Electrical charge on protein regulates its absorption from the rat small intestine

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Received 10 August 2001; accepted in final form 12 December 2001

Electrical charge on protein regulates its absorption from the rat small intestine. Am J Physiol Gastrointest Liver Physiol 282: G711–G719, 2002. First published December 19, 2001; 10.1152/ajpgi.00358.2001.—The effect of the electrical charge on the intestinal absorption of a protein was studied in normal adult rats. Chicken egg lysozyme (Lyz), a basic protein with a molecular weight of 14,300, was selected and several techniques for chemical modification were applied. Then the intestinal absorption of Lyz derivatives was evaluated by measuring the radioactivity in plasma and tissues, after the administration of an 111In-labeled derivative to an in situ closed loop of the jejunum. After the administration of 111In-Lyz, the level of radioactivity in plasma was comparable with the lytic activity of Lyz, supporting the fact that the radioactivity represents intact Lyz. 111In-cationized Lyz showed a 2–3 times higher level of radioactivity in plasma, whereas the radioactivity of 111In-anionized Lyz was much lower. The absorption rate of 111In-Lyz derivatives calculated by a deconvolution method was correlated for the strength of their positive net charge. A similar relationship was observed using superoxide dismutase. These findings indicate that the intestinal absorption of a protein is, at least partially, determined by its electrical charge.

intestinal absorption; chicken egg lysozyme; pharmacokinetics; chemical modification; superoxide dismutase

INTestinal permeability is known to be altered in disease states such as Crohn’s disease, celiac disease, viral infection, and multiple organ failure (38, 42, 47). Under these abnormal conditions, not only low-molecular weight compounds but also macromolecules, such as proteins, can pass transcellularly or paracellularly through the intestinal epithelium and be absorbed into the circulation. Altered properties of the intestinal epithelial cells would explain the difference in permeability. Such changes lead to the increased nonspecific adsorption of proteins to the cell (42). Increased permeability of macromolecules is related to the symptoms observed in these diseases. However, in healthy adults, it is well known that the intestinal permeability of a solute through the intercellular junction (paracellular pathway) is highly dependent on its molecular size (6, 15). Molecules that hardly interact with intestinal tissue might exhibit a simple correlation between their molecular size and rate and extent of absorption. Therefore, the intestinal epithelium of healthy adults is generally considered to be virtually impermeable to macromolecules. In addition to this mechanical barrier presented by the tissue, the enzymatic barrier, i.e., a rapid and extensive degradation of proteins by digestive enzymes, highly restricts the entry of intact, undigested proteins into the body (42). However, some studies support the idea that there is a small, but significant, degree of transport of biologically and/or antigenically active peptides and proteins through the epithelial cells of the intestines (2, 5, 17, 25, 41). The mechanism of this transport through the intestines has received little attention to date.

Intestinal epithelial cells possess a negatively charged cell surface as do other types of cells. The charged surface provides sites of interaction for positively charged compounds. Because of this charge-based interaction, cationic macromolecules have been used to increase the delivery of drugs and genes to target cells (3, 4). Positive charges on the molecular surface can be electrostatically attracted and adsorbed to the negatively charged cell surface glycoproteins, followed by increased cellular uptake of the positively charged molecules. These findings suggest that electrostatic interaction of protein with the intestinal epithelial cells may be a factor determining its intestinal absorption. However, there are few investigations of the intestinal absorption of proteins that have considered the electrical charge. Proteins formulated in oral dosage forms include positively charged ones, such as lysozyme, bromelain, and pancreatopeptidase E. The cationic nature of these protein drugs could facilitate their interaction with intestinal tissue, resulting in detectable absorption from the intestine (5). The effect of the electrical charge of proteins on their intestinal absorption needs to be quantitatively investigated.

