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Analysis of the molecular interaction between mannosylated proteins and serum mannan-binding lectins

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Abstract

The kinetics and specificity of the molecular interaction between proteins modified with varying numbers of mannose residues and isolated rabbit mannan-binding lectin (MBL) were characterized by using surface plasmon resonance spectroscopy (SPR). Mannosylated bovine serum albumin (Man-BSA) with different numbers of mannoses and other mannosylated derivatives of lysozyme (LZM), soybean trypsin inhibitor (STI), superoxide dismutase (SOD) and bovine γ-immunoglobulin (IgG) were synthesized. Rabbit MBL was isolated by affinity column chromatography and immobilized on the SPR sensor chip via avidin–biotin binding. Binding of Man-BSAs to immobilized rabbit MBL increased with an increase in the number of mannose residues, primarily due to the reduction in dissociation rate. On the other hand, the association rate constant was similar for five mannosylated proteins investigated, whereas the dissociation rate constant differed markedly in spite of the same degree of mannosylation. Specific binding of mannosylated proteins to MBL may depend on the number of mannose residues and their steric configurations.

Keywords: Serum mannan-binding lectins; Surface plasmon resonance; Mannosylated proteins; Intermolecular interaction

1. Introduction

Serum mannan-binding lectin (MBL) is a large oligomeric serum protein of hepatic origin, which belongs to the family of Ca^{2+}-dependent collagenous lectins. MBL plays an important role in the innate immune system (Holmskov et al., 1994), which can activate the complement system in an antibody and C1-independent manner when being associated with microbial surfaces (Ikeda et al., 1987; Matsushita, 1996). MBL can bind to a variety of pathogens by recognizing D-mannose, *N*-acetylglucosamine or L-fucose on their surface (Kawasaki et al., 1989). Besides these pathogens, MBL is known to interact with endogenous glycoproteins. Taylor and Summerfield (1984) who isolated human MBL from the serum found that MBL inhibits the in vitro uptake of glycoproteins by hepatic mannose receptors.

Much attention has been paid to mannosylation of biologically active proteins for targeted delivery to liver non-parenchymal cells (NPC) that are involved in various diseases of the liver (Wheeler, 2003; Rymsa et al., 1991). Mannosylated proteins are believed to be effectively taken up via mannose receptors expressed in NPC such as Kupffer cells and liver endothelial cells. Our previous studies demonstrated that mannosylated catalase (Man-CAT) is a promising approach to prevent the initial phase of hepatic ischemia/reperfusion injury (Yabe et al., 1999, 2002). Besides, mannosylated human serum albumin (Man-HSA) was found to be an effective carrier of an immunoactivator muramyl dipeptide in activating macrophage and enabled eradiation of lung metastases in mice (Sarkar and Das, 1997). In our recent studies, however, we found that mannosylated proteins exhibited a biphasic dose-dependent pharmacokinetic profiles: hepatic uptake of mannosylated bovine serum albumin (Man-BSA) and mannosylated immunoglobulin G (Man-IgG) was retarded not only at high dose but at very low dose (Opanasopit et al., 2001). The peculiar dose-dependence on pharmacokinetics of mannosylated ligands can be well explained by the binding of the ligands to MBL in the serum. However, binding characteristics between mannosylated ligands and MBL remained to be elucidated.

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The recent availability of optical biosensors, such as surface plasmon resonance (SPR) spectroscopy, allows for real-time detection of macromolecular interactions and determination of binding kinetics and affinities without using radioactive or fluorescent labeled ligands. This method has been widely used as a tool to study biomolecular interactions including receptor–ligand (Wu and Chaiken, 2004), DNA–protein (Wegner et al., 2003; Schubert et al., 2003) and protein–protein interactions (Stenlund et al., 2003). In this study, we have used SPR spectroscopy to analyze the molecular interaction involving rabbit serum MBL. To assess multivalent binding characteristics of MBL, we prepared Man-BSA with a different number of mannosylations. Furthermore, to clarify the binding characteristics, we synthesized mannosylated lysozyme (Man-LZM), mannosylated soybean trypsin inhibitor (Man-STI), mannosylated superoxide dismutase (Man-SOD), Man-BSA and Man-IgG, in which the degree of modification was similar. Following isolation and immobilization onto an SPR sensor chip of rabbit serum MBL, we evaluated the association and dissociation kinetics of each mannosylated protein with the lectin. We demonstrate that the specific binding of mannosylated proteins to MBL was due to multivalent interactions, and affected by the number of mannose residues and their configuration on the surface of proteins.

