Physicochemical and Pharmacokinetic Characterization of Highly Potent Recombinant L-Methionine γ-Lyase Conjugated with Polyethylene Glycol as an Antitumor Agent

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Abstract

A highly potent recombinant L-methionine γ-lyase (METase) conjugated with polyethylene glycol (PEG) was characterized physicochemically and pharmacokinetically *in vivo* and *in vitro*. Pegylated METase (PEG-METase), which contains pyridoxal 5′-phosphate (PLP) as a cofactor in the molecule, is a potent anticancer agent that can deplete L-methionine from plasma. Although pegylation decreased its specific activity, dithiothreitol (DTT) treatment increased it over three times with the detachment of one PEG moiety modified with a cysteine residue. We can produce DTT-treated PEG-METase on a large scale in sufficient quality for therapeutic use. The superiority of DTT-treated PEG-METase was confirmed by the enhancement of L-methionine depletion and amelioration of pharmacokinetics in mice. The holoenzyme of DTT-treated PEG-METase gave a several times larger area under the plasma concentration curve than that of DTT-untreated PEG-METase, not because of an increase of the half-life but because of high specific activity. Conversely, simultaneous PLP infusion led to a greatly increased half-life of the holoenzyme. DTT-treated PEG-METase administration with PLP infusion was the most useful combination for maximizing the potency of the enzyme. We showed that serum albumin interfered with holoenzyme activity *in vitro*. The decrease of holoenzyme activity was dependent on the type of serum albumin. We concluded that PLP was released from PEG-METase by serum albumin *in vivo* and *in vitro*. The deleterious effect of PLP dissociation from PEG-METase could be improved by supplementing PLP and oleic acid. Their synergistic effect in preventing a decrease of the holoenzyme activity was also observed. (Cancer Res 2006; 66(5): 2807-14)

Introduction

Numerous human cancer cell lines and primary tumors have an absolute requirement for L-methionine, an essential amino acid (1–4). Upon L-methionine depletion, L-methionine-dependent cancer cells are not able to divide and became arrested in the late S-G_2 phase of the cell cycle (5–7). On the other hand, normal human cells are relatively resistant to exogenous L-methionine restriction. Thus, depletion of L-methionine is effective for cancer therapy (8, 9).

L-Methionine γ-lyase (METase, methioninase, EC 4.4.1.11) is a pyridoxal 5′-phosphate (PLP)–dependent enzyme that cleaves L-methionine (10, 11). The gene from *Pseudomonas putida* has been cloned and expressed in *Escherichia coli* (12, 13). The enzyme is composed of 398 amino acid residues corresponding to the subunit of the homotetramer. The crystal structure of the enzyme was determined by X-ray diffraction analysis (14). A holoenzyme has a covalent bond via Schiff's base between PLP and the ε-amino group of an active site, Lys^{211} (15). The kinetics of the entire enzyme reaction was analyzed and the assay method of accurate enzyme activity was developed in an earlier study on the basis of the reaction mechanism (16). We (17) were able to develop an efficient manufacturing process for the recombinant enzyme by introducing bulk crystallization.

A therapeutic approach to L-methionine depletion using the enzyme has been reported. When the enzyme was i.v. administered to cancer patients, Tan et al. (18, 19) noted a dramatic decrease of plasma L-methionine levels without significant toxicity. Tan et al. (20) also documented the efficacy of METase in combination with cisplatin against human colon tumors *in vivo*. Yoshioka et al. (21) showed that METase and 5-fluorouracil displayed synergistic antitumor efficacy against Lewis lung carcinoma *in vivo*. An immunoenzymometric assay for METase in biological fluids has been developed (22). However, significant anaphylactic shock was observed by challenge injection of METase after repeated dosing of the enzyme at 8-hour intervals for 14 consecutive days in monkeys (23).

Biological macromolecules conjugated with polyethylene glycol (PEG) have been developed for a variety of therapeutic applications and reported to exhibit more superior properties than their corresponding unmodified parent molecules (24–26). Whereas pegylation equips them with the advantage of greater resistance to proteolytic degradation, higher solubility, longer circulating half-life, lower clearance, and lower immunogenicity, it ordinarily leads to diminished biological activity (26–28). Pegylated METase (PEG-METase) showed prolonged half-life and attenuated immunogenicity (29, 30). However, its specific activity when first prepared on a laboratory scale was relatively low (31). As the preparation and characterization of PEG-METase with high specific activity had not been reported in detail, our primary goal in the present study was to develop a highly potent PEG-METase with improved specific activity. We constructed an efficient production process of highly

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potent PEG-METase on a large-scale, then characterized it for therapeutic application, and evaluated its superiority *in vivo*.

