J. Biochem. 132, 223–228 (2002)

Optimum Modification for the Highest Cytotoxicity of Cationized Ribonuclease[1]

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Received April 29, 2002; accepted May 21, 2002

Cationization of a protein is considered to be a powerful strategy for internalizing a functional protein into cells. Cationized proteins appear to adsorb to the cell surface by electrostatic interactions, then enter the cell in a receptor- and transporter-independent fashion. Thus, in principle, all cell types appear to take up cationized proteins. Since ribonucleases (RNases) have a latent cytotoxic potential, cationized RNases could be useful cancer chemotherapeutics. In this study, we investigated the effect of the degree of cationization on the cytotoxicity of RNase A by modifying carboxyl groups with ethylenediamine. We found that there is an optimum degree of modification for cytotoxicity, in which 5 to 7 out of 11 carboxyl groups in RNase A are modified, toward MCF-7 and 3T3-SV-40 cells. More interestingly, the cytotoxicity of cationized RNase As correlates well with the value of [RNase activity] × [estimated concentration of RNase free from RNase inhibitor], mimicking the practical enzymatic activity of cationized RNase As in cytosol. The results indicate that cationization of a protein to an optimum level is important for maintaining protein function in the cytosol. Sophisticated protein cationization techniques will help to advance protein transduction technology.

Key words: cationization, cytotoxicity, protein transduction, ribonuclease.

The biological actions of ribonucleases (RNases) are now receiving considerable attention because RNase family proteins have chemotherapeutic potential due to their ability to degrade RNA and thus inhibit protein synthesis (*1–4*). Bovine RNase A [EC 3.1.27.5] is a prototype member of this family whose structure and function relationships have bean studied extensively (*5*). Although RNase As are generally non-toxic, two classes within the RNase A family, bovine seminal RNase (BS-RNase) and frog RNases, are highly toxic to cancer cells (*6–9*). Furthermore, 2′,5′-oligoadenylate-dependent RNase L, another RNase family member, plays a central role in the interferon-induced anti-viral cellular response by degrading viral and cellular mRNAs (*10–12*). Consequently, control of cellular RNA pools is a reasonable target for regulating cellular growth.

On the basis of their chemotherapeutic potential, nontoxic human RNases have been engineered to endow cell type–specific cytotoxic properties by chemical conjugation or genetic fusion with internalizing cell-binding ligands (*13–21*). These engineered RNases are potentially promising chemotherapeutic agents, however, the clinically targeted cells do not always have suitable internalizing ligands. Protein cationization is an alternative method for internalizing RNases into cells because cationic protein is efficiently adsorbed to the negatively charged cell surface by electrostatic interaction, resulting in highly efficient internalization (*22, 23*). Because the electrostatic interaction has no selectivity with respect to cell-type, cationization of an RNase may limit its chemotherapeutic application. However, the protein cationization method, which provides highly efficient and virtually transient protein transduction into every cell, is of great interest for its applications *in vitro* and *in vivo*. In this study, we used bovine RNase A as a model protein to optimize the protein cationization method based on cytotoxicity, because, in this case, cytotoxicity reflects the extent to which the functional protein is manifested in cytosol.

Other methodologies for protein transduction into cells have been developed. Several recently identified small cationic regions of proteins, called protein transduction domains (PTDs), as well as poly-Arg peptides have the ability to traverse biological membranes efficiently (*24–30*). For example, the Tat peptide from human immunodeficiency virus possesses 8 cationic amino acids out of 11 residues. Although the detailed mechanism remain unclear, the cationic property is likely to be essential in every case.