2. Materials and methods

2.1. Materials

BSA (fraction V) and IgG were purchased from Sigma (St. Louis, MO, USA). Recombinant human SOD was supplied by Asahi Kasei (Tokyo, Japan). D-Mannose was obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were of the highest grade available.

2.2. Synthesis of glycosylated proteins

Coupling of mannose moieties to protein was carried out by the method of Lee et al. (1976). Briefly, cyanomethyl 2,3,4,6-tetra-*O*-acetyl-1-thiomannoside was prepared from the pseudothiourea derivative and chloroacetonitrile. The nitrile group in these cyanomethyl thioglycosides can be converted to a methyl imidate group by treatment with sodium methoxide in dry methanol to yield 2-imino-2-methoxyethyl 1-thioglycosides. Cyanomethyl 1-thiomannoside was treated with 0.01 M sodium methoxide at room temperature for 24 h, and a syrup of 2-imino-2-methoxyethyl-1-thiomannoside was obtained after evaporation of the solvent. A quantity of the resultant syrup was added to protein in borate buffer (pH 9.5). The number of mannose residues per protein molecule was controlled by the molar ratio of the starting reagents. After 24 h at room temperature, the reaction mixture was dialyzed to remove any unreacted compound and then lyophilized. The characteristics of synthetic mannosylated proteins with different sugar densities are summarized in Table 1. The apparent molecular mass of the mannosylated proteins was estimated by SDS-PAGE. The number of mannose residues was determined by calculating the mannose content of the mannosylated protein solution using the anthrone-sulfuric acid method (Gray, 1974). The protein content was calculated by subtracting the weight of mannose from that of mannosylated protein. The final number of mannose residues was obtained by dividing the molar amount of mannose by the molar amount of protein.

Table 1
Characteristics of mannosylated proteins used in this study

Compound	Molecular weight	Mannose residue (mol/mol)	Mannose content (w/w, %)
$Man_{9.8}$-BSA	67000	9.8	2.63
Man_{16}-BSA	68400	16.2	4.26
Man_{23}-BSA	68400	22.8	6.00
Man_{37}-BSA	69800	36.9	9.52
$Man_{5.6}$-LZM	14900	5.6	6.77
$Man_{9.3}$-STI	20800	9.3	8.05
Man_{15}-SOD	40000	14.8	6.66
Man_{28}-BSA	69000	28.5	7.43
Man_{70}-IgG	174600	69.7	7.19

2.3. Isolation of MBL from rabbit serum

MBL was isolated from normal rabbit serum (Japan Biosupply) using a Sepharose-Man-IgG column according to the procedure reported by Kawasaki et al. (1989). Briefly, Man-IgG was coupled to *N*-hydroxysuccinimide (NHS)-activated Sepharose 4 Fast Flow (Pharmacia Biotech, Uppsala, Sweden) according to the instructions of the manufacturer. The rabbit serum was diluted with an equal volume of a buffer consisting of 40 mM imidazole-HCl, pH 7.8, 40 mM $CaCl_2$ and 2.5 M NaCl. The mixture was applied to a Sepharose-mannan or Sepharose-Man-IgG column, which had been equilibrated with loading buffer (20 mM imidazole-HCl, pH 7.8, 20 mM $CaCl_2$ and 1.25 M NaCl). The binding protein was eluted with an elution buffer (20 mM imidazole-HCl, pH 7.8, 1.25 M NaCl and 2 mM EDTA) and the eluate was applied to a second and third smaller affinity column. The final column was washed with the loading buffer and eluted with more loading buffer containing 100 mM mannose. All procedures were carried out at 4 °C. The isolated protein was subjected to SDS-PAGE (10%, w/v acrylamide) under reducing and nonreducing conditions by the method of Laemmli (1970). Molecular masses were estimated by comparison with Rainbow marker proteins (Amersham Biosciences, Uppsala, Sweden).

2.4. Biotinylation of rabbit serum MBL

Rabbit serum MBL was dissolved at a concentration of 5 mg/ml in a loading buffer containing 100 mM mannose. NHS-biotin was dissolved in DMSO and then added to the rabbit serum MBL solution in a molar ratio of 1:10 (rabbit serum MBL:NHS-biotin). The mixture was incubated on ice for 4 h. Unreacted compound was removed using CentriconYM-30 (Millipore, Bedford, USA). The apparent biotinylation ratio was determined by reacting biotinylated MBL with an excess of 2-(4′-hydroxyphenyl)-azobenzoic acid (HABA) and avidin (Pierce Chemical Co, Rockford, USA) by the method of Green (1970).