Simultaneous PLP administration greatly enhanced L-methionine depletion dose-dependently and prolonged the plasma half-life of holoenzyme activity *in vivo* (29, 32). We hypothesize that PLP was rapidly dissociated and lost from PEG-METase after injection (30, 32). Our secondary aim in this study was the elucidation of PLP interaction *in vivo* and *in vitro*. We determined the pharmacokinetic parameters of PEG-METase with and without PLP infusion in mice and revealed the interference of serum albumin with PEG-METase, and in this paper describe the release mechanism of PLP from PEG-METase.

Materials and Methods

Chemicals and reagents. The sources of materials and reagents used in this study were as follows: methoxy-PEG succinimidyl glutarate with molecular weight of ~5 k (MEGC-50HS) as an activated PEG from Nippon Oil Fat (Tokyo, Japan), PLP and potassium oleate from Nacalai Tesque (Kyoto, Japan), dithiothreitol (DTT) and L-methionine were from Wako Pure Chemical (Osaka, Japan), human serum albumin (HSA, A1653, fraction V, A1887, fatty acid free), murine serum albumin (MSA, A3139, fraction V), bovine serum albumin (BSA, A6003, fatty acid free), human serum γ-globulin (G4386), and bovine serum γ-globulin (G7516) were from Sigma-Aldrich (St. Louis, MO). METase as a parent molecule was prepared in Shionogi & Co., Ltd. (Osaka, Japan), as described previously (17). All other chemicals used in this study were of the highest quality commercially available.

Production of highly potent recombinant METase conjugated with PEG. METase (30 mg/mL), which was purified except for the final gel filtration process (17), was diluted to 10 mg/mL with 120 mmol/L sodium borate buffer (pH 9.0). The solution was concentrated using a tangential flow filtration system with an ultrafiltration membrane (10 kDa, Sartorius, Göttingen, Germany). The protein concentration was adjusted to 100 mg/mL. A total of 275 g MEGC-50HS was tripartitioned and added stepwise to 1.5 L concentrated METase solution every 15 minutes with mild agitation at 37°C. The final molar ratio of MEGC-50HS to METase was 60. After the reaction, 100 mmol/L NaH_2PO_4 (pH 4.5) was added to the pegylation mixture to adjust the pH to 7.2. DTT was added to the solution at final concentration of 0.5% and the solution was incubated for 2 hours with mild agitation at 37°C. The following procedures were done at 15°C unless otherwise stated. To reduce the concentrations of impurities, such as hydrolyzed PEG moiety, *N*-hydroxysuccinimide, and DTT, the reaction mixture was subjected to diafiltration with 50 mmol/L sodium phosphate buffer (pH 7.2) and then concentrated to 30 mg/mL by a tangential flow filtration system with an ultrafiltration membrane (50 kDa, Sartorius). After the concentrated solution had been applied to a 1 L anion-exchange column (DEAE-Sepharose FF, 14 × 6.5 cm, Amersham Biosciences, Piscataway, NJ), which had been equilibrated with 50 mmol/L sodium phosphate buffer (pH 7.2), the active pass-through fractions that were eluted with the same buffer at a flow rate of 7.5 L/h were collected and filtered with a series of microfiltration membranes (0.45-0.20 μm; Sartorius). Subsequently, 12 L of the solution was applied to a 170 L gel filtration column (Sephacryl S-200 HR, 60 × 30 cm in series, Amersham Biosciences), which had been equilibrated with 10 mmol/L sodium phosphate buffer (pH 8.0). DTT-treated PEG-METase was eluted with the same buffer at a flow rate of 30 L/h. Active fractions were pooled and concentrated to 3.0 L using a tangential flow filtration system with an ultrafiltration membrane (30 kDa, Sartorius). After 1 mmol/L PLP had been added to the solution to a final concentration of 0.1 mmol/L, the solution was filtered with a microfiltration membrane (0.22 μm; Millipore, Billerica, MA). DTT-untreated PEG-METase was also produced in the same manner except for DTT treatment.

Degree of modification of the primary amine. The degree of modification of the primary amine in PEG-METase was determined using fluorescamine (33). METase was used as a control.

Content of the sulfhydryl group. The sulfhydryl content in PEG-METase was quantified by the method of Ellman (34) using 5,5′-dithiobis(2-nitrobenzoic acid) after PEG-METase had been denatured by boiling for 5 minutes with 0.5% SDS in 10 mmol/L sodium phosphate buffer (pH 7.6) containing 1 mmol/L EDTA.

Amount of conjugated PEG moiety. PEG-METase was hydrolyzed with 100 mmol/L NaOH for 10 minutes (35) and neutralized to pH 8.0 with aqueous HCl. The amount of the released PEG moiety was determined by size exclusion HPLC (10 AVp, Shimadzu, Kyoto, Japan) on TSKgel SuperAW5000, AW4000, and AW3000 columns (6 × 150 mm, Tosoh, Tokyo, Japan) in series eluted with 10 mmol/L sodium phosphate buffer (pH 7.2) containing 120 mmol/L NaCl at a flow rate of 0.23 mL/min with measurement of differential refractive index detector. Authentic PEG (4120 Da, Polymer Laboratories, Church Stretton, Shropshire, United Kingdom) was used as a standard.