To this end, we chose chicken egg lysozyme (Lyz) as a model positively charged protein (isoelectric point of

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11) with a molecular weight of 14,300, because it can be absorbed from the intestines in small quantities (53, 54). Its electrical charge is altered by chemical modification, i.e., coupling with hexamethylenediamine or succinic anhydride to endow Lyz with an additional positive charge or negative charge, respectively. In addition, galactose or glucose can be covalently attached to Lyz to give glycosylated derivatives, because the intestinal epithelial cells possess glucose transporters, and some reports (23, 30) suggested their involvement in the enhanced absorption of glycosylated molecules. Pharmacokinetic profiles of these derivatives radiolabeled with $^{111}$In were studied in rats after intrajejunal administration or intravenous injection. The absorption rate was estimated by a deconvolution method using the profiles of the concentrations in plasma after intravenous and intrajejunal administration, and the relationship between the electrical charge of the protein derivatives and their absorption rate from the intestine was examined. In addition, to examine whether the relationship obtained can be applied to other proteins, we also report the altered intestinal absorption properties of recombinant human superoxide dismutase (SOD) that is different from Lyz in its physicochemical properties such as the electrical charge (negative, isoelectric point of ~5) and molecular weight (32,000) after cationization.

MATERIALS AND METHODS

Animals. Male Wistar rats (180–210 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Rats were fasted for 20 h before experimentation. All procedures were examined by the Ethics Committee on Animal Experimentation at Kyoto University.

Chemicals. Chicken egg Lyz and FITC were purchased from Sigma (St. Louis, MO). Recombinant human SOD (114Ser) was supplied by Asahi Chemical (Shizuoka, Japan). Diethylenetriaminepentaacetic acid (DTPA) anhydride was purchased from Dojindo Laboratory (Kumamoto, Japan). $^{111}$InCl$_3$ was supplied by Nihon Medi-Physics (Takarazuka, Japan). Micrococcus lysodeikticus was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were obtained commercially as reagent-grade products.

Synthesis of Lyz and SOD derivatives. Anionized Lyz (An-Lyz) was synthesized by succinylation (51), i.e., by reacting succinic anhydride with the amino group of Lyz. Coupling 1,6-hexamethylenediamine to Lyz was performed with 1-ethyl 3-(3-dimethylaminopropyl)carbodiimide to obtain highly positively charged Lyz [cationized Lyz (Cat-Lyz)] (49). Glucosylated (Glc-Lyz) and galactosylated Lyz (Gal-Lyz) were synthesized by reacting Lyz with 2-imino-2-methoxy-ethyl 1-thiogalactoside or thioglucoside, respectively, according to the method of Lee et al. (24). Highly negatively charged SOD [anionized SOD (An-SOD)] and cationized SOD (Cat-SOD) were synthesized by the same method as An-Lyz and Cat-Lyz, respectively.

The number of amino groups in each derivative was determined by trinitrobenzene sulfonic acid using glycine as a standard (11). The number of sugar residues was determined by the anthron-sulfuric acid method. The molecular weight of Lyz derivatives was estimated by SDS-PAGE using a standard curve obtained with marker proteins (low-range marker; Wako, Osaka, Japan), and that of SOD derivatives was estimated by HPLC gel-filtration chromatography using Shim-pack Diol-300 column (Shimadzu, Kyoto, Japan). Electrophoretic mobility of Lyz and SOD derivatives was measured with a laser electrophoresis-zeta potential analyzer (LEZA-500T; Otsuka Electronics). The lytic activity of Lyz derivatives was measured using M. lysodeikticus according to the method of Selsted and Martinez (45). Labeling. Lyz and SOD derivatives were radiolabeled with $^{111}$In using the bifunctional chelating agent, DTPA anhydride, according to the method of Hnatowich et al. (16). In brief, protein (2 mg) was dissolved in 1 ml 4-(2-hydroxyethyl)-1-piperazinethane sulfonic acid buffer (0.1 M, pH 7.0) and a twofold molar excess of DTPA anhydride in 10 µl dimethyl sulfoxide was added. After stirring for 30 min at room temperature, the mixture was purified by gel-filtration chromatography using a Sephadex G-25 column (1 × 40 cm) and eluted with acetate buffer (0.1 M, pH 6.0) to separate unreacted DTPA. Fractions containing DTPA-coupled protein were selected using spectrophotometry and concentrated by ultrafiltration. Thirty microliters $^{111}$InCl$_3$ solution was added to 50 µl sodium acetate buffer (1 M, pH 6.0), and 60 µl DTPA-protein derivative was then added to the mixture. After 30 min at room temperature, the mixture was purified by gel filtration chromatography using a PD-10 column (Amersham Pharmacia Biotech, Upsala, Sweden) and eluted with acetate buffer (0.1 M, pH 6.0). The appropriate fractions were selected based on their radioactivity and concentrated by ultrafiltration. The specific activity of the obtained samples was ~40 MBq/mg protein.