As HABA bound to avidin is displaced by biotin-MBL, the 500 nm absorbance associated with HABA bound to avidin is reduced.

2.5. Real-time SPR spectroscopy

SPR measurements were performed using a BIAcore X system and a sensor chip SA (BIAcore, Uppsala, Sweden). The sensor chip consists of a gold surface to which a carboxymethylated dextran layer is bound. The dextran layer is activated with streptavidin by the manufacturer. Prior to the experiment, the streptavidin surface was first cleaned three times with a solution of 50 mM NaOH in 1 M NaCl, each cleaning lasting 1 min. Biotinylated rabbit serum MBL was injected manually to a single flow cell at a flow rate of 5 μl/min until a resonance unit value of approximately 2000 was obtained. An untreated sensor chip SA with MBL was mounted onto a blank flow cell to monitor the background level of the refractive index. Each mannosylated protein was diluted in a running buffer (300 mM NaCl, 40 mM $CaCl_2$, 10 mM HEPES and pH 7.4) at a flow rate of 20 μl/min at 25 °C for 3 min. Each concentration was separated by an interval of 3 min and regeneration was carried out by injection of 20 μl 20 mM EDTA (pH7.4). All buffers were filtered and deoxygenated. Repeated experiments indicated that the regeneration procedure using 20 mM EDTA did not change binding characteristics of ligand proteins. The resulting curves were transformed to concentration units using the molecular mass of the injected species, the equivalence of 1000 RU per 1 ng/mm^2, and a matrix thickness of 100 nm. Each sensorgram was then analyzed by a global fitting procedure using a 1:1 (Langmuir-type) binding model-derived equation that is available in the BIAevaluation 3.0 software. The association constant (K_a) was determined from k_a/k_d. It was assumed that the flow in the cell was sufficiently high to prevent any depletion of mannosylated proteins in solution and that its concentration remained constant.

3. Results

3.1. Specificity of the binding of mannosylated proteins to immobilized rabbit serum MBL

The proteins isolated from rabbit serum according to the above-mentioned procedure were subjected to SDS-PAGE (data not shown). Only a single band (~30 kDa) was detected under reducing conditions, whereas several high-molecular mass bands were seen under nonreducing conditions. These observations were consistent with the reports of Kawasaki et al. (1989) who first isolated a rabbit serum MBL. The isolated rabbit serum MBL was biotinylated using NHS-biotin in the presence of excess mannose that protects amino residues in the carbohydrate recognition domain from being coupled with the biotinylation agent. The apparent biotinylation ratio of the protein was approximately 1.3 mol/mol and this was determined by reaction with avidin bound HABA reagent [2-(4′-hydroxyphenyl) azobenzoic acid]. Biotinylated rabbit serum MBL was immobilized onto the surface of a sensor chip SA coated with streptavidin. In the SPR spectroscopic investigation, nonspecific binding of an analyte and any change in bulk refractive index change were ruled out by using a native sensor chip SA as a control. The ability of the immobilized rabbit serum MBL to interact with mannosylated proteins was investigated using Man_{16}-BSA and native BSA. As illustrated in Fig. 1, Man_{16}-BSA readily bound to immobilized rabbit serum MBL in the presence of Ca^{2+}, as shown by the increase in RU during the association phase of the sensorgram. Bound Man_{16}-BSA could be completely eluted at the end of dissociation phase by a pulse injection of EDTA. In contrast, native BSA showed no ability to bind to rabbit serum MBL immobilized onto the surface of a sensor chip. These results indicate that immobilization of biotinylated MBL do not abolish the binding capacity of MBL. Further investigations were conducted by a competition experiment using mannose which is a known ligand of MBL. The positive response of Man_{16}-BSA was reduced in the presence of mannose, suggesting that the binding of Man-BSA to rabbit serum MBL was inhibited by mannose. Thus, these SPR results suggest that the interaction between mannosylated proteins and rabbit serum MBL occurs in a specific manner.