Molecular mass. The molecular mass of PEG-METase was determined with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/MS) equipped with a nitrogen laser at 337 nm in the linear mode (Reflex III, Bruker Daltonics, Billerica, MA). Sinapinic acid was used as a matrix.

Protein concentration. PEG-METase was hydrolyzed with 100 mmol/L NaOH for 10 minutes (35) and neutralized to pH 8.0 with aqueous HCl. Protein concentration was determined using a pyrogallol red–molybdate complex formation (36) with a protein assay kit (Wako Pure Chemical), with crystalline BSA as a standard protein. In the pegylation and purification process, protein concentration was monitored by measuring the absorbance at 220 or 280 nm with a spectrophotometer (UV-1600, Shimadzu).

Endotoxin concentration. Endotoxin concentration was determined using a *Limulus* amebocyte lysate reagent with a *Limulus* HS-F single test Wako kit (Wako Pure Chemical).

Animals. BALB/cAnNCrj mice (female, 8-10 weeks of age, 20-22 g body weight) were purchased from Charles River Japan (Yokohama, Japan).

L-Methionine depletion efficacy and pharmacokinetics in mice. All experiments examined three mice per group. BALB/cAnNCrj mice were s.c. implanted with a mini-osmotic pump (Model 2001, Alzet, Cupertino, CA) filled with 200 μL of 300 mg/mL PLP dissolved in 0.1 mol/L sodium phosphate buffer (pH 7.5). After implantation for 24 hours, 140 mg/kg of the respective PEG-METase was i.v. injected into the tail veins of the mice. Blood samples were drawn from the heart of each mouse using an EDTA-treated syringe under ether anesthesia before injection and 1, 8, 24, 32, 48, and 72 hours, or 1, 4, 6, 8, 24, and 48 hours, respectively, in the presence of PLP pump or not. The blood samples were rapidly centrifuged (12,000 × *g*, 5 minutes, 5°C) and plasma samples were stored at −80°C until use. Pharmacokinetic parameters were determined using WinNonlin software (Pharsight, Mountain View, CA).

L-Methionine concentration in plasma. Plasma L-methionine concentrations were determined by reversed-phase HPLC (10 AVp, Shimadzu) using a C18 column (Supelcosil LC-18-DB, 4.6 × 250 mm, Sigma-Aldrich) equipped with a fluorescence detector (excitation at 350 nm, emission at 450 nm) after derivatization of plasma amino acids with *o*-phthaldialdehyde (37).

PLP concentration in plasma. Plasma PLP concentrations were determined by reversed-phase HPLC (10 AVp, Shimadzu) using C18 column (Cosmosil 5C18-AR-II, 4.6 × 150 mm, Nacalai Tesque) equipped with a fluorescence detector (excitation at 300 nm, emission at 400 nm) after derivatization of plasma PLP with sodium bisulfite (38).

Enzyme activity of PEG-METase. The enzyme assay method for METase activity as previously described (16) was applied to PEG-METase activity. One unit was defined as the amount of enzyme that produced one μmol of α-ketobutyrate per minute at an infinite concentration of L-methionine. The specific activity was represented as the enzyme activity in units per milligram of protein.

Two kinds of enzyme activity in plasma were evaluated and designated as the actual enzyme activity derived from the holoenzyme and the total enzyme activity derived from the holoenzyme and the apoenzyme. Both enzyme assay procedures have been described previously (30, 32). Briefly, the actual enzyme activity was determined by allowing the plasma enzyme solution to react with L-methionine as a substrate in the absence of PLP after preparing it in the absence of PLP and DTT. The total enzyme activity was determined by allowing the plasma enzyme

Table 1. Synthesis and purification of highly potent PEG-METase

Step	Total activity (MU)	Total protein (g)	Specific activity (U/mg)	Protein yield (%)	Endotoxin (EU/mg)
Concentration	8,560	150	57	100	2
Pegylation	1,590	150	11	100	3
DTT treatment	6,110	150	41	100	2
Diafiltration	5,910	147	40	98	2
DEAE-Sepharose FF	5,820	145	40	97	0.8
Sephacryl S-200 HR	5,330	132	40	88	0.8

solution to react with L-methionine as a substrate in the presence of 10 μmol/L PLP after it had been prepared in the presence of 10 μmol/L PLP and 0.01% DTT.

Serum albumin interference *in vitro*. DTT-treated PEG-METase at final concentration of 2.4 mg/mL was incubated with 40 mg/mL of various serum albumins and 10 mg/mL of various serum γ-globulins dissolved in 100 mmol/L sodium phosphate buffer (pH 7.4) at 37°C. If necessary, L-methionine, PLP, and potassium oleate were supplemented. At selected time points, the reaction mixture was promptly cooled on ice and both the actual enzyme activity and total enzyme activity were estimated.