Separately, FITC was coupled to Lyz derivatives by the method of Monsigny et al. (31) for confocal fluorescence microscopic studies.

Biodistribution after intravenous injection. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). The urinary bladder and bile duct were cannulated for the collection of bile and urine samples. $^{111}$In-Lyz derivative was injected into the femoral vein at a dose of 0.1 mg/kg. At predetermined time points, blood, urine, and bile were collected for the entire experimental period (3 h). At the end of the rats, were killed, and the liver, kidney, and spleen were sampled. Blood samples were centrifuged at 2,000 g for 2 min, and 100 µl plasma was assayed for radioactivity.

Intestinal absorption from an in situ closed loop. Intestinal absorption of the test compound was examined in the in situ closed loop of the jejunum. A midline abdominal incision was made, and the lumen of the jejunum was washed with saline three times. A jejunal loop, 5 cm in length, was prepared by closing both ends with sutures. Each protein derivative was dissolved in 700 µl phosphate buffer (0.15 M, pH 6.5) and then administered into the jejunal loop at a dose of 1 or 10 mg/kg body wt. Blood, urine, and bile samples were collected for 3 h.

Degradation of $^{111}$In-Lyz derivatives in the loop was examined in different rats. At 30 min, 1 or 3 h after intrajejunal administration, the contents of the loop were subjected to gel-filtration chromatography on a Sephadex G-50 column (1 × 40 cm) and eluted with MES buffer (0.05 M, pH 6.0).

Determination of $^{111}$In radioactivity and lytic activity of Lyz derivatives. $^{111}$In radioactivity in each sample was measured in a well-type NaI scintillation counter (ARC-500, Pharmacia, Uppsala, Sweden) and eluted with acetate buffer (0.1 M, pH 6.0). The specific activities were selected based on their radioactivity and concentrated by ultrafiltration. The specific activity of the obtained samples was ~40 MBq/mg protein. 

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Determination of $^{111}$In radioactivity and lytic activity of Lyz derivatives. $^{111}$In radioactivity in each sample was measured in a well-type NaI scintillation counter (ARC-500, Aloka, Tokyo). Lytic activity of Lyz and Cat-Lyz in plasma was measured using M. lysodeikticus as described above.

Confocal microscopic images. FITC-Lyz derivatives were introduced into the loop in the same manner as in the in situ absorption experiment. At 1 h after administration, rats were killed and the loop was excised, washed with PBS, and frozen. Cryosections 10-µm thick were made using a cryostat.
(CM3000-Kryostat; Leica, Heidelberg, Germany) and fixed with 4% formaldehyde; the nucleus was stained with propidium iodide. Slices were scanned with a confocal laser microscope (ACAS 570 interactive laser cytometer; Meridian Instruments).

Estimation of amount absorbed by deconvolution method. Plasma concentration of $^{111}$In-protein after intrajejunal administration $[C_{po}(t)]$ is expressed as follows (39)

$$C_{po}(t) = \int_0^t f(\theta)C_{vo}(t-\theta)d\theta$$

where $f(\theta)$ is an absorption rate at time $t$ and $C_{vo}(t)$ is the plasma concentration at time $t$ after intravenous injection of an unit dose (an impulse input). When the amount of $f(\theta)\Delta \theta$ is rapidly injected to the systemic circulation at time $\theta$, plasma concentration associated with the pulse is $f(\theta)\Delta \theta C_{vo}(t-\theta)$ at time $t$. Equation 1 is derived, considering that an absorption rate-time profile is composed of an infinite number of the input pulses. Absorption profiles of $^{111}$In-protein were estimated by deconvoluting $C_{po}(t)$ with $C_{vo}(t)$ in Eq. 1 (20). In applying the computation algorithm (20), the plasma concentration-time profile of radioactivity after intravenous injection of $^{111}$In-protein was approximated with a biexponential equation.

Statistical analysis. Differences were statistically evaluated by one-way ANOVA followed by the Student-Newman-Keuls multiple comparison test. The level of significance was set at * $P < 0.05$ and ** $P < 0.01$.

