this covalent binding of Adan–RZX to tubulin was greatly reduced by addition of RZX (Table 2), indicating that the covalent binding is the RZX-binding site-specific. The yield of photoaffinity labeling was estimated to be approximately 3% from the fluorescence intensity.

HPLC analysis of proteolytic digests

Proteolytic digests of the Adan–RZX-labeled carboxymethylated tubulin were analysed on a C-18 reverse-phase HPLC column under the conditions described in Materials and Methods. HPLC profiles of absorbance at 215 nm and fluorescence intensity are shown in Fig. 2. On digestion with either endoproteinase Lys-C or Asp-N, labeled tubulin gave a single major fluorescent peak whose intensity was markedly reduced when the photoaffinity labeling was performed in the presence of RZX (retention time; 36.4 min for the Lys-C-digest and 34.5 min for the Asp-N-digest, Fig. 2a and b, respectively). The results suggest that only one residue in tubulin reacts with photo-activated Adan–RZX bound at the RZX-binding site.

On the other hand, upon digestion with clostripain, labeled tubulin gave two fluorescent peaks (retention times; 35.1 and 36.6 min) (Fig. 2c). It was possible that Adan–RZX reacted at two sites. However, the Lys-C- and Asp-N-digest contained one labeled fragment, so we considered that Adan–RZX-labeled at only one site, rather than two sites. The fluorescence intensity ratio of these peaks varied, depending upon the enzymatic hydrolysis conditions, suggesting that susceptibility to this enzyme of one of the Arg residues near the labeled site is rather low. We tentatively concluded that these two peaks contain the same photoaffinity labeled region.

All HPLC profiles indicate major peak(s) at 15 min. Absorbance and fluorescence intensity of these peaks were not reduced at all by the addition of RZX before irradiation, so these peaks were non-specific. Whether these represent non-specific labeled fragments or others was not determined.

Movement of the fluorescent peak upon successive peptidase digestions

To identify the covalent binding site of Adan–RZX, we used successively two peptidases differing in their substrate-specificity (Fig. 3). As described above, only one or two major fluorescent peak(s) were found in the enzymatic digests by HPLC (Fig. 2). The fluorescent peak fraction was collected and digested with another enzyme. If the fluorescent product isolated from the digest with the first enzyme contains a recognition site for the second enzyme, the isolated fluorescent fragment should be cleaved further, resulting in a different retention time of the fluorescent peak on HPLC. Thus, from the movement of the fluorescent HPLC peak upon successive enzymatic digestions, we should be able to determine the relative positions of the peptidase-recognition sites around the photoaffinity-labeled site, and thus be able to map the Adan–RZX-binding site.

Initially we used endoproteinase Lys-C as the first enzyme. The fluorescent peak in the digest (the retention time, 36.4 min) was isolated, and then digested with endoproteinase Asp-N or clostripain (Arg-C). As shown in Fig. 3a, no movement of the fluorescent peak position after the second digestion was observed, indicating that the Adan–RZX-bound fragment generated by Lys-C digestion contains no Asp-N or Arg-C sites.

Next, we used endoproteinase Asp-N as the first enzyme. The subsequent digestion of the isolated fluorescent peak from the Asp-N-digest with endoproteinase Lys-C or clostripain (Arg-C) resulted in movement of the fluorescent peak position (Fig. 3b). The results indicated that the Adan–RZX-bound fragment generated by the Asp-N digestion contains both Lys-C and clostripain (Arg-C) recognition sites. The retention time of the fluorescent peak produced by the second digestion with endoproteinase Lys-C (Fig. 3b, middle) coincided with that of the major peak generated by the first digestion with endoproteinase Lys-C of labeled tubulin (Fig. 3a, top). Clostripain (Arg-C) also digested the isolated fragment from the first digest of endoproteinase Asp-N to give two major peaks (Fig. 3b, bottom). Both the retention times of these two peaks produced by the second digestion with clostripain (Fig. 3b, bottom) coincided with those of the corresponding two major peaks in the clostripain digest of labeled tubulin (Fig. 3c, top).

Finally, we used clostripain (Arg-C) as the first enzyme, and endoproteinase Lys-C or endoproteinase Asp-N as the second enzyme. Labeled tubulin gave two fluorescent peaks on digestion with clostripain (Arg-C). We considered that these two peaks contained the same labeled region, so the fragments isolated from both peaks were usable for this study. Therefore, we selected the peak at 35.1 min to isolate the labeled fragment for second digestion, because the fluorescence intensity of this peak was more greatly reduced than that of the other peak. (Unless these two peaks contained the same region, the major labeled fragments were isolated from the peak at 35.1 min. Therefore this peak was better for the study of the RZX site.) The fragment isolated from the first digestion was cleaved by endoproteinase Lys-C, but not by endoproteinase Asp-N (Fig. 3c). The retention time of the peak produced by endoproteinase Lys-C as the second enzyme (Fig. 3c, middle) coincided with the major fluorescent peak generated by the first Lys-C digestion of labeled tubulin (Fig. 3a, top). Therefore, Adan–RZX bound fragment generated by clostripain (Arg-C) digestion is considered to contain a Lys-C-recognition site but no Asp-N-recognition site.