Protein cationization with amine, such as with ethylenediamine, can be accomplished by modifying carboxyl groups *via* a carbodiimide reaction [Fig. 1a (*31*)]. Although the cationized protein will acquire a new ability to pass through

[1] This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydorochloride; RI, RNase inhibitor.

cell membranes, unfavorable effects of the extensive modification of carboxyl groups on protein function and stability limit the usefulness of this method. Thus, we investigated the relationship between the degree of chemical modification of RNase A and cytotoxicity. First, cationized RNase A (ethylenediamine-modified RNase A) was fractionated into several portions by net-charge (that is, by the degree of modification) by means of cation-exchange column chromatography (Fig. 1b). Since RNase-mediated cytotoxicity is suggested to depend on several factors (*2, 3*), cationized RNase As possessing various degrees of modification were then assessed with respect to their cytotoxicity, enzymatic activity, and affinity for cytosolic RNase inhibitor. We found that most of cationized RNases were strongly toxic to malignant cells, and more significantly, that there was an optimum level of modification for cytotoxicity. The cytotoxicity of cationized RNases appeared to correlate with the estimated value of their practical RNases activity manifested in cytosol under the chosen set of experimental conditions. These results indicate that the achievement of an optimum cationization level is important for a protein to enter and function in cytosol most efficiently.

EXPERIMENTAL PROCEDURES

Materials—Bovine RNase A (Type XII-A), yeast tRNA (Type X), and human serum albumin (×1 crystallized) were purchased from Sigma. Recombinant RapLR1 expression vector plasmid 236–11, in which RapLR1 cDNA is cloned into the pET22b(+) expression vector, was provided by Dr. S.M. Rybak (*32*). The recombinant RapLR1 was expressed in *Escherichia coli* BL21(DE3) as an inclusion body, and purified according to the methods described previously (*33, 34*). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydorochloride (EDC) was purchased from Dojin (Kumamoto). Ethylenediamine dihydrochloride was obtained from Tokyo Kasei (Tokyo), and recombinant human placental RNase inhibitor (RI) was from Wako Chemicals (Osaka).

Chemical Modification and Fractionation of Variously Modified RNase A—The coupling reaction was carried out under three different conditions to obtain variously modified RNase A. Briefly, three solutions were prepared by dissolving 50 mg of RNase A in 5 ml of 2 M ethylenediamine dihydrochloride–NaOH, pH 5.0. The coupling reactions were initiated by adding 10 mg and 50 mg of EDC to the first and second solutions with stirring at room temperature, leading to low-level and mid-level modification, respectively. To prepare a highly modified RNase A, 80 mg of EDC was added to the third solution, then 80 mg of EDC was added at 3 h, and 40 mg at 18 h. All reaction mixtures were stirred continuously at room temperature for 21 h, then dialyzed against distilled water at 4°C. To fractionate ethylenediamine-cationized RNase A by the degree of modification (that is, the degree of cationization), the dialysates were combined and applied to an open column of a carboxylic cation-exchanger, CM-Toyopearl 650M (Tosoh, Tokyo; 12 × 560 mm), and eluted with a linear gradient from 500 ml of 50 mM phosphate buffer, pH 6.5, to 500 ml of the same buffer containing 1.0 M NaCl. The pooled fractions were fractionated into 6 portions by net-charge, exhaustively dialyzed against distilled water for 3 days at 4°C, and then lyophilized to give RNaseA-ED1 to RNaseA-ED6, respectively. The numbers of carboxyl groups modified by ethylenediamine in RNaseA-ED1 to RNaseA-ED6 were determined by MALDI-TOF mass spectrometry with a Perseptive Voyager-DE PRO mass spectrometer using native RNase A and native hen egg lysozyme for calibration.

Cytotoxicity Assays—Cytotoxicity assays were conducted with Swiss mouse albino 3T3 cells transformed with SV40 (3T3-SV-40; Dainippon Pharmaceutical, Tokyo) and a human breast carcinoma cell line, MCF-7 (*35*). Both cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 70 μg/ml of kanamycin. The protocol for cytotoxic assay was described previously (*22*).