RESULTS

Physicochemical properties of Lyz derivatives. The physicochemical characteristics of Lyz derivatives are summarized in Table 1. All synthesized Lyz derivatives had a similar molecular size to unmodified Lyz. The number of free amino groups in Lyz fell from 7.8 to 1.4 for An-Lyz and to 2.5 for Glc- and Gal-Lyz, whereas the number increased to 10.5 for Cat-Lyz. Lyz had an enzymatic activity of 100, whereas An-Lyz was 1.4, Glc-Lyz 15,000, and Gal-Lyz 2.5 for Glc- and Gal-Lyz, whereas Cat-Lyz was 10.2. The enzymatic activity was almost unchanged for Cat-, Glc-, and Gal-Lyz. However, succinylation of Lyz (An-Lyz) resulted in a complete loss of enzymatic activity, suggesting the cationic charge of Lyz is critical for its lytic activity.

These determinations confirmed that Cat-Lyz is a highly cationic derivative of Lyz, whereas An-Lyz is an anionic derivative. Glycosylation slightly reduced the positive charge of Lyz. Lytic activity was almost unchanged for Cat-, Glc-, and Gal-Lyz. However, succinylation of Lyz (An-Lyz) resulted in a complete loss of enzymatic activity, suggesting the cationic charge of Lyz is critical for its lytic activity.

Dispersion of $^{111}$In-Lyz derivatives after intravenous injection. The tissue disposition of $^{111}$In-Lyz derivatives was examined after intravenous bolus injection into rats. Figure 1A shows the plasma concentration-time profile of radioactivity after intravenous injection at a dose of 0.1 mg/kg into rats. $^{111}$In-Lyz rapidly disappeared from plasma. Chemical modification of Lyz slightly altered the elimination rate from plasma and had a marked effect on the tissue disposition of $^{111}$In-Lyz (Fig. 1, B–F). $^{111}$In-Lyz was recovered largely in the kidneys (53% of dose) and urine (20%) but not in the liver (1.9%), reflecting its high susceptibility to glomerular filtration and reabsorption (18, 26). $^{111}$In-An-Lyz was largely excreted into urine instead of accumulating in the kidney, whereas $^{111}$In-Cat-Lyz was recovered in both the liver (31%) and kidney (54%). $^{111}$In-Gal-Lyz and $^{111}$In-Glc-Lyz also showed high hepatic recovery in addition to recovery in the kidney and urine, suggesting their recognition by the asialoglycoprotein receptors on hepatocyte (34).

Tissue disposition of $^{111}$In-Lyz after intrajejunal administration. $^{111}$In-Lyz was administered into the jejunal loop, and the contents of the loop were applied to a column to check the molecular size of $^{111}$In-Lyz. $^{111}$In-Lyz recovered at 0.5 and 1 h after its administration into the jejunal loop showed a similar chromatographic profile to that of intact $^{111}$In-Lyz. At the end of the 3-h experiment, ~3% of the radioactivity was eluted in fractions different from those of $^{111}$In-Lyz (data not shown).

Figure 2A shows the plasma concentration-time profile of radioactivity after intrajejunal administration of $^{111}$In-Lyz derivatives at a dose of 1 mg/kg. Radioactivity was detected in plasma after intrajejunal administration of $^{111}$In-Lyz, indicating that $^{111}$In-Lyz can be

Table 1. Physicochemical properties of Lyz and SOD derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Weight</th>
<th>Number of amino (Sugar) Groups</th>
<th>Enzymatic Activity, % with respect to original</th>
<th>Electrophoretic Mobility at pH 6.5, $10^{-4}$ cm$^2$V$^{-1}$s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyz</td>
<td>14,300</td>
<td>7.8</td>
<td>100</td>
<td>0.137 ± 0.058</td>
</tr>
<tr>
<td>An-Lyz</td>
<td>14,000</td>
<td>1.4</td>
<td>ND</td>
<td>-0.517 ± 0.169</td>
</tr>
<tr>
<td>Cat-Lyz</td>
<td>15,000</td>
<td>10.5</td>
<td>100</td>
<td>0.556 ± 0.060</td>
</tr>
<tr>
<td>Glc-Lyz</td>
<td>15,000</td>
<td>2.5(4.3)</td>
<td>105</td>
<td>0.052 ± 0.021</td>
</tr>
<tr>
<td>Gal-Lyz</td>
<td>15,000</td>
<td>2.5(4.4)</td>
<td>106</td>
<td>0.048 ± 0.009</td>
</tr>
<tr>
<td>SOD</td>
<td>32,000</td>
<td>24</td>
<td>100</td>
<td>-0.461 ± 0.025</td>
</tr>
<tr>
<td>Cat-SOD</td>
<td>34,000</td>
<td>35</td>
<td>47</td>
<td>0.106 ± 0.056</td>
</tr>
</tbody>
</table>

