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The Unusual Stability of Saporin, a Candidate for the Synthesis of Immunotoxins[1]

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Saporin, a monomeric protein extracted from the seeds of *Saponaria officinalis*, is an enzyme capable of specific depurination of the eukaryotic ribosomes. Because of its toxicity, saporin proved useful for the synthesis of immunotoxins, chimeric conjugates of a toxin and an antibody specifically directed against cancer cells or other targets. In this paper we report a study of the structural properties of saporin in the presence of denaturing agents and/or proteolytic enzymes. We found that saporin is extremely resistant to denaturation by urea or guanidine (up to 4 M), even at relatively high temperature (up to 55 °C). Moreover a structural change detected as a reduction of the fluorescence emission of the single Trp residue is reversible and is not paralleled by changes of the far UV CD spectrum, suggesting that even under harsh experimental conditions unfolding is limited. In good agreement with these results, guanidine-treated saporin is not attacked by proteolytic enzymes. The remarkable resistance of saporin to denaturation and proteolysis suggests this protein as an ideal candidate for biotechnological applications.

Immunotoxins, the anti-cancer conjugates between a toxin and a specific immunoglobulin directed against antigens from cancer cells, are gaining wide interest as innovative therapeutic agents (1,2). Several toxins have been employed in the synthesis of immunotoxins. A large experience is available on ricin, the heterodimeric toxin extracted from the seeds of castor bean (*Ricinus communis*), constituted by the enzymatically active A chain which catalyses the hydrolysis the N-glycosidic bond of adenine 4324 on the 28S rRNA, and the B chain, a lectin which binds to galactose or N acetyl galactosamine residues of membrane glycoproteins and glycolipids (2).

Only a few plants produce ricin-like toxins, but a class of related monomeric toxins is widespread; the dimeric and monomeric toxins are both called Ribosome Inactivating Proteins (RIPs; see 2). Monomeric (or type I) RIPs are devoid of the carrier lectin, and hence are poorly toxic to intact cells, but very active as protein synthesis inhibitors when tested on cell lysates. Amongst these, saporin and gelonin have been employed as the toxic moiety of chimeric molecules containing as the carrier an immunoglobulin, a hormone or transferrin, which is internalised *via* its specific membrane receptor (3).

The endocytosis and intracellular routing of chimeric molecules containing a plant toxin may prove a limiting step in their action (4), because the toxin's target is most often a nuclear or cytoplasmatic molecule, whose inactivation demands that the toxin struggles its way out of the endosome. Presumably because of incorrect intracellular transport, the immunoconjugates of ricin are less toxic than the native molecule, while toxicity is retained in the immunoconjugates of whole ricin (4). Moreover there is evidence that ricin A chain is processed by proteolytic enzymes inside secondary endosomes (5,6), a factor which may play a role in reducing the toxicity of immunotoxins.

Since the stability of the toxin employed for the synthesis of the chimera is an important factor in determining the toxic activity on target cells, we have investigated on the stability of saporin, the monomeric RIP extracted from seeds of *Saponaria officinalis* against unfolding by chemical denaturing agents, and digestion by several animal and plant proteases. Our results show that saporin is characterised by an unusual resistance to guanidine hydrochloride and to proteolysis, supporting the opinion that it is a suitable candidate for the synthesis of artificial conjugates, to be eventually employed in the treatment of cancer.

[1] This paper is dedicated to Alessia Paglia, prematurely deceased on January 10, 1997.