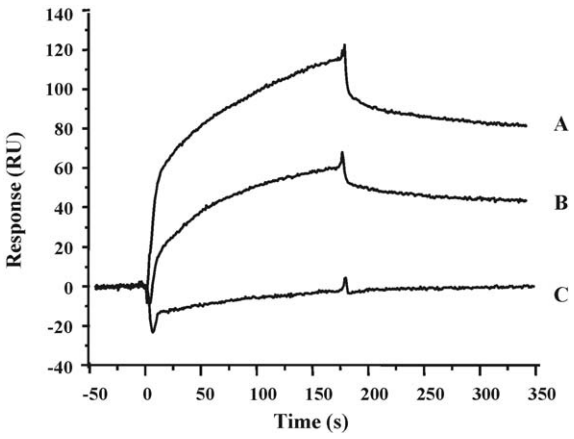

Fig. 1. Specificity of the binding of mannosylated proteins to rabbit serum MBL immobilized on SPR sensor chip. 0.8 μM Man_{16}-BSA (A), 0.8 μM Man_{16}-BSA in presence of 0.8 mM mannose (B) and 100 μg/ml BSA (C) were injected over immobilized rabbit MBL. Each SPR sensorgram was overlaid and zeroed on the *y*-axis to the average baseline. The start injection time for each sample was set to zero on the *x*-axis.

3.2. Effect of numbers of mannose residues on the interaction between Man-BSAs and rabbit serum MBL

The binding patterns of $Man_{9.8}$-, Man_{16}-, Man_{23}- and Man_{37}-BSA to rabbit serum MBL were studied using SPR spectroscopy (Fig. 2). These experiments were performed on the same day, confirming that the binding activity of rabbit MBL remained constant throughout the entire experiment. Various concentrations (0.1, 0.2, 0.4, 0.6 and 0.8 μM) of Man-BSAs were infused into the system. As the number of mannose residues of Man-BSAs increased, the SPR response signal also increased (Fig. 2). Furthermore, in the sensorgrams of Man_{23}- and Man_{37}-BSA, the change in response with their concentration was smaller, suggesting that the binding of these

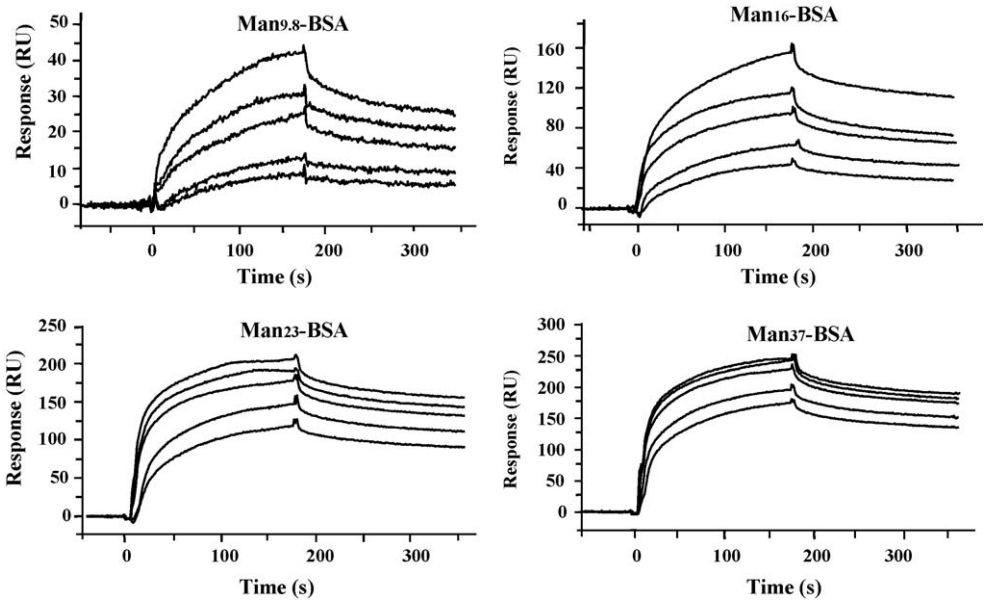

Fig. 2. Effect of numbers of mannose residues on the interaction between Man-BSAs and rabbit serum MBL. Increasing concentrations (0.1, 0.2, 0.4, 0.6 and 0.8 μM) of Man-BSAs were injected over immobilized rabbit MBL.

BSA derivatives to MBL was saturated at relatively lower concentrations.