RNase Activity and Interaction with RNase Inhibitor—The RNase activity of native and ethylenediamine-modified RNase As was determined using tRNA as a substrate (*36*). The activity was measured in a final volume of 0.3 ml of 0.33 mg/ml yeast tRNA, 10 mM Tris-HCl, pH 7.5, or 10 mM MES-NaOH, pH 6.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.17 mg/ml human serum albumin, and appro-

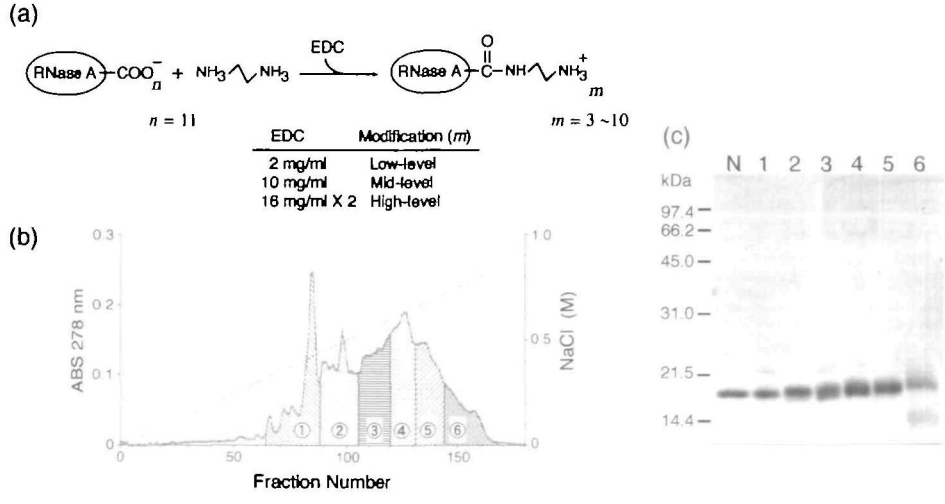

Fig. 1. **Preparation and fractionation of ethylenediamine-cationized RNase A by net charge.** (a) The conjugation of ethylenediamine and RNase A was carried out under three different conditions. (b) Heterogeneously modified ethylenediamine-RNase A was fractionated on a cation-exchange column of CM-Toyopearl 650M at pH 6.5 by a linear NaCl gradient. The pooled fractions from 1 to 6 were designated as RNaseA-ED1 to RNaseA-ED6, respectively. (c) SDS-PAGE analysis of native (lane N) and ethylenediamine-modified RNase As (lanes 1–6). Samples (1.5 μg/lane) were analyzed by SDS-PAGE under reducing conditions using a 15% polyacrylamide gel. The lane numbers 1–6 correspond to RNaseA-ED1 to RNaseA-ED6 grouped according to their net charge (b). The gel was stained with Coomassie Brilliant Blue R250.

priate concentrations of RNases (dilutions were made in 0.5 mg/ml human serum albumin). The mixtures were incubated for 15 min at 37°C and the reactions were then terminated with 700 μl of 3% ice-cold perchloric acid. The mixtures were left on ice for 10 min and microcentrifuged at 13,000 rpm for 10 min at 4°C. The absorbance at 260 nm of the supernatant was determined as a measure of the digested products. Assays were performed in the linear range of each protein.

To determine the affinity of RI for each RNase, the RNase activity was measured in the presence and absence of commercial RI (1 unit of RI is that amount required to inhibit 50% the activity of 5 ng of RNase A) in the range of 20- to 1,500-fold molar excess over RNase. Values of K_i, the inhibition constant, were calculated from the remaining activity, assuming RI to be a competitive inhibitor of cationized RNase A or RapLR1 bound to RI at 1:1 stoichiometry, as in the case of native RNase A (*37*).

