

# *In Vivo* Efficacy of Recombinant Methioninase Is Enhanced by the Combination of Polyethylene Glycol Conjugation and Pyridoxal 5'-Phosphate Supplementation

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## ABSTRACT

Recombinant methioninase (rMETase) is an enzyme active in pre-clinical mouse models of human cancer. The efficacy of rMETase is due to depletion of plasma methionine, an amino acid for which tumors generally have an abnormally high methionine requirement. Furthermore, transient methionine depletion results in a markedly increased sensitivity of the tumors to several chemotherapeutic agents. This study characterized methods to prolong the half-life of rMETase to extend the *in vivo* period of depletion of plasma and tumor methionine. In the present study, rMETase was coupled to methoxypolyethylene glycol succinimidyl glutarate-5000 in order to prolong the half-life of rMETase and thus extend the *in vivo* period of depletion of plasma and tumor methionine. Matrix-assisted laser desorption ionization mass spectrometry indicated that one sub-unit of rMETase was modified by approximately 4, 6 and 8 PEG molecules when rMETase was PEGylated at molar ratios of PEG/rMETase of 30/1, 60/1, and 120/1, respectively. PEG-rMETase (120/1) had a serum half-life increase of 20-fold, and methionine depletion time increased 12-fold compared to unmodified rMETase. The increase in *in vivo* half-life depended on the extent of PEGylation of rMETase. In addition, a remarkable prolongation of *in vivo* activity and effective methionine depletion by the PEG-rMETase was achieved by the simultaneous administration of pyridoxal 5'-phosphate. PEGylation also reduced the immunogenicity of rMETase. The extent of reduction in immunogenicity depended on the number of residues PEGylated. PEG-rMETase 30/1 had a 10-fold decrease in IgG titer while PEG-rMETase 120/1 had a 10<sup>4</sup>-fold decreased titer compared to naked rMETase. Thus, the molecular modification of PEGylation confers critical new properties to rMETase for development as a cancer therapeutic.

## INTRODUCTION

L-methionine  $\alpha$ -deamino- $\gamma$ -mercaptomethane lyase (methioninase, METase) [EC 4.4.1.11] from *Pseudomonas putida* (1) has been previously cloned and produced in *Escherichia coli* (2–4) to target the methionine dependence of tumor cells. rMETase<sup>5</sup> is found in *Pseudomonas* (Pp), *Aeromonas*, and *Clostridium*, but not in yeast, plants, or mammals (5). rMETase is a homotetrameric PLP enzyme of 172-kDa molecular mass. The biochemical reaction catalyzed by rMETase is shown below (Scheme 1).

rMETase has 398 amino acid residues per subunit. The amino acid sequence of rMETase is homologous to the  $\gamma$ -family of PLP enzymes that catalyze  $\alpha$ , $\gamma$ -elimination and  $\gamma$ -replacement reactions, such as cystathionine  $\gamma$ -lyase, cystathionine  $\gamma$ -synthase, and *O*-acetylhomoserine *o*-acetylserine sulphydrylase (6). In rMETase,

tyrosine 114 has been shown to be important in  $\gamma$ -elimination of the substrate (6).

rMETase has been crystallized (5, 7). The structure of rMETase has been determined at 1.7 Å resolution using synchrotron radiation diffraction data and found to be a homotetramer with 222 symmetry. Two monomers associate to build the active dimer. The spatial fold of the subunits have three functionally distinct domains. Their quaternary arrangement is similar to those of L-cystathionine  $\beta$ -lyase and L-cystathionine  $\gamma$ -synthase from *E. coli* (5).

Previous studies have extensively documented that a broad range of human tumors are sensitive to rMETase *in vitro*. The IC<sub>50</sub> was several fold less for a wide variety of cancer cell lines compared to non-neoplastic cells. Sensitivity was particularly exquisite for breast, kidney, colon, lung, and prostate tumor cell lines (2, 8). Subsequent evaluation of rMETase on a variety of tumor cell lines in mouse xenograph models demonstrated a similar sensitivity to rMETase (8). In addition, plasma methionine depletion by rMETase resulted in a remarkable increased sensitivity of the tumors to several different types of chemotherapeutic agents (9).