3.3. Interaction of various mannosylated proteins with rabbit serum MBL

The binding patterns of $Man_{5.6}$-LZM, $Man_{9.3}$-STI, Man_{15}-SOD, Man_{28}-BSA and Man_{70}-IgG to rabbit serum MBL were studied using SPR spectroscopy in experiments in which the degree of modification was similar (Fig. 3). The concentrations of mannosylated proteins infused into the system were adjusted on a mass basis (10, 20, 40, 60 and 80 μg/ml), in order to keep the apparent concentration of mannose units consistent. It should be noted that the mannosylated proteins were designed to have the same mannose content per molecular weight. The sensorgrams of Man-IgG showed the highest response and the slowest dissociation from rabbit serum MBL of all the proteins investigated. The dissociation of Man-SOD was much faster than that of any other mannosylated protein. On the other hand, Man-STI exhibited a higher response in spite of having a smaller molecular

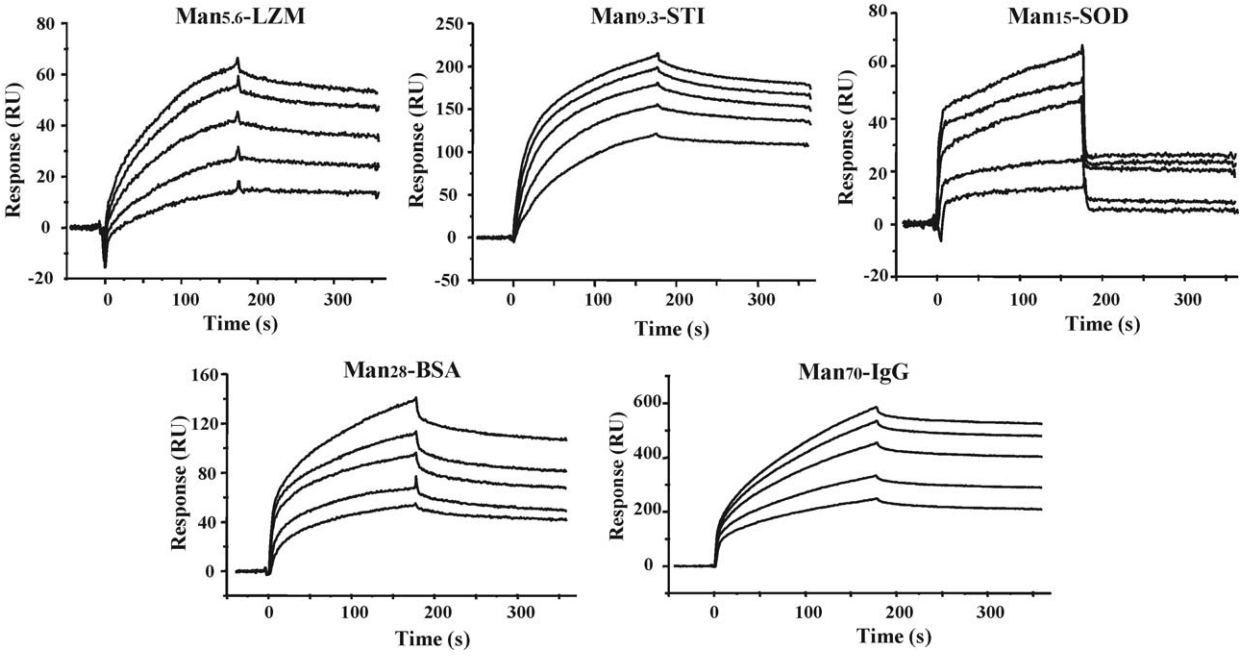

Fig. 3. Interaction of various mannosylated proteins with rabbit serum MBL. Increasing concentrations (10, 20, 40, 60 and 80 μg/ml) of $Man_{5.6}$-LZM, $Man_{9.3}$-STI, Man_{15}-SOD, Man_{28}-BSA and Man_{70}-IgG were injected over immobilized rabbit MBL.

Table 2
Rate constants and affinity constants (mean ± S.D.) for the interaction between immobilized rabbit MBL and mannosylated BSAs

	Rate constant		Affinity constant
	k_a[a] $\times 10^{-4}$ ($M^{-1}\,s^{-1}$)	k_d[b] $\times 10^{3}$ (s^{-1})	$K_a \times 10^{-6}$ (M^{-1})
$Man_{9.8}$-BSA	1.82 ± 0.19	3.22 ± 0.03	5.63 ± 0.64
Man_{16}-BSA	3.47 ± 0.33	2.62 ± 0.04	13.30 ± 1.41
Man_{23}-BSA	5.90 ± 0.63	2.10 ± 0.01	28.10 ± 3.04
Man_{37}-BSA	8.93 ± 1.13	0.72 ± 0.09	124 ± 2.65

[a] Association rate constant.
[b] Dissociation rate constant.