RESULTS

Fractionation of Variously Modified RNase—The carboxyl groups of RNase A (the number of carboxyl groups, n = 11) were amidated with ethylenediamine to covert negative charges of the carboxylate anions to positive charges by a carbodiimide reaction. Since the three reaction conditions varied in the amount of EDC used, the combined products were mixtures of diversely modified proteins. As shown in Fig. 1b, a series of ethylenediamine-modified bovine RNase As was fractionated according to net charge by cation-exchange chromatography. The pooled fractions were grouped into 6 fractions from RNaseA-ED1 to RNaseA-ED6, which were then assessed with respect to their cytotoxicity, enzymatic activity, and affinity for cytosolic RI.

SDS-PAGE analysis under reducing conditions of the modified RNases (Fig. 1c) indicated that they migrate slightly more slowly than unmodified RNase, as expected from the increase in molecular mass as well as the increase in positive charge due to the modification. In the case of RNase-ED6, a small portion of the protein was found by a protein sequencer to be nicked mainly between Asp37 and Ser38. The net charge of each fractionated protein at neutral pH (Table I) was calculated from the average number of modified carboxyl groups as determined by mass spectrometry. The number of modified groups ranged from an average of 3.7 in RNase-ED1 to 9.6 in RNase-ED6 (data not shown).

Cytotoxicity—The cytotoxicities of modified RNases and RapLR1 toward MCF-7 and 3T3-SV-40 cells were compared. RapLR1 is a cytotoxic RNase derived from the liver of gravid female amphibian *Rana pipens*, and is related to the antitumor protein Onconase™ with four amino acid differences (Leu11 to Ile, Asn20 to Asp, Thr85 to Lys, and His103 to Ser) (*32*). Since both enzymatic and cytotoxic properties are equivalent to Onconase™ (Dr. S.M. Ryback,

TABLE I. **Characteristic properties of native and modified RNases.**

RNase molecule	Net charge[a]	Relative RNase activity (%)[b]	K_i (nM)[c]	IC_{50} (nM)[d]	
				MCF-7	3T3-SV-40
RNaseA	+4	100 (0.61)	n.d.	n.d.	n.d.
RNaseA-ED1	+11.4	12.8	0.014	10000	3000
RNaseA-ED2	+13.4	8.57	1.4	95	230
RNaseA-ED3	+15.6	5.0	19	85	75
RNaseA-ED4	+18.8	1.56	56	105	120
RNaseA-ED5	+21.0	0.59	125	300	355
RNaseA-ED6	+23.2	0.11	200	1200	670
RapLR1	+6	0.06 (0.30)	207	1150	950

[a]The net charge of a modified RNase A is the average value calculated from the mass spectrum. [b]RNase activity against yeast tRNA was determined at pH 7.5 (or at pH 6.5 as shown in parenthesis) and 37°C and is expressed relative to that of RNase A. [c]The value of K_i was determined for the inhibition of RNase activity by RNase inhibitor at pH 7.5 (or at pH 6.5 for RapLR1) and 37°C. [d]The value of IC_{50} was determined from the cytotoxicity (Fig. 2). Values that could not be obtained are shown as n.d.