However, the short *in vivo* half-life of rMETase and evidence of immunogenicity indicated the need to prolong the survival of the enzyme, prolong the period of methionine deprivation, and reduce potential immunogenicity that might result from repeated administration of the enzyme.

Conjugation of protein therapeutics with PEG has been shown to confer important therapeutic benefits—most importantly, increased serum half-life and reduced antigenicity (10). PEG-proteins have enhanced solubility, decreased antigenicity, decreased proteolysis, and reduced rates of kidney clearance as well as enhanced selective tumor targeting. To couple PEG to a protein, it is first necessary to activate the polymer by converting the hydroxyl terminus to a functional group capable of reacting typically with lysine and N-terminal amino groups of proteins (10).

Each ethylene oxide unit of PEG associates with two to three water molecules, which results in the molecule behaving as if it were five to ten times as large as a protein of comparable molecular weight (10). The clearance rate of PEGylated proteins is inversely proportional to molecular weight (11). Below a molecular weight of approximately 20,000, the molecule is cleared in the urine. Higher-molecular-weight PEG proteins are cleared more slowly in the urine and the feces (11).

The FDA has approved the PEGylated forms of the protein therapeutics adenosine deaminase, asparaginase,  $\alpha$ -IFN and a growth hormone antagonist (12). PEG- $\alpha$ -IFN for treatment of hepatitis C (10, 13) has recently been approved in two forms. Patients with refractory or recurrent acute lymphoblastic leukemia (ALL) are treated with a combination of PEG-asparaginase and methotrexate, vincristine, and prednisone (14). A genetic defect of adenosine deaminase (ADA) deficiency inhibits the development of the immune system making patients vulnerable to almost any type of infection. PEG-ADA strengthened the immune system considerably in these patients (15, 16).

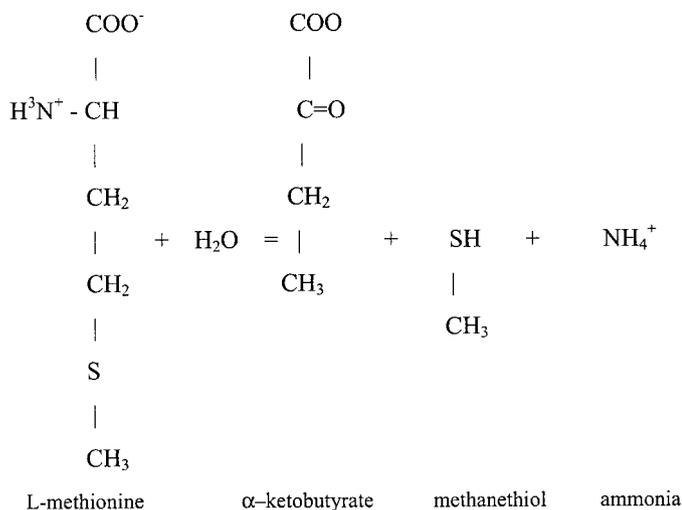
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<sup>5</sup>The abbreviations used are: rMETase, recombinant methioninase; PLP, pyridoxal 5'-phosphate; MALDI, matrix-assisted laser desorption ionization; PEG, polyethylene glycol.



Scheme 1. The biochemical reaction catalyzed by rMETase.

Initially, to prevent immunological reactions, which might be produced by multiple dosing of rMETase, and to prolong the serum half-life of rMETase, the N-hydroxysuccinimidyl ester of methoxypolyethylene glycol propionic acid (M-SPA-PEG 5000) was coupled to rMETase (17). MALDI mass spectrometry indicated the conjugation of two PEG molecules per subunit of rMETase and eight per tetramer. PEGylation of rMETase increased the serum half-life of the enzyme in rats to approximately 160 min compared to 80 min for unmodified rMETase. PEG-rMETase could deplete serum methionine levels to less than 1  $\mu\text{M}$  for approximately 8 h compared to 2 h for rMETase in rats (17).

We report here the conjugation of methoxypolyethylene glycol succinimidyl glutarate PEG (MEGC-PEG) to rMETase with resulting remarkable prolongation of effective enzyme half-life and duration of methionine depletion *in vivo*. Simultaneous *in vivo* administration of pyridoxal 5'-phosphate along with MEGC-PEG-rMETase is shown to be a novel approach to improve the efficacy of the PEGylated enzyme.