Table 3
Rate constants and affinity constants (mean ± S.D.) for the interaction between immobilized rabbit MBL and mannosylated proteins

	Rate constant		Affinity constant
	$k_a \times 10^4$ ($\mu g^{-1}\,ml\,s^{-1}$)	$k_d \times 10^3$ (s^{-1})	$K_a \times 10^2$ (μg^{-1} ml)
$Man_{5.6}$-LZM	6.23 ± 0.98	0.67 ± 0.001	9.28 ± 1.44
$Man_{9.3}$-STI	7.71 ± 0.47	0.37 ± 0.02	20.7 ± 7.01
Man_{15}-SOD	9.29 ± 1.93	100.67 ± 8.81	0.92 ± 0.11
Man_{28}-BSA	8.22 ± 1.48	1.79 ± 0.01	45.9 ± 8.4
Man_{70}-IgG	7.98 ± 0.57	0.12 ± 0.02	680 ± 153

size than Man-SOD and Man-BSA. It is generally known that the SPR response signal is proportional to the molecular mass (Persson et al., 1991). Man-LZM exhibited a similar response intensity to Man-SOD but its dissociation was slower.

3.4. Kinetic analysis of the interaction between mannosylated proteins and rabbit serum MBL

The association and dissociation rate constants were estimated by global fitting to sensorgrams using a 1:1 (Langmuir-type) binding model-derived equation. The association rate k_a ($M^{-1}\,s^{-1}$) and the affinity constants K_a (M^{-1}) of all Man-BSAs increased according to the number of mannose residues, and the dissociation rate constants k_d (s^{-1}) decreased (Table 2). Such in vitro binding corresponded well to the in vivo binding estimated in previous pharmacokinetic analysis (Opanasopit et al., 2001).

Other mannosylated proteins in which the degree of modification was similar were also subjected to SPR analysis (Table 3). All tested mannosylated proteins exhibited comparable association rate constants but widely different dissociation rate constants. The dissociation rate constant was smallest for Man-IgG and largest for Man-SOD among the tested mannosylated proteins.

4. Discussion

MBL is a C-type lectin (collectin) that has a binding affinity for *N*-acetyl-D-glucosamine (GlcNAc) and mannan (Kawasaki et al., 1989). Furthermore, MBL is the immune active factor that circulates in serum and recognizes pathogens that have mannose residues on their surface (Holmskov et al., 1994; Ikeda et al., 1987; Matsushita, 1996). The existence of MBL in blood makes biodistribution of mannosylated proteins more complicated (Opanasopit et al., 2001). Considering the difficulty in interpreting complex in vivo distribution data, we have tried to elucidate the direct molecular interaction between MBL and mannosylated ligands by using SPR spectroscopy.

SPR spectroscopy has been found to be a useful tool as far as accurate molecular interaction analysis is concerned and it has been widely used for many purposes and many kinds of the analytical substrates, including chemicals (Rich et al., 2002) and liposomes (Laukkanen et al., 1994). Recently, it has been used for the screening of antibodies from crude hybridoma samples (Canziani et al., 2004) or endocrine disrupting chemicals (Asano et al., 2004) and for the characterization of selected ligands using phage peptide display technology (Gu et al., 2004). In addition, SPR spectroscopy has been used to confirm that the human cytokeratin peptide sequence that mimics *N*-acetyl-D-glucosamine, a known ligand of MBL, specifically binds to MBL (Montalto et al., 2001). Thus, SPR spectroscopy has increasingly been applied to molecular interaction studies. In the present study, rabbit serum MBL was immobilized on the SPR sensor chip to examine the binding characteristics of mannosylated proteins with different degrees of modification. Preliminary experiments (Fig. 1) revealed a Ca^{2+}- and mannose-dependence of the binding between mannosylated protein and MBL immobilized on the SPR sensor chip.