The molecular weight of lysozyme (Lyz) and superoxide dimutase (SOD) derivatives was estimated by SDS-PAGE or by HPLC gel chromatography, respectively. The number of amino groups was measured with trinitrobenzene sulfonic acid using glycine as a standard (11). The number of sugar residues was determined by the anthron-sulfuric acid method. Enzymatic activity of Lyz was measured using Micrococcus lysiodeikiticus (45), and that of SOD was determined by a nitroblue tetrazolium reduction method. Electrophoretic mobility was measured using a laser electrophoresis-zeta potential analyzer (model LEZA-500T, Otsuka Electronics). An, anionized; Cat, cationized; Glc, glucosylated; Gal, galactosylated; ND, not detected.

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absorbed from the intestine. Compared with $^{111}$In-Lyz, $^{111}$In-Cat-Lyz showed a two- to threefold higher level of radioactivity in plasma ($P < 0.05$ at 2 h, $P < 0.01$ at 1 and 3 h). On the other hand, $^{111}$In-An-Lyz showed much less radioactivity, although the difference was not significant. The plasma level of radioactivity after the administration of the $^{111}$In-glycosylated Lyz derivatives was a little lower than that of $^{111}$In-Lyz. The tissue disposition profiles of radioactivity after intrajejunal administration of the $^{111}$In-Lyz derivatives were similar to those obtained after administration by the intravenous route (Fig. 2, B–F). These tissue disposition data after intrajejunal administration suggest that Lyz derivatives retain their structural characteristics even after passage through the intestinal epithelial cells into the blood circulation.

When the tissue disposition was normalized with respect to the dose (%dose or %dose/ml), no significant differences were observed in the tissue disposition of radioactivity after intrajejunal administration of $^{111}$In-Lyz or $^{111}$In-Cat-Lyz at a dose of 1 and 10 mg/kg (Fig. 3). For both derivatives, the disposition profiles at the 10 mg/kg dose could be superimposed on those obtained at the 1 mg/kg dose, indicating that the intestinal absorption of these Lyz derivatives is proportional to the dose over the range examined.

To see whether the absorbed fraction of $^{111}$In-Lyz remains intact, the lytic activity of Lyz in plasma after intrajejunal administration of (nonradiolabeled) Lyz was examined at a dose of 10 mg/kg. The concentration of lytic activity in plasma was comparable with, and not significantly different from, the concentration of $^{111}$In radioactivity after $^{111}$In-Lyz administration, after their normalization to the administration dose (Fig. 4). These results suggest that the radioactivity in plasma after administration of $^{111}$In-Lyz is due to intact Lyz.
Confocal microscopic images of rat jejunum after administration of FITC-Lyz derivatives. Figure 5 shows the confocal microscopic images of rat jejunum cryosections after intrajejunal administration of FITC-Cat-Lyz. Green fluorescence derived from FITC-Cat-Lyz was mainly observed around the luminal surface of the tissue. In addition, fluorescence could be detected near the nucleus of the epithelial cells. Fluorescent intensity associated with the intestinal tissue depended on the electrical charge of the Lyz derivative, and FITC-An-Lyz had a much weaker signal (data not shown).

Intestinal absorption rate of 111In-Lyz derivatives calculated by deconvolution method. Figure 6 shows the intestinal absorption-time profiles after intravenous and intrajejunal administration. 111In-Lyz derivatives were linearly absorbed from the jejunum with time. The amount of 111In-Cat-Lyz absorbed was significantly greater than that of 111In-Lyz (P < 0.05 at 1, 2, and 3 h), which resulted in a greater absorption rate for 111In-Cat-Lyz (0.46% dose/h) (Table 2). On the other hand, the absorption of 111In-An-Lyz was much slower than that of the cationic derivatives, although the difference was not significant. The rates of 111In-Gal-Lyz and 111In-Glc-Lyz were calculated and found to be comparable with that of 111In-Lyz, indicating that glycosylation has no effect on the intestinal absorption of Lyz.