## MATERIALS AND METHODS

**Materials.** rMETase was provided by Shionogi Co., Ltd. (Osaka, Japan, Lot No. 8Y003). The production protocols for rMETase were as described previously (2, 17). rMETase was formulated in 50 mM sodium phosphate buffer, pH 7.2, containing 10  $\mu\text{M}$  PLP, with protein concentration 31 mg/ml and specific activity 50.7 U/mg. rMETase was more than 95% pure by HPLC with tetramer/oligomer ratio 96.7/3.3 and endotoxin 0.06 EU/mg.

Methoxypolyethylene glycol succinimidyl glutarate-5000 (MEGC-50HS-PEG or MEGC-PEG) (NOF Corporation, Kawasaki-shi, Kanagawa, Japan, Lot No. M21514) had a polydispersity of 1.02, substitution 94.2%, dimer content 0.84% and purity by  $^1\text{H-NMR}$  of 98.4%. The average molecular weight was 5461 Da. The chemical structure of MEGC-PEG is shown in Fig. 1.

DEAE Sepharose FF was purchased from Amersham Pharmacia Biotech (Piscataway, New Jersey, USA). 10% pre-cast Tris-glycine gels were from NOVEX (San Diego, CA, USA). Mini-osmotic pumps (Model 2001), with a reservoir volume of 200  $\mu\text{l}$ , pumping rate of 1.0  $\mu\text{l/hr}$  and duration of 7 days, were purchased from Durect Corporation (Cupertino, CA, USA). PLP and other chemicals were purchased from Sigma (St. Louis, MO, USA).

**Preparation and Purification of PEGylated rMETase.** The activated PEG derivative was used at a molar excess (1–4 fold) of PEG to free lysines in rMETase (32 per rMETase molecule), which corresponds to molar ratios of PEG to rMETase of 30–120/1. For each reaction, 120 mg/ml rMETase in 100 mM borate buffer (pH 8.8) was used. Based on 30–120/1 molar ratios of activated PEG *versus* rMETase (equal to 0.87–

3.5/1 weight ratio of activated PEG *versus* rMETase), a given amount of the activated PEG was added to the rMETase solution with three stepwise additions at 30 min intervals. The PEGylation reactions were carried out at 20–25°C under gentle stirring for 90 min.

To eliminate an excess of unreacted activated PEG, the resulting PEG-rMETase conjugate was applied on a Sephacryl S-300 HR gel filtration column (HiPrep 26/60, Amersham Pharmacia Biotech, Piscataway, NJ, USA) immediately after the PEGylation reaction. PEG-rMETase was eluted with 80 mM sodium chloride in 10 mM sodium phosphate, pH 7.4, containing 10  $\mu\text{M}$  PLP at a flow rate of 120 ml/h.

The fractions containing the PEG-rMETase conjugate were further purified by DEAE Sepharose FF column (XK 16/15, Amersham Pharmacia Biotech, Piscataway, NJ, USA) to remove trace amounts of un-PEGylated rMETase. The column was equilibrated and eluted with 80 mM sodium chloride in 10 mM sodium phosphate pH 7.2, containing 10  $\mu\text{M}$  PLP at a flow rate of 180 ml/h. The fractions containing the PEG-rMETase conjugate were collected. PEG-rMETase-containing fractions were concentrated with an Amicon centrprep YM-30 (Millipore Corp, Bedford, MA, USA) and sterilized by filtration with a 0.22  $\mu\text{m}$  membrane filter (Fisher Scientific, Tustin, CA, USA). Storage was at -80°C.

**Determination of Protein Content.** Protein was measured with the Wako Protein Assay Kit (Wako Pure Chemical, Osaka, Japan) according to the instruction manual with slight modification (18). 50  $\mu\text{l}$  of each sample or standard protein (BSA) was added to 3 ml of chromophore solution (pyrogallol red-molybdate complex) and vortexed well. The mixture was incubated at room temperature for 20 min without shaking and then measured for absorbance at 600 nm. The protein content of the sample was determined from the BSA standard calibration curve.