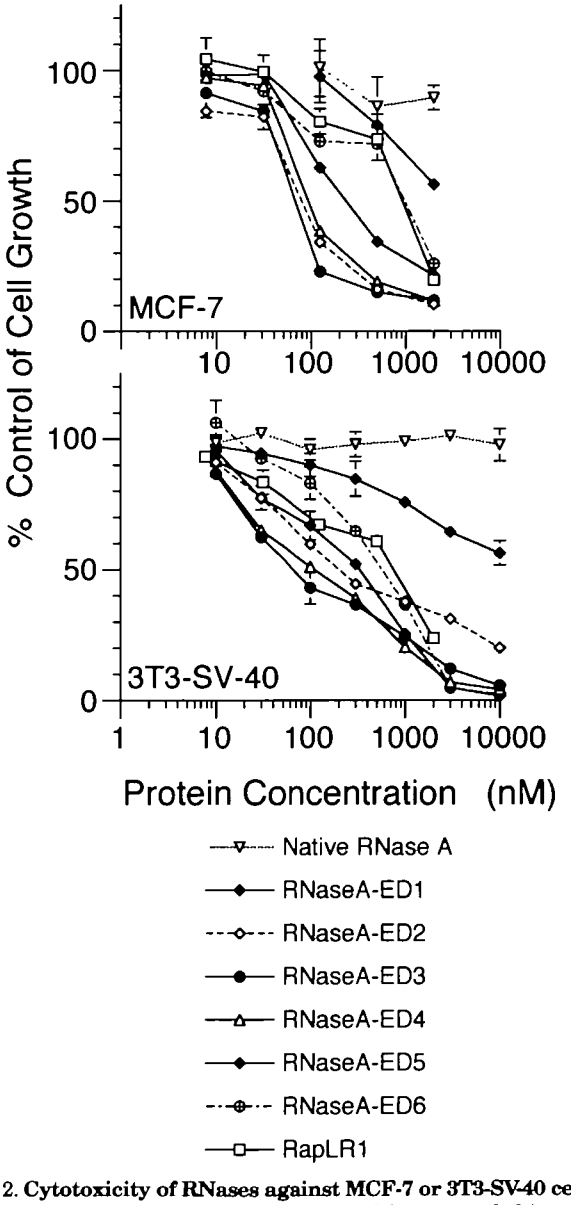

Fig. 2. **Cytotoxicity of RNases against MCF-7 or 3T3-SV-40 cells.** Cells in DMEM supplemented with 10% FBS were seeded into 96-well plates (1500 cells/well), left to adhere for 12 h, and then treated with various concentrations of each protein sample for 3 days. Cell growth in each well was monitored by MTT assay. Values are the mean of three cultures and are reported as a percentage of the buffer-treated control cells, which was the mean value from medium without protein sample.

personal communication), we used RapLR1 as a positive control for cytotoxic RNase. As shown in Fig. 2, modified RNases and RapLR1 were cytotoxic to both MCF-7 and 3T3-SV-40 cells showing IC_{50} values (the concentration required for inhibition of 50% of cell growth) to be 75–10,000 nM, while native RNase A was not cytotoxic (Table I). Among the cytotoxic RNases, RNaseA-ED3, a mixture of RNases with 5–7 carboxyl groups modified by ethylenediamine, was the most cytotoxic, indicating the presence of an optimum degree of modification for cytotoxicity (Fig. 3). Interestingly, all modified RNases except RNase A-ED1 were 1–16 fold more cytotoxic than RapLR1.

RNase Activity and Interaction with RNase Inhibitor—RNase activities were assessed using yeast tRNA as a substrate (Table I). As the number of modified carboxyl groups increased, the specific activity of RNase A decreased markedly. Since the optimum pH for activity differed between RNase A and RapLR1, the activity of RapLR1 was only 0.06% that of RNase A at pH 7.5, but 50% at pH 6.5.

The interaction between each RNase and RI was quantitated by the inhibition by RI of the enzymatic activity of RNase against yeast tRNA at pH 7.5 (or at pH 6.5 for RapLR1) and 37°C (K_i values in Table I). Due to the extraordinarily tight binding of RI to native RNase A, the K_i value could not be determined by this assay, but has been reported to be 4.4×10^{-14} M (*38*). As for modified RNases, the affinity for RI was substantially reduced, and the K_i values correlated well with the levels of modification (Table I). The high K_i value for RapLR1 (207 nM) is consistent with the fact that frog RNases have a low affinity for RI (*39*).