Intestinal absorption of 111In-SOD derivatives. Table 1 shows the physicochemical characteristics of SOD and Cat-SOD. After intrajejunal administration of 111In-SOD and 111In-Cat-SOD, low but significant radioactivity was detected in plasma (data not shown). However, in the case of 111In-An-SOD, there was no detectable radioactivity in plasma throughout the experiment that lasted 3 h. Figure 7 shows the intestinal absorption-time courses of 111In-SOD and 111In-Cat-SOD. Although 111In-Cat-SOD tended to be more absorbed from the intestine than 111In-SOD, the amount absorbed was not significantly different except for the first time point (P < 0.05). The absorption rate calculated for 111In-Cat-SOD was twofold greater than that for 111In-SOD (Table 2), but these values were less than those exhibited by the 111In-Lyz derivatives, probably reflecting the differences in the properties of SOD and Lyz, e.g., the molecular size (32,000 SOD; 14,300 Lyz).

DISCUSSION

Cationization is a universal approach applied to increase the interaction of compounds with negatively charged biological components. Since Felgner (8) reported efficient gene expression using cationic lipids for the transfection of cells with plasmid DNA, cationic molecule-based delivery systems for plasmid DNA have been extensively investigated in an attempt to achieve nonviral gene transfer to target cells (33). In addition, enzymes such as SOD (27, 28, 40, 48), glucose
oxidase (21), and catalase (21, 43), as well as serum albumins (32), immunoglobulins (52), and ferritin (7), all of which are negatively charged at physiological pH, have been directly modified with diamines to obtain cationized derivatives. Cationized proteins exhibit increased cellular uptake by brain microvascular endothelial cells, hepatocytes, kidney epithelial cells, and enterocytes. On interaction with the negatively charged surface of cells, cationized proteins are believed to be endocytosed or transcytosed through an adsorptive endocytosis/transcytosis process. However, to our knowledge, there have been few studies to examine the effect of the electrical charge of a protein on its intestinal absorption. Intestinal epithelial cells possess a negatively charged surface like other cells. Therefore, in the present study, the effect of the physicochemical properties, especially the electrical charge, of the protein on its intestinal absorption was explored using an in situ closed loop of rat jejunum. In this system, the pH of the solution in the loop could hardly change from the initial value of pH 6.5 during experiment, and the local luminal pH might not be so different from the pH value of the bulk solution (22).

A reliable detection method is required to evaluate the intestinal absorption of proteins. We carried out 111In labeling using DTPA anhydride to monitor the disposition of proteins because of the better stability of these radiolabeled proteins compared with their iodinated counterparts (13, 36). A possible radioactive metabolite, 111In-DTPA-lysine (9), has only a limited capacity to cross biological membranes and to escape from cells where the labeled protein is degraded after endocytosis. When Lyz was radiiodinated and administered to the rat jejunal loop, the concentration of trichloroacetic acid-precipitable 125I radioactivity in plasma was higher than that of 111In radioactivity after the administration of 111In-Lyz (data not shown). When radiiodinated Lyz was used for experiments, the concentration of radioactivity in plasma was much greater than one obtained with 111In counterpart (P < 0.01). Finding no significant differences between the lytic activity of Lyz and 111In radioactivity strongly supports the idea that the transport of intact Lyz derivatives through the intestinal epithelium can be assayed by monitoring 111In radioactivity. These considerations suggest that radiiodination might overes-

Table 2. Intestinal absorption rate of 111In-Lyz and 111In-SOD derivatives following intrajejunal administration to rats calculated by a deconvolution method