**rMETase Activity Assay.** rMETase activity was determined from  $\alpha$ -ketobutyrate produced from L-methionine according to the method of Tanaka *et al.* (1) with slight modification. 0.5 ml of sample diluted in 100 mM potassium phosphate buffer pH 8.0, containing 0.01% DTT, 1 mM EDTA Na<sub>2</sub>, 10  $\mu\text{M}$  PLP and 0.05% Tween 80, was mixed with 0.5 ml substrate solution containing 100 mM potassium phosphate buffer, pH 8.0, 25 mM L-methionine and 10  $\mu\text{M}$  PLP in a glass test tube. The reaction mixture was vortexed immediately and incubated at 37°C without shaking for precisely 10 min. The reaction was stopped by adding 0.5 ml of 50% TCA. The suspension was centrifuged at 13,000 rpm for 2 min. The supernatant (0.5 ml) was collected in a glass tube containing 1 ml 1 M acetate buffer, pH 5.0. Then, 0.4 ml MBTH solution containing 0.1% 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (Wako Pure Chemical, Osaka, Japan) was added to the tube, mixed well and incubated at 50°C for 30 min. The absorbance of the reaction mixture was measured at 320 nm. The assay was carried out in triplicate.  $\Delta\text{E}$  was calculated by subtracting the average absorbance of blanks from the average absorbance of the reaction mixture. The enzyme activity was calculated by the following equation: Activity (U/ml) = 0.548 (1.07 + 2.2 $\Delta\text{E}$ )  $\Delta\text{E}$ . One unit of enzyme is defined as the amount of enzyme which produced 1  $\mu\text{M}$   $\alpha$ -ketobutyrate per minute at an infinite concentration of L-methionine.

**SDS-Electrophoresis Analysis.** SDS-PAGE analysis of PEG-rMETase was carried out using 10% Novex polyacrylamide-precaster tris-glycine gels in Novex tris-glycine buffer with SDS according to the instruction manual. Gels were stained with Coomassie brilliant blue.

**Determination of PEGylation Degree of rMETase.** The degree of modification of PEGylated rMETase was estimated both by the fluorescamine assay (19) and by MALDI. For the fluorescamine assay, various amounts of rMETase and PEGylated rMETase in 2 ml of 0.1 M sodium phosphate

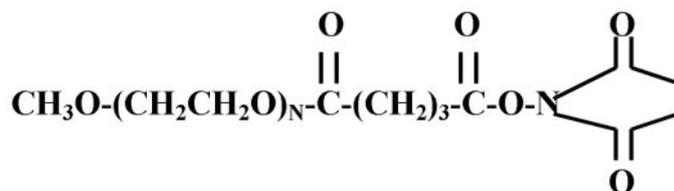


Fig. 1. Chemical structure of MEGC-50HS-PEG (methoxypolyethylene glycol succinimidyl glutarate-PEG).

buffer, pH 8.0 were mixed with 1 ml fluorescamine solution (0.3 mg/ml in acetone) and incubated for 5 min at room temperature. Samples were then assayed with a fluorescence spectrometer at 390 nm excitation and 475 nm emission. Results were plotted as fluorescence units *versus* concentration, with the slope of the line being determined by linear regression. The percent of PEGylated primary amines was determined according to the following formula:  $1 - (\text{slope PEGylated rMETase} / \text{slope naked rMETase}) \times 100$ . MALDI analysis of naked and PEG-rMETase was performed at the Scripps Research Institute using a PerSeptive Biosystems Voyager-Elite mass spectrometer.