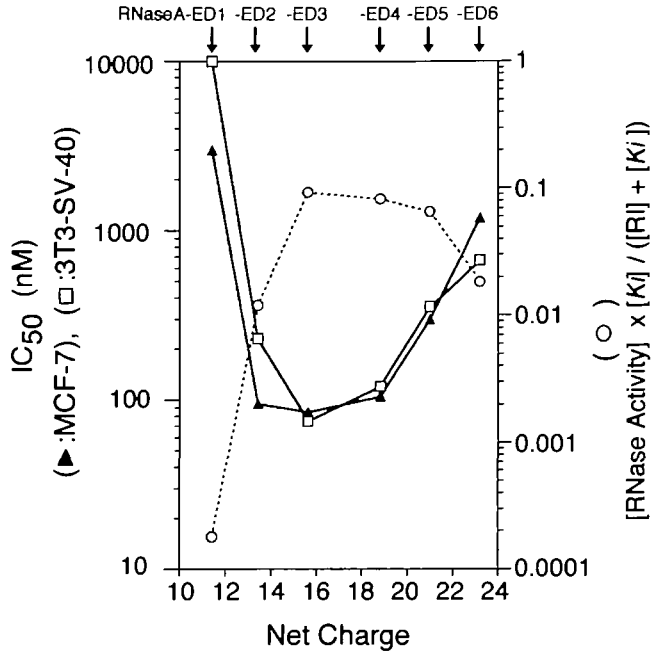

Fig. 3. **Correlation between cytotoxicity and RNase activity under cytosolic conditions.** The cytotoxicities (IC_{50}) and the estimated practical RNase activity under cytosolic conditions of ethylenediamine modified RNase As (RNaseA-ED1 to RNaseA-ED6) are plotted against net charge. The value of [RNase activity (%)] × [K_i (nM)/(1,000 + K_i (nM))] was assumed to be proportional to the practical RNase activity. All data are from Table I.

DISCUSSION

The mechanism of RNase-mediated cytotoxicity is not known in detail. Intuitively, the cytotoxicity of RNase must be manifested by RNA degradation in cytosol. To have access to the cytosol, RNase must cross a cellular membrane. In the previous study, we demonstrated that when added to the culture medium, the amount of RNase A derivative associated with cells increases with an increase in the net positive charge of the derivative, thus suggesting that more cationic proteins can internalize more efficiently into cytosol (*22*). Here, we prepared a series of variously cationized RNase A derivatives (RNaseA-ED1 to RNaseA-ED6) by amidation of the carboxyl groups with ethylenediamine. Thus, if the efficiency of internalization is the only factor for RNase cytotoxicity, the most cationic RNase A derivative should always be the most cytotoxic. However, this was not the case, because there was an optimum level of modification for cytotoxicity (Figs. 2 and 3).

Obviously, cytotoxic RNase should be enzymatically active. However, as shown in Table I, the specific activities of RNase A derivatives cationized by this modification decreased substantially with the increase in the extent of modification. This result is consistent with the observation that extensively modified RNase A (for example RNaseA-ED6) is less cytotoxic than a moderately modified one (RNaseA-ED3), suggesting that the extensive chemical modification of the protein leads to a serious decline in their essential functions.

On the other hand, the RI gene is ubiquitously expressed in human tissues (*40*), and the cytosolic concentration of RI has been roughly estimated to be about 1,000 nM (*41*). Since RI usually interacts with pancreatic type RNases with high affinity (K_i less than the pM range), most RNases can not be active in cytosol unless RNase in excess of RI enters the cytosol. Thus, the sensitivity of RNase to RI should be an important factor for RNase cytotoxicity (*42*). In fact, naturally occurring cytotoxic RNases, such as BS-RNase, and Onconase™, have been shown to be relatively insensitive to RI (*39, 43*). RapLR1, which is an RNase homologous to Onconase™, also showed a low affinity for RI and was cytotoxic (Table I). As for cationized RNase As, the affinity for RI decreased with the increase in the extent of modification (K_is in Table I). This tendency explains why a lightly modified RNase A such as RNaseA-ED1 was less cytotoxic than a more moderately modified one.