Results

Production of highly potent PEG-METase. We found an increase in the specific activity of PEG-METase with DTT treatment. Table 1 summarizes the synthesis and purification of highly potent PEG-METase. The specific activity of PEG-METase decreased ~20% to that of METase at the end of pegylation. After DTT treatment, however, the specific activity of PEG-METase recovered to ~70% to that of METase. The concentration of DTT was preferably above 0.1% during treatment. When a low DTT concentration was used, a long time was needed to raise the specific activity of PEG-METase. DTT-treated PEG-METase was not activated further by DTT retreatment. METase was unaffected by DTT treatment.

The effects of various compounds on the recovery of PEG-METase activity were investigated. Compounds containing the sulfhydryl group, such as DTT, 2-mercaptoethanol, *N*-acetyl L-cysteine, and reduced glutathione, made it possible to increase the specific activity of PEG-METase. DTT was the most effective compared with other sulfhydryl compounds at the same concentrations.

The conditions of pegylation and purification were optimized for large-scale production. We achieved the production of 132 g of highly potent PEG-METase per batch with an overall protein yield of 88% and endotoxin level of 0.8 EU/mg. METase was not detected in the PEG-METase on SDS-PAGE stained with Coomassie brilliant blue R-250 (data not shown). The purified highly potent PEG-METase could be stored at −80°C for several months with no significant loss of activity.

Physicochemical characterization of highly potent PEG-METase. PEG-METase was characterized by DTT treatment from the viewpoint of the degree of primary amine modification, content of the sulfhydryl group, amount of the conjugated PEG moiety, and molecular mass (Table 2). The degree of primary amine modification remained constant regardless of DTT treatment. On the other hand, the sulfhydryl group content of PEG-METase was reduced by approximately one cysteine residue after pegylation, but completely recovered to that of METase after DTT treatment. The number of conjugated PEG moieties of PEG-METase decreased by approximately one moiety after DTT treatment. Mass spectrometric analysis was used to evaluate the reduction of the molecular mass of PEG-METase corresponding to one PEG moiety by DTT treatment. These results indicated that DTT treatment detached one PEG moiety from the sulfhydryl group, which was modified with pegylation, without influencing the PEG moieties attached to the primary amines.

L-Methionine depletion efficacy and pharmacokinetics *in vivo*. Comparison of L-methionine depletion efficacy and pharmacokinetics between DTT-untreated PEG-METase and DTT-treated PEG-METase was examined with and without PLP infusion in mice. The profiles of L-methionine concentration in plasma are presented in Fig. 1*A*. After administration of 140 mg/kg of the respective PEG-METases, L-methionine concentrations in plasma rapidly decreased. When the DTT-treated PEG-METase was injected, the duration of L-methionine depletion of <5 μmol/L was prolonged from 1 to 6 hours without PLP infusion and from 8 to 24 hours with PLP infusion, respectively, in comparison with the injection of DTT-untreated PEG-METase. We concluded that DTT-treated PEG-METase, as a highly potent PEG-METase, has an advantage over DTT-untreated PEG-METase for cancer therapy

Table 2. Characteristic properties of METase and PEG-METases

	Primary amine modification (%)	SH group per subunit	Conjugated PEG moiety per subunit	Molecular mass (Da)
METase	0	3.7	0	42,564 [42,626]
DTT-untreated PEG-METase	75	3.0	8.4	82,496, 86,701
DTT-treated PEG-METase	74	3.7	7.5	77,512, 82,496

NOTE: The calculated average molecular mass of METase is in brackets.