**Plasma Methionine Determination.** The methionine level in the plasma was measured by pre-column derivatization, followed by HPLC separation (20). Briefly, 10  $\mu$ l of plasma sample or methionine standard was precipitated with 30  $\mu$ l of acetonitrile, followed by centrifugation at 10,000 rpm for 5 min. 10  $\mu$ l of the supernatant was mixed with 5  $\mu$ l of a fluoraldehyde derivative reagent, *o*-phthalaldehyde, for 1 min at room temperature, followed by addition of 150  $\mu$ l of 0.1 M sodium acetate, pH 7.0. 20  $\mu$ l of the reaction mixture was loaded on a reversed-phase Supelcosil LC-18DB column (25 cm  $\times$  4.8 cm, particle size 5  $\mu$ m (Supelco, Bellefonte, PA, USA). The amino acid derivatives were separated by using a gradient elution of 40–60% solution B (Methanol) in solution A (tetrahydrofuran/methanol/0.1M sodium acetate, pH 7.2; 5/95/900) at a flow rate of 1.5 ml/minute. A fluorescence spectrophotometer was used for detection: excitation at 350 nm and emission at 450 nm. The plasma methionine was identified by the retention time of a methionine standard solution and quantitated according to a methionine standard curve.

**Determination of Plasma PLP.** PLP in plasma was determined by HPLC using derivatization with sodium bisulfite in the mobile phase (21). Briefly, the plasma sample and PLP standard solutions were mixed with an equal volume of 0.8 M HClO<sub>4</sub> as deproteinizing agent, and vortexed vigorously. After centrifuging at 15000 rpm for 5 min at 4°C, the supernatants were taken and transferred to new vials. 50  $\mu$ l of the supernatant was loaded on a reversed phase Cosmosil 5C18-AR-II column (4.6  $\times$  150, Nacalai Tesque, Japan). The column was eluted with a gradient elution of 20–80% mobile phase B (30% acetonitrile/water v/v) in mobile phase A (0.1 M potassium dihydrogen phosphate buffer containing 0.1 M sodium perchlorate and 0.5 g/L sodium bisulfite, pH 3.0) at a flow rate of 1.0 ml/minute. A fluorescence spectrophotometer was used for detection: excitation at 300 nm and emission at 400 nm. The PLP peak was identified by the retention time of a PLP standard. The concentration of plasma PLP was calculated using a calibration curve.

**Pharmacokinetics and Methionine Depletion Efficacy *in Vivo*.** Athymic nude (*nu/nu*) mice aged 4 weeks (20–25g) were used for the study with 4 mice per group. Osmotic mini-pumps filled with 250  $\mu$ l PLP (0.5g/ml) were implanted s.c. Twenty-four hours after pump implantation, 80 units native rMETase or PEG-rMETase in 0.5 ml PBS, pH 7.4, were injected via the tail vein in mice with or without PLP pumps. 400  $\mu$ l blood was collected from the retroorbital plexus of each animal using heparinized capillary tubes. Blood was collected prior to injection, and 1 h, 2 h, 4 h, 8 h, 24 h, 48 h, 72 h, 96 h and 120 h post injection. The plasma was separated and stored in small aliquots at -80°C. The plasma enzyme activity, methionine concentration and PLP level in the plasma, collected at different time points, were measured as described above.

**Determination of Plasma Anti-rMETase Antibody.** Normal BALB/c male mice were grouped randomly at 5 per group. Each mouse received three i.p. injections of 0.2 ml (200  $\mu$ g) naked or PEG-rMETase emulsified in Freund's complete adjuvant (FCA) at weekly intervals. Two weeks following the last injection, a booster injection of the rMETase or PEG-rMETase was given to each mouse. Blood samples were collected two weeks after the booster injection, and plasma was separated and stored at -80°C.

Plasma anti-rMETase antibody was measured using a Sandwich ELISA technique (22). 100  $\mu$ l 200  $\mu$ g/ml rMETase in 0.1 M carbonate coating buffer, pH 9.5, was added to each well of a 96-well microplate and incubated at 4°C overnight. The plate was washed three times with PBS washing buffer, pH 7.4, containing 0.05% Tween-20, and blocked for 2 h at room temperature with 200  $\mu$ l of PBS assay buffer, pH 7.4, containing 10% FBS. After washing three times, 100  $\mu$ l of 10-fold serial dilutions of the plasma samples in PBS assay buffer were added to appropriate wells