The crystal structure of the complex of porcine RI and RNase A revealed regions in each protein that contribute to their strong association (*44*). The insensitivity of BS-RNase, and Onconase™, as well as RapLR1, to RI can be explained as follows. Frog RNases (Onconase™ and RapLR1) have substitutions at most residues corresponding to inhibitor binding residues in RNase A (*32*), and BS-RNase assumes a dimeric form that prevents inhibitor binding (*45*). Structural inspection of the complex of RNase A and RI (*44*) suggests that 3 (Asp38, Glu86, and Glu111) out of 11 carboxyl groups are mainly responsible for the decrease due to modification of the affinity of modified RNase As for RI.

Since the cytotoxicity of cationized RNase A is suggested to depend on the practical enzymatic activity in cytosol, as described above, we estimated this activity roughly under the conditions where cytotoxicity was determined. The con-

centration of RNase free from RI in the cytosol was assumed to be proportional to the value of $K_i/([RI] + K_i)$, where [RI] is the concentration of RI in the cytosol. Thus, the practical RNase activity of the respective cationized RNase A in cytosol was estimated to be proportional to the value of [RNase activity] $\times K_i/([RI] + K_i)$. The value of [RI] was assumed to be 1,000 nM (*41*), and the values were calculated and plotted in Fig. 3. A good correlation between this value and cytotoxicity was obvious, that is, moderately modified RNase As showed a higher practical RNase activity than either lightly or extensively modified RNase As. These data strongly suggest that the extent of RNA degradation caused by the internalized RNase determines the cytotoxicity of RNase.

On the basis of the results of the present study and other results obtained previously, we can postulate the following mechanism for the cytotoxicity of cationized RNases. First, cationized RNases internalize into the cytosol efficiently *via* adsorption to the anionic cell surface through electrostatic interaction; second, the cytotoxicity is caused by cellular RNA degradation that depends on the practical enzymatic activity in the presence of cytosolic RI. The optimum cationization to make RNase most cytotoxic should be the one that maintains as much enzymatic activity as possible under the cytosolic conditions, and this is the most important conclusion of this study. Other proposed factors for the cytotoxicity of RNase, such as proper translocation (*46, 47*) and conformational stability against proteolytic degradation (*34, 48*), will be examined further.

Over a decade ago, the methods for protein cationization to enhance cellular uptake by adsorptive-mediated endocytosis were developed (*22, 23, 49–56*). Recently, several small regions of proteins, called PTDs, have been identified to possess the ability to traverse cellular membranes (*24–30*). Although the mechanism is unknown (*27*), one of the characteristics of PTDs is that they generally consist of a Lys- or Arg-rich sequence of 10–16 amino acid residues. Consequently, the cationic properties of PTDs may be responsible for protein transduction. In the case of TAT-PTD fusion proteins, it has been demonstrated that partial or complete denaturation of the protein prior to delivery is required for efficient protein transduction (*24, 28*). As for the ethylenediamine-cationization method, denaturation of the protein is not required, although the side chain structure can be altered along with the chemical modification. Whether or not there is a difference in the mechanism by which a protein crosses the cellular membrane between a cationized protein and a PTD-fusion protein, the cationic properties seem to be commonly important.

Prospectus—The development of a method to deliver biologically active proteins freely into living cells *in vitro*, *in vivo*, or *ex vivo* would be immense importance in biological studies and medical applications of proteins. Because attempts to deliver proteins to intracellular targets have often failed due to poor cellular uptake, traditional small-drug molecules are emphasized in current drug design. Now, in the post-genomics era, the discovery of potential new protein therapeutics is anticipated than ever. Because of its simplicity and convenience, traditional chemical cationization for protein transduction into cells may be tested to screen the cellular functions of proteins. Understanding the mechanism of the cellular uptake of cationized proteins in detail would further improve the chemical modification techniques and greatly advance the potential use of proteins as both experimental and therapeutic tools. The findings of this study suggest that sophisticated chemical modification to retain maximum protein function is important for this cationization method. This principle suggests a useful starting point for the next cationization project to achieve the entry of a protein into a cell with retained function.

We thank Dr. Takahiro Hohsaka for the mass spectrometry measurements.

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