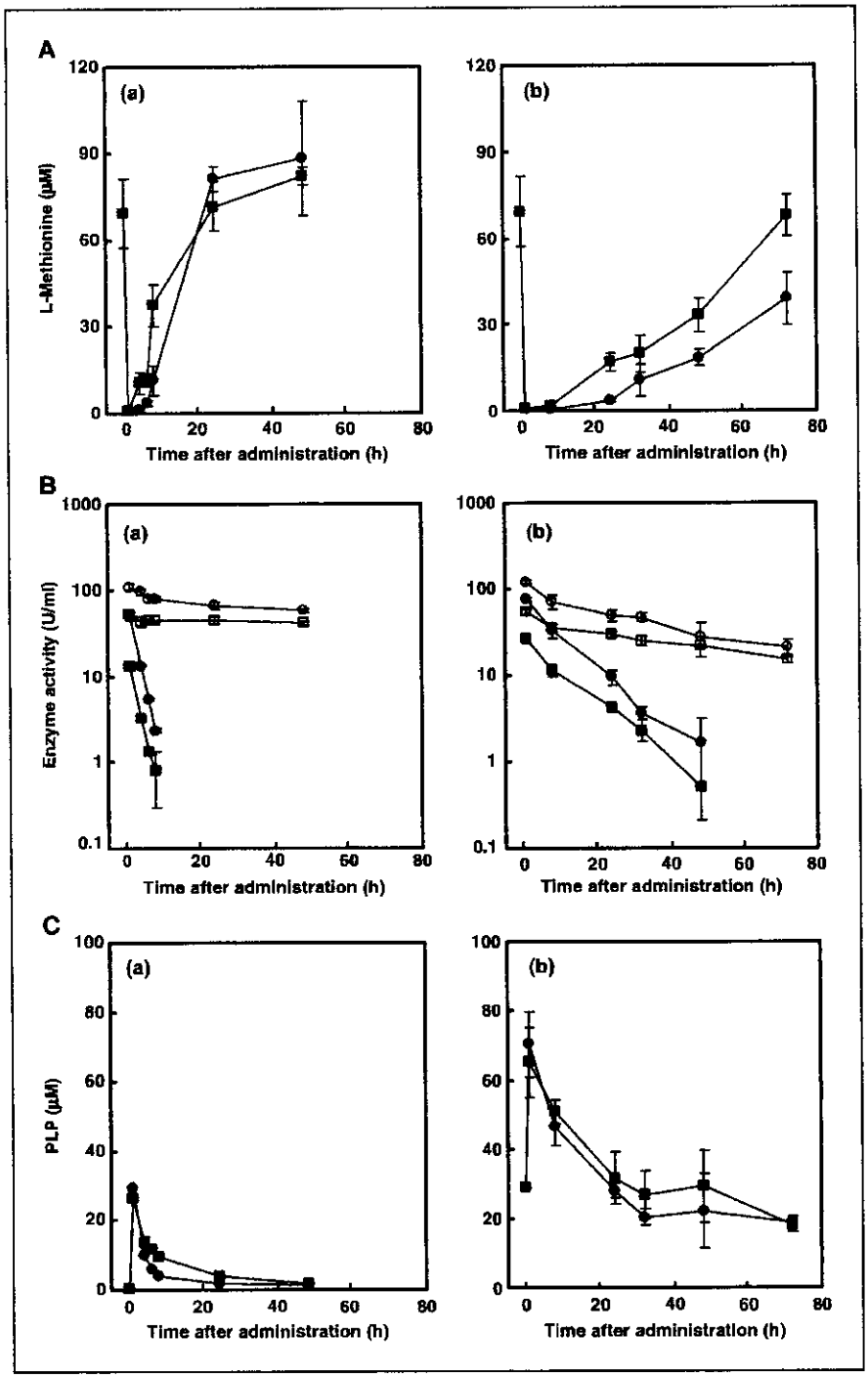

Figure 1. Time course of L-methionine concentration (*A*), residual enzyme activity (*B*), and PLP concentration (*C*) in mice administered DTT-treated PEG-METase and DTT-untreated PEG-METase. *A*, plasma L-methionine concentration profiles by PEG-METase administration without PLP infusion (*a*) and with PLP infusion (*b*). ●, DTT-treated PEG-METase; ■, DTT-untreated PEG-METase. *Points*, mean; *bars*, SD. *B*, plasma enzyme activity profiles by PEG-METase administration without PLP infusion (*a*) and with PLP infusion (*b*). ●, actual enzyme activity of DTT-treated PEG-METase; ■, actual enzyme activity of DTT-untreated PEG-METase; ○, total enzyme activity of DTT-treated PEG-METase; □, total enzyme activity of DTT-untreated PEG-METase. *Points*, mean; *bars*, SD. *C*, plasma PLP concentration profiles by PEG-METase administration without PLP infusion (*a*) and with PLP infusion (*b*). ●, DTT-treated PEG-METase; ■, DTT-untreated PEG-METase. *Points*, mean; *bars*, SD.

in vivo. PLP infusion effectively maintained a low L-methionine concentration in plasma for both DTT-untreated PEG-METase and DTT-treated PEG-METase. The most useful combination for depleting L-methionine from plasma was DTT-treated PEG-METase administration with PLP infusion.

Figure 1*B* presents the profiles of the actual enzyme activity and the total enzyme activity in plasma. A discrepancy was obviously observed in the profiles of the actual enzyme activity and the total enzyme activity. The enzyme activity responsible for controlling L-methionine concentration in plasma was not the total enzyme activity but the actual enzyme activity. The summary of mean pharmacokinetic parameters is found in Table 3. The actual enzyme activity of DTT-treated PEG-METase had 2.7 and 3.8 times greater areas under the plasma concentration curve (AUC), respectively, than that of DTT-untreated PEG-METase with and without PLP infusion. The half-life and mean residence time (MRT) of the actual enzyme activity were not considerably different between DTT-untreated PEG-METase and DTT-treated PEG-METase. On the other hand, PLP infusion prolonged the half-life and MRT of the actual enzyme activity by several times

Table 3. Pharmacokinetic parameters of PEG-METases in mice

	$T_{1/2\lambda z}$ (h)	CLt (mL/h/kg)	$AUC_{0\to\infty}$ (U h/mL)	Vss (mL/kg)	MRT (h)
DTT-untreated PEG-METase					
Without PLP					
Actual enzyme activity	2.0	40	50	84	2.1
Total enzyme activity	200	0.15	14,000	42	280
With PLP					
Actual enzyme activity	7.7	5.8	340	65	11
Total enzyme activity	50	0.67	3,000	47	71
DTT-treated PEG-METase					
Without PLP					
Actual enzyme activity	1.6	26	190	51	1.9
Total enzyme activity	88	0.47	11,000	59	130
With PLP					
Actual enzyme activity	9.2	5.5	910	57	10
Total enzyme activity	36	1.1	4,400	56	50

Abbreviations: $T_{1/2\lambda z}$, half-life at terminal elimination; CLt, total systemic clearance; $AUC_{0\to\infty}$, area under the plasma concentration curve; Vss, distribution volume; MRT, mean residence time.

irrespective of DTT treatment. DTT treatment of PEG-METase and PLP infusion had an influence on AUC of actual enzyme activity independently. As a result, there was a combinational effect for improving the pharmacokinetic profiles of the actual enzyme activity when DTT-treated PEG-METase and PLP were coadministrated. As for the total enzyme activity, decreases in the half-life and MRT were observed due to DTT treatment and these were enhanced by PLP infusion. The volumes of distribution of PEG-METases were similar to the volume of plasma.

The implanted mini-osmotic pump filled with PLP sustained a high PLP concentration in plasma (Fig. 1*C*). We observed no significant difference of PLP concentration in plasma between DTT-untreated PEG-METase and DTT-treated PEG-METase.

Serum albumin interference *in vitro*. DTT-treated PEG-METase, as a highly potent PEG-METase, was incubated with MSA (A3139), HSA (A1653), and human γ-globulin (G4386) supplemented with or without 30 μmol/L L-methionine. Time courses of the actual enzyme activity and the total enzyme activity are displayed in Fig. 2. The actual enzyme activities diminished with incubation time in the presence of MSA and HSA. MSA had an influence on the decrease of the actual enzyme activity of PEG-METase rather than HSA. Moreover, L-methionine promoted reduction of the actual enzyme activity, especially when incubated with HSA. Incubation with human serum γ-globulin was independent of the actual enzyme activity. In contrast, the total enzyme activities remained constant without serum albumin interference.

PLP is absolutely essential for expressing the enzyme activity. Fonda et al. (39) reported that oleic acid inhibited the binding of PLP to serum albumins. To prevent reduction of the actual enzyme activity of PEG-METase in contact with serum albumins, we investigated the effect of addition of PLP and potassium oleate to the mixtures. DTT-treated PEG-METase was incubated with HSA (A1887) and BSA (A6003) supplemented with 50 μmol/L L-methionine for 3 hours in the presence of PLP and potassium oleate of varying concentrations. In our observation, the actual enzyme activity of PEG-METase was susceptible to HSA rather than BSA (Fig. 3). PLP and potassium oleate were helpful to PEG-METase for protecting against interference by both HSA (Fig. 3*A*)