<table>
<thead>
<tr>
<th>Compound</th>
<th>Absorption rate, % of dose/h</th>
<th>Apparent permeability coefficient × 10⁻⁷ (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyz</td>
<td>0.184 ± 0.002</td>
<td>0.539</td>
</tr>
<tr>
<td>An-Lyz</td>
<td>0.065 ± 0.006</td>
<td>0.191</td>
</tr>
<tr>
<td>Cat-Lyz</td>
<td>0.462 ± 0.009</td>
<td>1.35</td>
</tr>
<tr>
<td>Glc-Lyz</td>
<td>0.165 ± 0.008</td>
<td>0.484</td>
</tr>
<tr>
<td>Gal-Lyz</td>
<td>0.187 ± 0.002</td>
<td>0.548</td>
</tr>
<tr>
<td>SOD</td>
<td>0.025 ± 0.002</td>
<td>0.0733</td>
</tr>
<tr>
<td>Cat-SOD</td>
<td>0.056 ± 0.003</td>
<td>0.164</td>
</tr>
</tbody>
</table>

Results are expressed as the means ± SD. The apparent permeability coefficient is calculated by dividing the estimated absorption rate by the area of exposed intestine (6.63 cm²) and the initial concentration (100% of dose/0.7 ml).
timate the intestinal absorption of proteins, although the reason for the discrepancy needs to be understood.

Chemical modification greatly changed the tissue disposition characteristics of Lyz after intravenous injection. As summarized in our reviews (14, 46, 50), the tissue disposition of macromolecules is determined mainly by the overall physicochemical properties, such as the electrical charge and molecular weight, as well as by the structure involved in the specific recognition, such as monoclonal antibody, lectin, and sugar. Macromolecules having a molecular size smaller than the threshold of the glomerular filtration of the kidney are easily filtered and then reabsorbed at the proximal tubules depending on their property (26). This is the case for Lyz and SOD, and the cationic nature of Lyz increases the susceptibility to glomerular filtration and reabsorption. When $^{111}$In-Lyz was injected intravenously, the radioactivity was mainly recovered in the kidney and urine (Fig. 2). $^{111}$In-Cat-Lyz, which has about fourfold greater mobility to the negative pole than Lyz, showed a relatively high accumulation in the liver, like other cationic proteins, such as cationized bovine serum albumin (32). No detectable oligomers formed during the cationization were found during SDS-PAGE (data not shown). On the other hand, succinylation of Lyz greatly altered the balance of radioactivity in the kidney and urine. Because the renal uptake of macromolecules occurs mainly from the luminal side of epithelial cells (26) and not from the capillary side, such changes indicate that the reabsorption of Lyz is inhibited by succinylation. Both $^{111}$In-glycosylated Lyz derivatives showed some hepatic uptake after systemic administration. Hepatocytes are known to possess asialoglycoprotein receptors on their surface that recognize compounds having galactose or N-acetylgalactosamine at the nonreducing end of the sugar chain (1). Synthetically galactosylated macromolecules are ligands for the receptor (34), and this is the reason for the high hepatic uptake of $^{111}$In-Gal-Lyz. Although asialoglycoprotein receptors have a much lower affinity for glucose than galactose, it binds to proteins modified with 2-imino-2-methoxyethyl 1-thio-glucoside as used in the present study. Therefore, asialoglycoprotein receptors are involved in the hepatic uptake of $^{111}$In-Glc-Lyz (34). Although the imidination used in this study to synthesize glycosylated Lyz has been reported not to alter the electrical charge of the protein (24), their electrophoretic mobility was a little lower than that of Lyz, suggesting a reduced positive charge after glycosylation. These glycosylated derivatives were prepared because: 1) these modified derivatives possess a positive charge intermediate between unmodified Lyz and An-Lyz and 2) some reports have suggested the involvement of specific mechanisms for sugars associated with enterocytes as far as the transport of glycosylated macromolecules is concerned (12, 23).

The tissue disposition profile of radioactivity after intrajejunal administration of each $^{111}$In-Lyz derivative was comparable with that after intravenous administration (Figs. 1 and 2), suggesting that Lyz derivatives entering the systemic circulation from the intestine possess unique physicochemical properties. Tissue uptake clearances, which can be calculated based on the area under the plasma concentration-time curve and the amount in tissue (35), were also comparable after intravenous and intrajejunal administration (data not shown). These results indicate that the radioactive molecules appearing in the plasma maintain the structures that determine their tissue disposition.