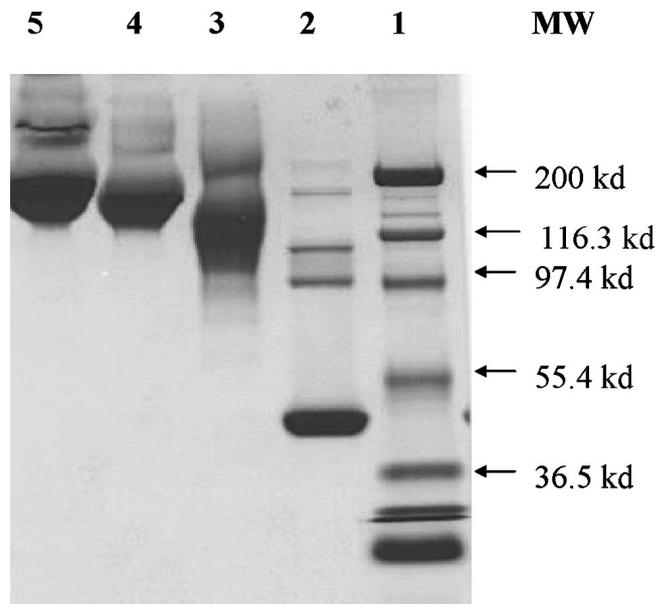


Fig. 2. SDS-PAGE of naked and PEGylated rMETase. The purified PEGylated rMETases were run on a 10% SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue. Numbers on the right indicate molecular mass. Lane 1, molecular mass markers; Lane 2, naked rMETase; Lane 3, PEG/rMETase synthesized at a ratio of 30/1; Lane 4, PEG/rMETase 60/1; Lane 5, PEG/rMETase 120/1 (see "Materials and Methods" for details).

and incubated for 2 h at room temperature, followed by washing. 100  $\mu$ l optimally diluted goat anti-mouse IgG and IgM subtypes conjugated with horseradish peroxidase (Sigma, St. Louis, MO) were added to each well. The plate was incubated for 1 h at room temperature and washed three times. 100  $\mu$ l substrate solution containing O-phenylenediamine dihydrochloride (OPD) and hydrogen peroxide (Sigma) were added to each well, followed by 30 min incubation at room temperature. 50  $\mu$ l 2N sulfuric acid were added to each well to stop the color reaction. The absorbance of each well was measured at 492 nm. The antibody titer was determined as the highest plasma dilution at which the extinction at 492 nm generated in the well of the immune plasma exceeded twice the extinction generated in those wells with negative control plasma.

## RESULTS

**SDS-PAGE Analysis of PEGylated rMETase.** Three rMETase conjugates were prepared using molar ratios of PEG/rMETase of 30/1, 60/1 and 120/1. The extent of PEG conjugation to rMETase and the purity of PEGylated rMETase conjugates were initially determined by SDS-PAGE (Fig. 2). All rMETase subunits were PEGylated after 90 min reaction at room temperature when the above molar ratios were used since no non-PEGylated rMETase subunits were detected by SDS-PAGE (Fig. 2) in the purified PEGylated rMETase conjugates. More PEG chains were conjugated to rMETase with increasing molar ratios of PEG to rMETase as seen by SDS-PAGE (Fig. 2). At low PEG/rMETase ratios, broad bands were observed on the gels, indicating the heterogeneity of PEGylated rMETase conjugates. When higher molar PEG/rMETase ratios were used in the reaction, less heterogeneity of PEGylated rMETase conjugates was observed (Fig. 2).

**Determination of PEGylation Degree by Fluorescamine Assay and MALDI.** The PEGylation degree of three PEGylated rMETase conjugates are shown in Table 1. The fluorescamine assay indicated approximately 33%, 61% and 81% of free lysines in rMETase were coupled with PEG chains at ratios of PEG/rMETase-30/1, PEG/rMETase-60/1 and PEG/rMETase-120/1, respectively. The data indicated corresponds to an average of 3, 5 and 7 PEGylated

Table 1 Determination of PEGylation degree of PEGylated rMETase conjugates

The degree of PEGylation of naked and PEGylated rMETase was determined using the fluorescamine assay and MALDI as described in "Materials and Methods." The results of fluorescamine assay were expressed as the percentage of PEGylated lysine groups in rMETase. The MALDI results were expressed as both the total molecular mass of PEGylated rMETase monomer and the calculated number of conjugated PEG polymers/rMETase monomer. Each PEG polymer attached to rMETase contributes approximately 5 kDa to the total molecular mass of PEGylated rMETase monomer.