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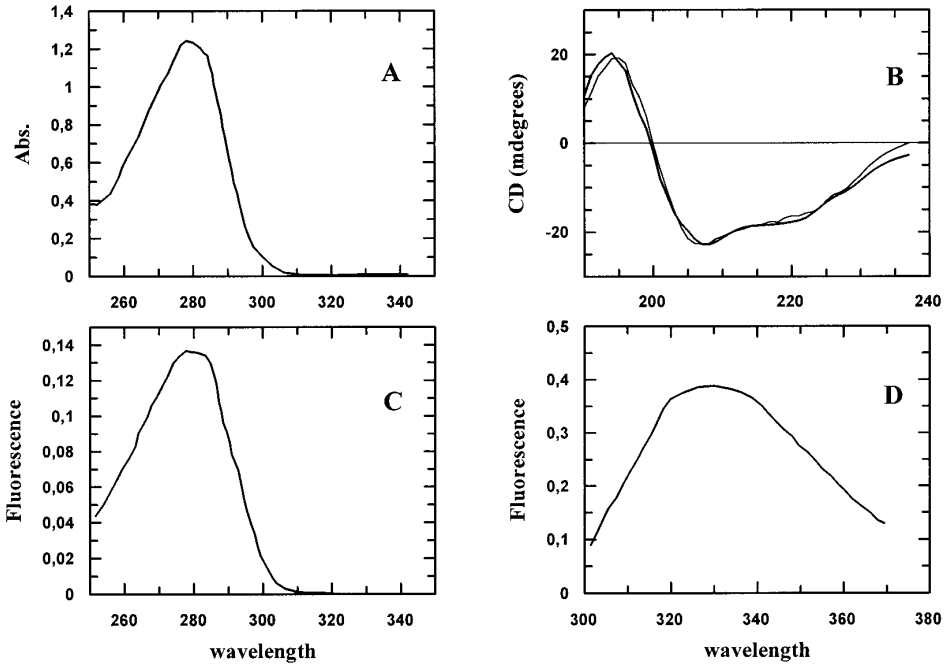

FIG. 1. Spectral properties of saporin. Panel A: Absorbance spectrum of 51 μM saporin in 0.1 M sodium phosphate pH=7, t=20°C, pathlength 1 cm. Panel B: Far UV circular dichroism spectrum of 3.3 μM saporin (bold line) and theoretical spectrum for ricin A chain (thin line); pathlength 0.1 cm, other experimental conditions as in panel A. Panel C: Fluorescence excitation spectrum of 0.5 μM saporin (emission was monitored at 330 nm). Panel D: Fluorescence emission spectrum of 0.5 μM saporin excited at 285 nm.

MATERIALS AND METHODS

The isoform 6 of saporin was purified from homogenates of the seeds of *Saponaria officinalis,* as described (7). Absorbance spectra were recorded with a Jasco V500 spectrophotometer; CD spectra with a Jasco J700 spectropolarimeter; fluorescence emission and excitation spectra with a Spex Fluoromax spectrofluorometer.

Samples for proteolytic digestion experiments were prepared by mixing 400 μl of the chosen buffer (either 0.1 M sodium phosphate pH 7.0 or 0.1 M tris HCl pH 9.0 or 0.02 M sodium formiate pH 3.0) with 50 μl of a 6.8 mg/ml solution of saporin and 50 μl of a 1 mg/ml solution of the chosen proteolytic enzyme (either trypsin, chimotrypsin, pepsin, papain, ficin or clostripain); this procedure correspond to a saporin:protease stoichiometric ratio of 5:1. The mixture was allowed to react for 5′ to 120′ at 25 °C and the reaction was stopped by addiction of the appropriate inhibitor (e.g. Pefabloc, pTosyl lysine chloro methyl chetone etc.). After addition of the inhibitor, the samples were denatured by dilution with an equal volume of tris-glycine buffer containing 2% SDS pH 6.8 and heated at 100°C in a boiling water bath for 5 minutes. A significant degradation of the toxin (positive control) was obtained by performing the denaturation in the presence of the uninhibited protease; the negative control was obtained by adding the inhibitor before the proteolytic enzyme. SDS electrophoresis war carried out as described by Laemli (8)

RESULTS AND DISCUSSION

The 3D structure of saporin is not available; its sequence has limited homology with other RIPs (30% with ricin A chain; see 9) and shows a single Trp residue at position 208, probably relevant for catalytic activity. The fluorescence of this single Trp has a broad emission with a maximum around 330 nm (Fig 1 C-D)

The far UV CD spectrum of saporin (shown in Fig. 1 B), is compatible with a secondary structure content of 29% α helix, 12% β sheet and 59% coil. The calculated spectrum was deduced from the secondary structure content of ricin A chain, as obtained from its 3D model (10,11); of course the far UV CD spectrum of purified ricin A chain is also very similar to those reported in fig. 1B. The good agreement suggests that the two protein share a similar fold, in spite of the low sequence homology.