There have been reports showing that some proteins can be absorbed from the intestinal tract, although

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**Fig. 7.** Intestinal absorption time courses of $^{111}$In-SOD and $^{111}$In-Cat-SOD calculated by a deconvolution method. ○, $^{111}$In-SOD; □, $^{111}$In-Cat-SOD. *P < 0.05, statistically significant difference from $^{111}$In-Lyz. SOD, superoxide dismutase.

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**Fig. 8.** Dependence of the intestinal absorption rate of $^{111}$In-Lyz and $^{111}$In-SOD derivatives on the electrophoretic mobility measured by a zeta sizer. ●, $^{111}$In-Lyz derivatives; ○, $^{111}$In-SOD derivatives. The absorption rate of a derivative calculated by the deconvolution method is plotted against its electrophoretic mobility.
skepticism about the experimental evidence of transmucosal absorption of proteins is common because of the limitations of the methodology used (44). Recently, Castell et al. (5) clearly showed that bromelain, a basic protein with a molecular mass of 24–26 kDa isolated from the stem of the pineapple plant, can be absorbed from the intestinal tract of healthy volunteers as an immunoreactive form of unchanged molecular mass. The consistency of their results indicates that the absorption of a certain protein molecule from the gastrointestinal tract is probably a common phenomenon in healthy adults. As discussed above, the cationic nature of this protein could help its intestinal absorption, although the route of its absorption, i.e., transcellular or paracellular, needs to be identified.

Cationization of Lyz did increase the intestinal absorption of Lyz, probably through the increase in the isoelectrical point of the enzyme whose positive charges result in an electrostatic attraction to the negatively charged proteoglycans of the cell surface, a process that can lead to adherence to the oppositely charged surfaces (4). Interaction of proteins with the intestinal tissues was facilitated by cationization (21, 40). Because binding to the surface can be considered as the first step in the intestinal transport of proteins, cationization might be one possible approach to increase the permeability of proteins. Increased binding of Cat-Lyz was detected in the specimens of intestinal tissues treated with FITC-labeled Lyz derivatives.

Recently, glycosylation has been applied to peptides to increase their transport across the intestinal tissues via a Na+/glucose cotransporter (30), as well as to produce increased stability to peptidases (29). Although some reports have suggested involvement of these mechanisms in the transport of glycosylated macromolecules (12, 23), no improved absorption was observed for glycosylated Lyz derivatives. Kim et al. (19) reported that bile acid-conjugated small peptides could bind to intestinal bile acid transporters without being transported.

The amount of 111In-Cat-Lyz absorbed was significantly greater than that of 111In-Lyz (P < 0.05 at 1, 2, and 3 h), whereas that of 111In-An-Lyz was smaller. The absorption rate of Lyz derivatives was proportional to their electrophoretic mobility (Fig. 8). These results clearly indicate that the electrical charge of Lyz determines its intestinal absorption from the jejunal loop. The apparent permeability coefficient of 111In-Cat-Lyz (0.135 × 10−6 cm/s, Table 2), however, was still much smaller than that of small molecules such as a vasopressin derivative (43 × 10−6 cm/s, molecular weight of 1,069) and inulin (4 × 10−6 cm/s, molecular weight of 5,200) (37). The relationship between the electrical charge and intestinal absorption could be applied to other proteins such as SOD. 111In-Cat-SOD tended to be absorbed faster than 111In-SOD, although the amount absorbed was not significantly different at later time points. With these protein derivatives, it is quite obvious that the molecular weight is a very important factor determining their intestinal absorption. Although 111In-Cat-SOD had a greater electrophoretic mobility to the negative pole than 111In-Lyz, its absorption rate was smaller than that of 111In-Lyz, showing the importance of the molecular weight of the protein as far as its intestinal absorption is concerned. It is difficult to conclude that these protein derivatives are absorbed through the transcellular or paracellular route. The absorption through the both routes could be enhanced by cationization (10, 42). Further studies are needed to clarify the contribution of each route to the intestinal absorption of the derivatives.

In conclusion, it has been shown that the intestinal absorption of a protein is regulated by its electrical charge, if the molecular size of protein is not altered. Enhanced adsorption of a cationic derivative to the surface of the tissue would result in its increased permeability through the barrier. These findings provide useful information about the intestinal absorption of proteins of immunological and/or pharmacological importance in normal, healthy subjects.

This work is supported, in part, by a grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES