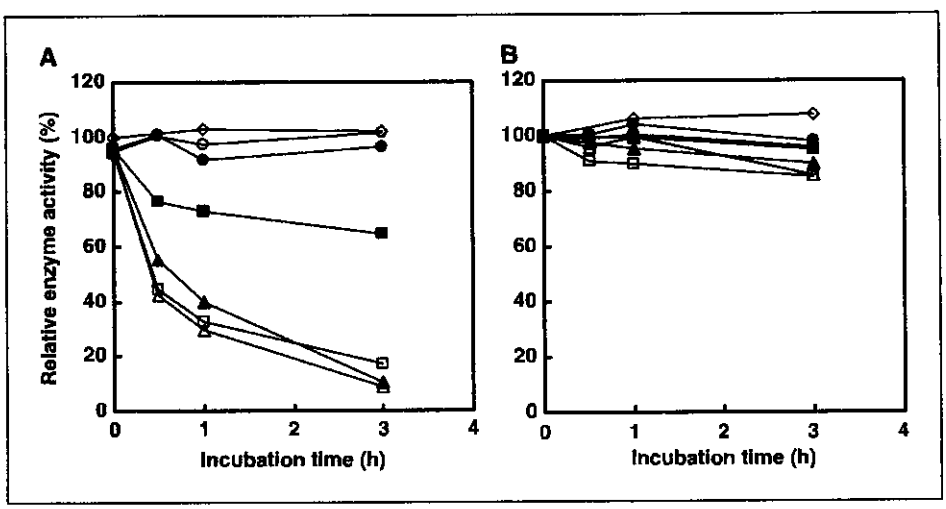

Figure 2. Effect of serum albumin on actual enzyme activity (*A*) and total enzyme activity (*B*) of PEG-METase. Enzyme activities of PEG-METase incubated with buffer (100 mmol/L sodium phosphate buffer, pH 7.4) were taken as 100%. ●, buffer as a control; ○, buffer and 30 μmol/L L-methionine; ■, HSA; □, HSA and 30 μmol/L L-methionine; ▲, MSA; △, MSA and 30 μmol/L L-methionine; ◇, human serum γ-globulin.

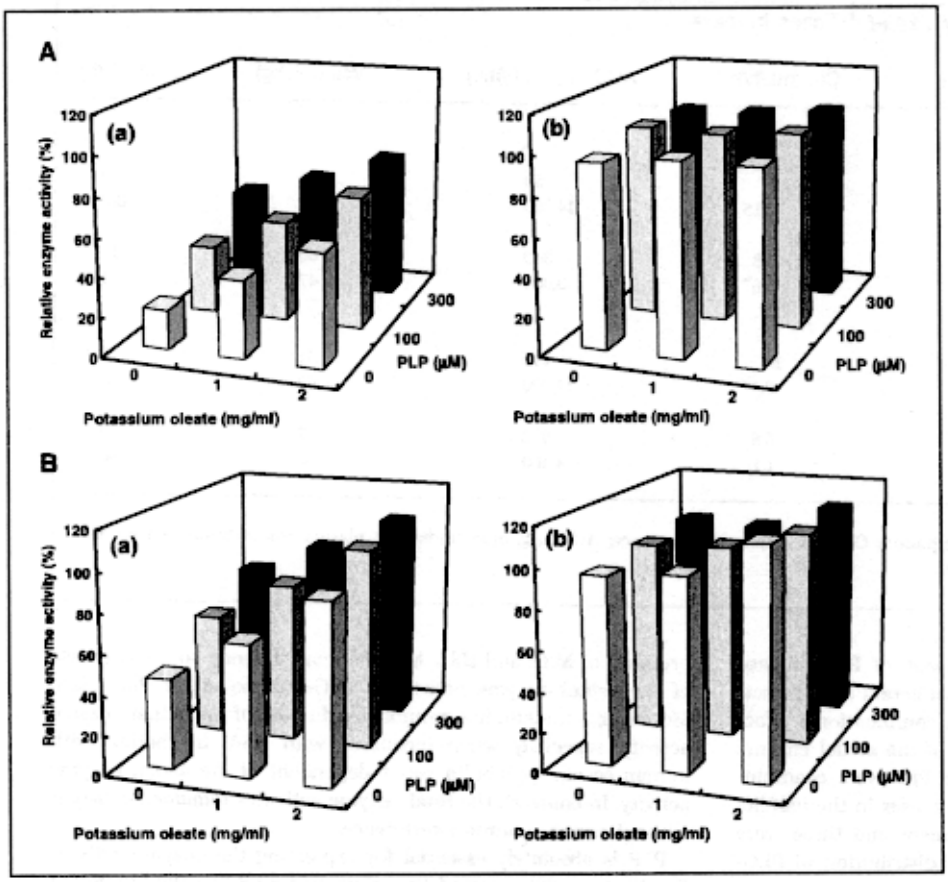

Figure 3. Effect of PLP and oleic acid on actual enzyme activity (*a*) and total enzyme activity (*b*) of PEG-METase in the presence of HSA (*A*) and BSA (*B*). Enzyme activities of PEG-METase incubated with buffer (100 mmol/L sodium phosphate buffer, pH 7.4) were taken as 100%.

and BSA (Fig. 3*B*) depending on their concentrations. They also exhibited a synergetic effect to hamper the decrease of actual enzyme activity. In contrast, the total enzyme activities were independent of the existence of serum albumins, PLP, and potassium oleate.

Discussion

Highly potent PEG-METase involved with DTT treatment, which has high specific activity, is useful for depleting L-methionine *in vivo* and improving the pharmacokinetics for cancer therapy. This enzyme can be manufactured on a large scale in high yield with sufficient quality for therapeutic use. However, PEG-METase is a heterogeneous mixture of species that are derived from number, site, and length of PEG moieties attached to METase. Several variables, in particular, protein concentration, pH, temperature, and reaction time at pegylation, affect the heterogeneity of the PEG-METase. The qualities of MEGC-50 HS as an activated PEG and METase as a parent molecule play important roles for the resulting PEG-METase. Under strictly controlled conditions, we were able to reproducibly produce several batches of highly potent PEG-METase.