Unfolding of saporin under various experimental conditions was followed through changes in the Trp emission and the far UV CD spectra. In the absence of denaturing agents, the fluorescence is stable over the pH range 2 to 10; urea up to 6 M is scarcely effective in promoting denaturation of saporin. Guanidine hydrochloride (Gnd HCl) is more effective, but at concentrations 2 to 4 M, long incubation times are required. Fig.2A reports the decrease of Trp fluorescence emission as a function of time, recorded at pH values from 5 to 9 in the presence of 4 M Gnd HCl.

Maximal stability was observed at pH 6, close to the pH of secondary endosomes (12), while at alkaline pH the exposure of Trp is faster ($t_{1/2}$ = 13 min); the lowest stability is observed at pH values comparable with the high isoelectric point of this protein (pI>9, see 2). The effect of temperature on the time course of the process detected by fluorescence was investigated between 20

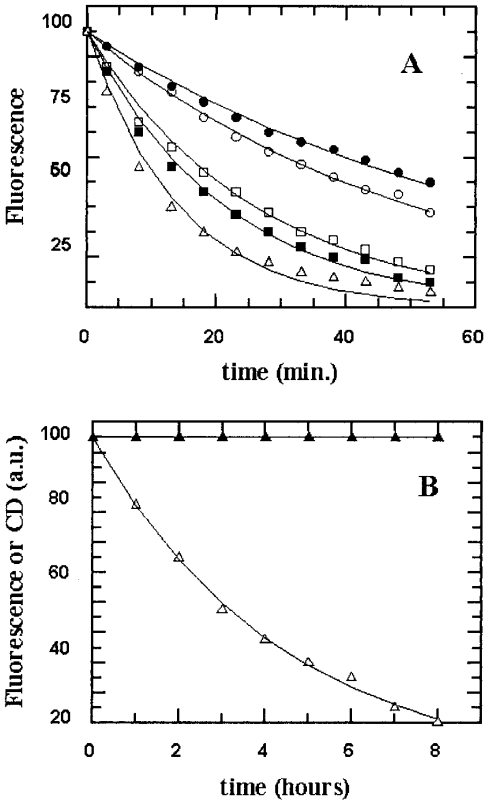

FIG. 2. Time course of Gnd HCl denaturation. Panel A: Decrease of fluorescence as a function of time of incubation in 4 M Gnd HCl at t=25°C; (○) pH = 5.0; (●) pH = 6.0; (□) pH = 7.0; (■) pH = 8.0; △ pH = 9.0. Panel B: Time evolution of the CD (220 nm▲) and fluorescence (330 nm △) signal in 2 M Gnd HCl, 0.1 M sodium phosphate pH 7.0; t=37°C.

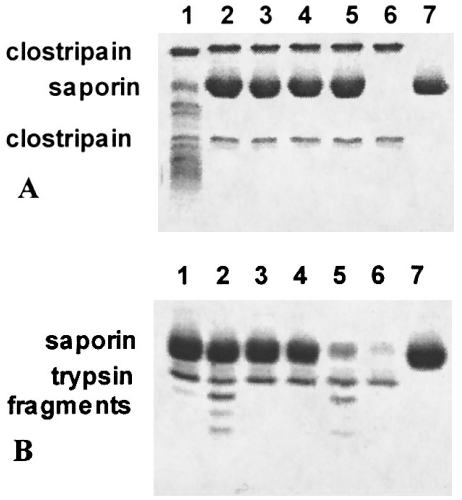

FIG. 3. Gel electrophoresis of the tryptic and clostripain digests of saporin. Panel A: 1) saporin + clostripain positive control (see text mat. and met.), 2) same as lane 1 inhibited immediately, 3) inhibited after 30′, 4) inhibited after 60′, 5) inhibited after 120′, 6) clostripain inhibited after 120′, 7) saporin. Panel B: 1) saporin + trypsin inhibited immediately, 2) same as lane 1 positive control, 3) inhibited after 5′, 4) inhibited after 15′, 5) inhibited after 30′, 6) inhibited after 5′ at 37°C, 7) saporin. Experimental conditions as described under materials and methods.

°C and 55 °C using a Gnd HCl concentration of 1 M; only at the highest temperatures a significant increase of the rate constant was observed (table 1).

The reversibility of the fluorescence decrease was tested. We found that rapid dilution in Gnd free buffer of saporin incubated at pH=7 and t=20 °C in 4 M Gnd HCl for up to 2 hours is associated to the recovery of over 80% of the initial emission; the recovery progressively decreases for longer incubation times (data not shown).

TABLE 1

Buffer	pH	Temperature °C	k
4 M Gnd 0.1 M acetate	5.0	25	0.023
4 M Gnd 0.1 M Na pho.	6.0	25	0.021
4 M Gnd 0.1 M Na pho.	7.0	25	0.043
4 M Gnd 0.1 M Na pho.	8.0	25	0.054
4 M Gnd 0.1 M tris HCl	9.0	25	0.086
1 M Gnd 0.1 M Na pho.	7.0	37	<0.001
1 M Gnd 0.1 M Na pho.	7.0	45	0.013
1 M Gnd 0.1 M Na pho.	7.0	55	0.046
1 M Gnd 0.1 M Na pho.	7.0	37	0.0038

Rate constants of the exposure of Trp 208 under various experimental conditions, expressed in min^{-1}.

The time course of fluorescence quenching was followed in parallel with the far UV CD spectrum as a function of time in 50 mM sodium phosphate buffer pH 7 containing 2 M Gnd HCl, a concentration which causes a significant decrease of fluorescence over a time range of several hours. As shown in fig.2B, no change in the ellipticity at 222 nm is observed while the fluorescence emission decays, suggesting that exposure of the single Trp is a local phenomenon and that protein unfolding requires considerably more drastic experimental conditions.

We tend to exclude that the state populated after several hours incubation with Gnd HCl is a molten globule intermediate, because no loss of secondary structure was observed contrary to the common notion that in molten globule states secondary structure, though present, is somewhat reduced compared to the native state; moreover as detailed below the protein is insensitive to proteolytic enzymes, while molten globule states are often good substrates for proteolytic enzymes (13, 14)

As a further step in the evaluation of the stability of saporin, we tested the resistance to exposure to six

different proteolytic enzymes: ficin, papain, trypsin, chymotrypsin, pepsin and clostripain. All were shown to be ineffective against saporin, and previous incubation of the protein with Gnd HCl (3 M for up to 3 hours at 37°C) was also ineffective. Fig. 3 shows a SDS-polyacrylamide gel electrophoresis of saporin exposed to trypsin (panel A) or clostripain (panel B) for time interval ranging from 5′ to 2 hours at 37°C.

The aim of these experiments was to assess the structural stability and resistance to proteolytic attack of saporin in view of its biotechnological applications. The protein proved so stable that only under extreme experimental conditions (incompatible with the cell interior) partial unfolding and some proteolysis occurred. The ricin AB heterodimer is relatively stable to common denaturing agents (15), but its isolated A chain is much less stable than saporin, in keeping with the higher toxicity of the latter in macrophages and its very limited digestion inside the cell (Fiani M. unpublished results).

In conclusion this study demonstrates that saporin is characterised by high resistance to denaturation and/or proteolysis, a property which makes it a good candidates for the synthesis and use of chimeric toxins (3). Moreover we observe that the very slow kinetics of denaturation, even at high concentration of guanidine, and the maintenance of native secondary structure are remarkable features of saporin, especially considering the high coil content (>50%). Elucidation of the three dimensional structure of saporin by X-ray diffraction may provide clues to explain the remarkable stability of the native state of this protein.

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