In SPR spectroscopic investigations of Man-BSAs, Man-BSA with a larger number of mannose residues exhibited a higher association rate constant (k_a, Table 2). As the number of mannose unit per protein increased, the likelihood of mannose residues interacting with MBL would increase. When the k_a value was divided by the mannose density to estimate the molecular interaction using the concentration of mannose units, it was comparable in Man-BSAs (1.9 ± 0.19, 2.2 ± 0.2, 2.6 ± 0.27 and $2.4 \pm 0.3 \times 10^3$ (mannose unit $M^{-1}\,s^{-1}$) for $Man_{9.8}$-, Man_{16}-, Man_{23}- and Man_{37}-BSA, respectively). Thus, the association of mannosylated protein with MBL appears to be determined primarily by simple probability. On the other hand, if the interaction occurred via a 1:1 (Langmuir-type) process, the dissociation rate constant would not have been independent of the concentration of mannose units. Nevertheless, the dissociation rate constant (k_d) became smaller on increasing the number of mannose residues of Man-BSA. These results can be explained by multivalent binding between Man-BSA and MBL. Although the monomer of MBL (32 kDa) contains only one carbohydrate recognition domain (CRD) at the COOH-terminus, three monomers are closely associated with the formation of a triple helix structure at the collagen domain, and two to six sets of trimer form the bouquet-like structure of MBL via intermolecular disulfide bonds (Weis et al., 1998; Presanis et al., 2003). This complicated structure of MBL allows multivalent interactions with mannosylated proteins. It is a matter of course that Man-BSA with a higher mannose density is more closely associated with MBL due to multivalent binding.

Various mannosylated proteins, in which the degree of modification was similar, were subjected to the SPR binding studies. Here, the degree of modification was reflected in the number of

mannose residues per molecular weight of protein. The association rate constants for all tested mannosylated proteins were almost identical (Table 3). When the k_a value was evaluated using the apparent concentration of mannose units, it was found to be 1.66 ± 0.26, 1.72 ± 0.11, 2.48 ± 0.52, 2.03 ± 0.37 and $1.99 \pm 0.14 \times 10^3$ (mannose unit $M^{-1}\,s^{-1}$) for $Man_{5.6}$-LZM, $Man_{9.3}$-STI, Man_{15}-SOD, Man_{28}-BSA and Man_{70}-IgG, respectively. These values were comparable with those mentioned earlier for Man-BSAs, supporting the hypothesis that the association was due to simple probability depending on the apparent concentration of mannose units.

In contrast, mannosylated proteins varied widely in terms of the dissociation pattern of the responses (Fig. 3) or dissociation rate constants k_d (s^{-1}). In particular, the sensorgram of Man-SOD exhibited a faster dissociation pattern than that of other mannosylated proteins. Our previous in vivo studies (Opanasopit et al., 2001) demonstrated that Man-SOD exhibited simple saturable kinetics with regard to hepatic uptake whereas Man-BSA and Man-IgG exhibited slow hepatic uptake at low doses (<1 mg/kg). As suggested by this study, the interaction of Man-SOD with serum MBL would be too weak to retard liver nonparenchymal cell uptake. These results support the proposal that hepatic targeting by mannosylation of SOD is a promising approach to protecting liver cells from ischemia/reperfusion injury (Yabe et al., 1999, 2002). On the other hand, the dissociation rate constant k_d (s^{-1}) of Man-IgG was the lowest. This result agrees with our previous in vivo study (Opanasopit et al., 2001) demonstrating that Man-IgG exhibited a concave dose–AUC relationship even under conditions of a low mannose content, i.e. the dose-dependent increase in AUC observed over a range of lower doses was likely to be associated with strong binding of Man-IgG to serum MBL (Opanasopit et al., 2001). It should be noted that, in spite of the degree of modification being similar for all mannosylated proteins, the binding to MBL differed widely. Weis and Drickamer (1994) have suggested that the ability of multipronged binding sites of MBL to recognize microbes might depend on the highly repetitive structure of ligands presented by microbes. Since amino acid residues having primary amine groups, such as lysine and arginine, are the site of mannosylation, the configuration of the mannose groups would differ from protein to protein. Thus, it is likely that the steric configuration of mannose residues might determine the efficacy of multivalent interactions with geometrically organized CRDs of MBL.

5. Conclusion

Binding characteristics of Man-SOD, Man-BSA and Man-IgG with isolated MBL were similar to in vivo binding evaluated by the pharmacokinetic model (Opanasopit et al., 2001). This result indicates that MBL can be one route of retarding plasma elimination of mannosylated proteins. In addition, the specific binding of mannosylated proteins to MBL is due to multivalent interactions, and affected by the number of mannose residues and their configuration on the surface of proteins.

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