We confirmed that pegylation occurred at the sulfhydryl group of the cysteine residue as well as at the primary amino group and that DTT treatment served to eliminate the PEG moiety from the cysteine residue in PEG-METase on the basis of stoichiometrical analyses, namely, sulfhydryl content, number of conjugated PEG moieties, and molecular mass. These were unexpected observations. In general, a derivative of PEG activated with *N*-hydroxysuccinimide ester reacts preferably with a primary amino group (28, 40). Recently, the major component of the positional isomer in recombinant human IFN α-2b conjugated with PEG, which was synthesized with methoxy-PEG succinimidyl carbonate, has been identified as a histidine-linked form (41). An unusual coupling by activated PEG with *N*-hydroxysuccinimide ester has been observed in the modification of the tyrosine residue in recombinant mouse epidermal growth factor (42). METase contains all the four free cysteine residues of Cys^{49}, Cys^{116}, Cys^{190}, and Cys^{270} (14, 15). The free sulfhydryl group in Cys^{116} as a nucleophilic residue plays a significant role in expressing the enzyme activity (15). It is reasonable to elevate the specific activity of PEG-METase by DTT treatment involved with site-specific depegylation at the cysteine residue. The pegylation sites and depegylation site at the cysteine residue through DTT treatment need to be identified by further study. Judging from the results with sulfhydryl compounds as well as DTT, the PEG moiety seems to be linked with the sulfhydryl group at the cysteine residue via a thioester linkage.

Our *in vivo* experiment revealed the effectiveness of highly potent PEG-METase for L-methionine depletion in plasma. The higher specific activity of DTT-treated PEG-METase enhanced the pharmacokinetics that led to higher AUC of actual enzyme activity. Because the half-life and MRT of the actual enzyme activity represented similar values between DTT-treated PEG-METase and DTT-untreated PEG-METase, DTT treatment had little effect on PLP dissociation from PEG-METase. Our *in vivo*

study also proved the effectiveness of PLP infusion for L-methionine depletion in plasma. PLP infusion clearly lengthened the half-life and MRT of the actual enzyme activity of PEG-METase. Because PLP was released from PEG-METase by serum albumin, it needs to be infused for keeping the holoenzyme. We believe that DTT-treated PEG-METase should be used together with PLP infusion to minimize the dose and the frequency of administration and to increase efficacy. Conversely, the half-life and MRT of the total enzyme activity decreased under the conditions where there was high potency and prolonged action. One possibility is that PEG-METase is susceptible to degradation by proteolytic enzymes due to increased turnover of PEG-METase under these conditions.

To summarize the serum albumin interference with PEG-METase, first, serum albumins disturb the expression of holoenzyme activity, especially in the presence of L-methionine. Second, the holoenzyme activity is inhibited depending on the kind of serum albumin involved. Third, PLP and oleic acid effectively prevent serum albumin interference. In general, most of the circulating PLP is linked to HSA in plasma (43). Because PLP bound to HSA is protected from hydrolysis by phosphorylases (43), HSA functions as a reservoir and mediates PLP transport to tissues. Binding sites for PLP in HSA have been identified as Lys^{190}, which has a high affinity, and are composed of two or more nonspecific residues (39, 44). We concluded that serum albumin detached PLP, which was coupled with the ε-amino group at Lys^{211} via a Schiff's base of holoenzyme *in vivo* and *in vitro*. L-Methionine accelerates PLP dissociation from the holoenzyme. We hypothesize that serum albumin is easily accessible to PLP during the catalytic reaction in the presence of L-methionine from the standpoint of the reaction mechanism. Specific serum albumin is present in animal blood. Fonda et al. (39) reported that the binding of PLP to BSA is not consistent with that to HSA. Accordingly, it is anticipated that PLP release from PEG-METase would be dependent on the type of serum albumins involved—MSA, BSA, and HSA. This information would be helpful for further preclinical studies. Yang et al. (32) reported increase of the half-life of holoenzyme activity with PLP concentrations in plasma. Our *in vitro* result correlated with a previous *in vivo* study. PLP supplementation plays a role in compensating for the deficiency. Serum albumin is known to bind fatty acids. The major bound fatty acid is oleic acid in HSA, which accounts for 33% of the total fatty acids associated with it (45). It was reported that oleic acid inhibited PLP binding to HSA (39). Oleic acid exhibited the restriction of PLP binding to serum albumins as shown in Fig. 3. Seven binding sites of oleic acid in HSA are determined by X-ray diffraction analysis using the crystal of the HSA complex (46). The inhibition of PLP binding by oleic acid is probably due to the conformational alternation induced by it, because both the binding sites of PLP and oleic acid are not identical in HSA. Concomitant administration of oleic acid as well as PLP may be useful for enhancing L-methionine depletion and for circulating half-life of active PEG-METase *in vivo*.

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