Mapping of the location of covalently incorporated Adan–RZX

The location of the covalent binding site of Adan–RZX could be determined from the results of successive digestions of the isolated fluorescent fragment and the enzymatic digestion map of α- and β-tubulins. As described above, the results of successive digestions using two of Lys-C, Asp-N and clostripain indicated that the Adan–RZX bound fragment generated by (i) Lys-C digestion contains no recognition site for the other two enzymes, (ii)

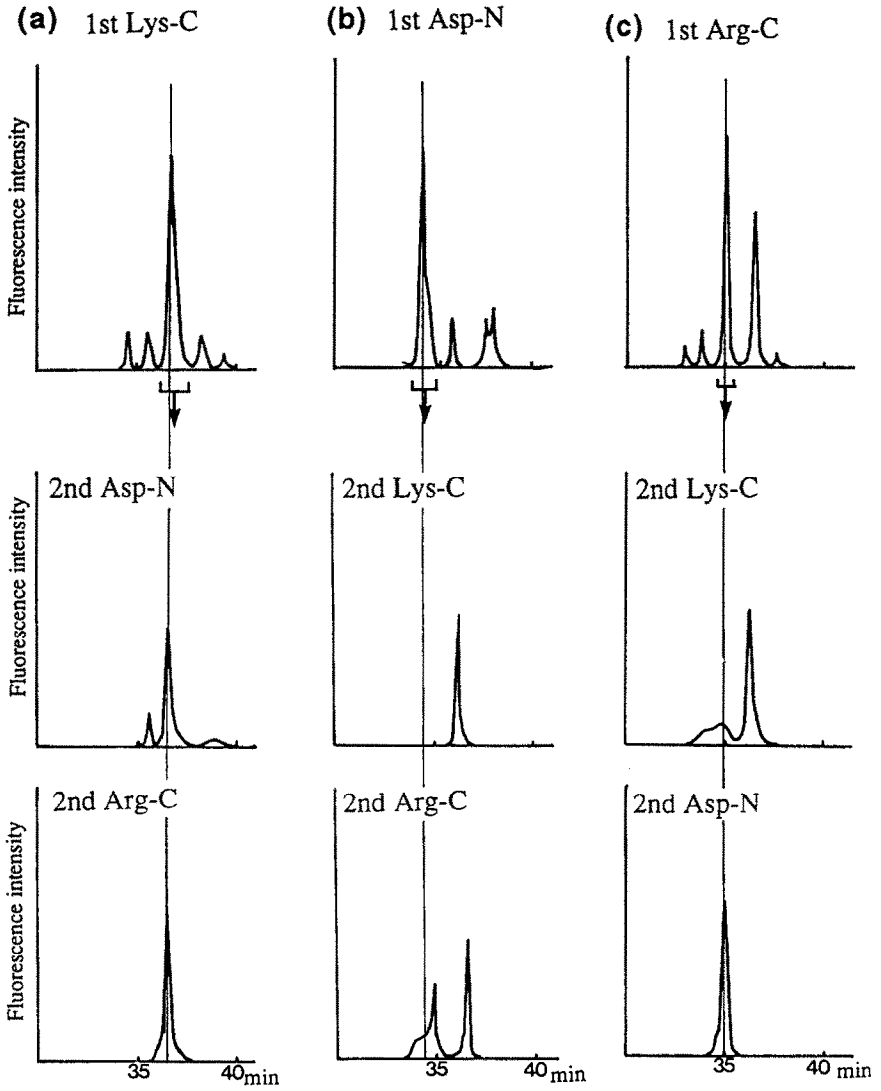

Fig. 3. Further digestion of Adan–RZX labeled fragments with a second enzyme. All HPLC profiles were monitored in terms of fluorescence intensity. The top profile shows HPLC of the digests obtained with the first enzymes. The major fluorescent fragment was isolated, and digested with a second enzyme. The vertical lines show the retention times of the fluorescent fragments from the digests with the first enzymes. The middle and bottom profiles are digests of the isolated fragments with the second enzymes.

Asp-N digestion contains both Lys-C and clostripain recognition sites, and (iii) clostripain digestion contains a Lys-C recognition site, but no Asp-N recognition site. These results suggested that the sequence around the Adan–RZX incorporation site should be Asp–Arg–Lys-(labeled site)–Lys–Arg–Asp. The use of endoproteinase Asp-N as the first enzyme and clostripain as the second enzyme produced two fluorescent fragments (Fig. 3b, bottom), which correspond to the two fluorescent peaks generated by the first clostripain digestion (Fig. 3c, top). This suggests that the fluorescent Asp-N fragment contains at least three Args. The sequence satisfying all these requirements was sought in the known amino acid sequence of porcine brain tubulin [18, 19]. The only such sequence is Asp-355–Arg-359–Lys-362–Lys-379–Arg-380–Arg-390–Asp-404 on β-tubulin (Fig. 4). Therefore, the Adan–RZX bound site should be in the Met-363–Lys-379 fragment on β-tubulin.

DISCUSSION

Adan–RZX has been synthesized as a potential photoaffinity labeling probe for part of the RZX-binding site of tubulin. It inhibits the *in vitro* assembly of microtubules, and competes with Dan–RZX, a fluorescent probe known to bind at the RZX-binding site.

Adan–RZX formed a covalent adduct with tubulin after photoactivation. As formation of this adduct was decreased by the addition of RZX, it is considered

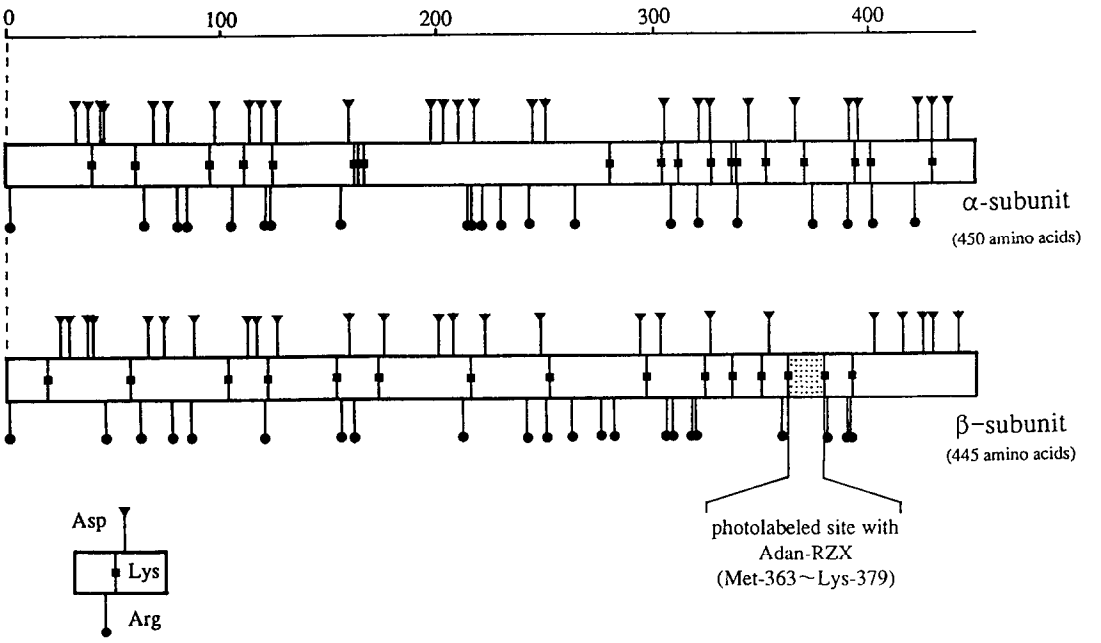

Fig. 4. Enzymatic digestion map of α- and β-tubulins. The abscissa indicates the number of amino acids. The vertical lines indicate the cleavage sites of the enzymes (the middle lines for Lys-C; the upper lines for Asp-N; the lower lines for Arg-C).

that Adan–RZX first bound noncovalently to the RZX-binding site, and then formed a covalent bond on irradiation. Since digestion of labeled tubulin with the endoproteinases Lys-C and Asp-N generated only one fluorescent peak (two peaks in the case of clostripain digestion; *vide infra*) on C-18 reverse phase HPLC analysis, Adan–RZX is considered to react at a unique site on tubulin under irradiation.

We have reported that RZX binding to tubulin is inhibited by the mutation of Asn-100 of the β-subunit to Val or Ile, suggesting that the RZX-binding site is located near Asn-100 of the β-tubulin. In the present work, we tried to determine the sequence of the fragment photoaffinity labeled with Adan–RZX. Amino acid sequence analysis of the peptide fragment by automatic Edman degradation was unsuccessful because of the small amount of the labeled fragment and of contamination. Therefore, we employed combinations of peptidases, and obtained results indicating that the labeled fragment is Met-363–Lys-379 of the β-subunit. This might suggest that the Met-363–Lys-379 region is in the vicinity of Asn-100 spatially, though we must await X-ray analysis for a definitive answer.

The function of Met-363–Lys-379 on β-tubulin is not known. An antibody against β: 339–417 which contained Met-363–Lys-379 was obtained previously, but it was not investigated whether this antibody affected microtubule functions [20]. On the other hand, the mutation of Thr-372, Ile-374, Gln-375 and Lys-390 on β-tubulin of *Saccharomyces cerevisiae* conferred benomyl supersensitivity, and the sequences of amino acid-363–379 have low homology between α- and β-tubulin [20]. In view of these results, it appeared that this region was important for the conformation of β-tubulin and its function.

We obtained a clear result by using combinations of highly specific peptidases, i.e. endoproteinase Lys-C, endoproteinase Asp-N and clostripain (Arg-C). Other highly specific enzymes should also be usable. This technique should be generally applicable for identification of the modified site in polypeptides whose primary structure is known.

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