	Fluorescamine assay (% of lysines conjugated)	MALDI	
		Molecular mass (kDa)	PEGs/rMETase monomer
Naked rMETase	0	42.3	0
PEG/rMETase 30	33	48.3–75.3	1–6
PEG/rMETase 60	61	59.8–81.0	3–7
PEG/rMETase 120	81	82.0–91.9	7–9

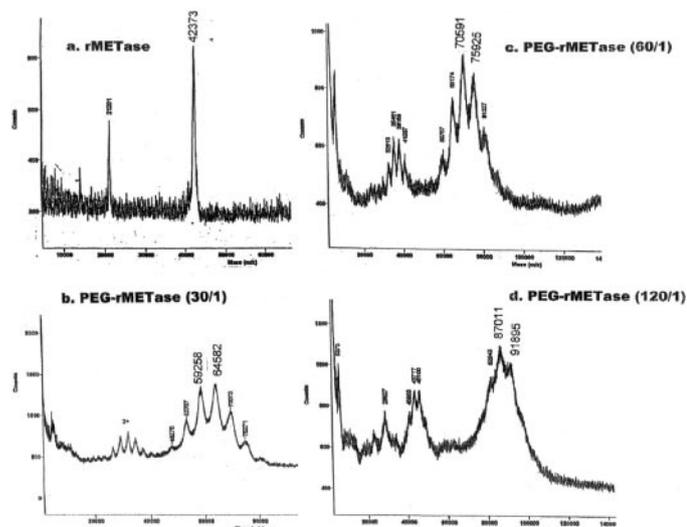


Fig. 3. MALDI spectrum of naked and PEGylated rMETase. MALDI analysis was performed using a PerSeptive Biosystem Voyager-Elite mass spectrometer. The last serial signal peaks at 42,373, 48,276–75,271, 59,757–81,027, and 82,043–91,895 Da represent naked rMETase, PEG/rMETase (30/1), PEG/rMETase (60/1), and PEG/rMETase (120/1) ion signals, respectively. The series of signal peaks before the naked rMETase and PEG-rMETase peaks are doubly charged species derived from the above parent ion signals.

lysines in each rMETase subunit, respectively, using the above coupling ratios.

MALDI analysis also demonstrated that a series of signal peaks were observed at an average of molecular mass of 64582, 70591 and 87011 Da for the three coupling ratios used, respectively (Fig. 3). These data are consistent with the covalent attachment of an average of 4, 6 and 8 PEG units to each rMETase subunit, respectively, for the three coupling ratios used. The signal peaks of PEGylated rMETase in the MALDI spectrum reflect the distribution heterogeneity arising from PEGylation of rMETase. It was found that at a higher PEGylation degree, fewer signal peaks in MALDI were observed, indicating less heterogeneity. For example, the PEG/rMETase 120 conjugate was the least heterogeneous (Fig. 3). Both MALDI and the fluorescamine assay demonstrate that an increase in PEG/rMETase molar ratios in the PEGylation reaction resulted in an increase in PEGylation of rMETase subunits.

**Plasma Circulating Half-Life of PEGylated rMETase.** Plasma enzyme activity of naked rMETase decreased rapidly and was undetectable in blood 24 h after injection of 80 units per mouse. However, PEGylated rMETase demonstrated a significant pharmacokinetic improvement. Plasma enzyme activity was detectable until 72 h when PEG/rMETase 60 and PEG/rMETase 120

were evaluated. The half-life for naked rMETase was 2 h in contrast to the half-life of 12 h, 18 h and 38 h for PEG/rMETase-30, PEG/rMETase-60 and PEG/rMETase-120, respectively (Fig. 4, Table 2A).

#### Plasma Methionine Depletion Efficacy of PEGylated rMETase.

Plasma methionine was depleted from a baseline of 40  $\mu\text{M}$  to less than 5  $\mu\text{M}$  within 1 h by 80 U naked and the three PEGylated conjugates (Fig. 5). However, PEG/rMETase-30, PEG/rMETase-60 and PEG/rMETase-120 depleted the plasma methionine level below 5  $\mu\text{M}$  for 8, 24, and 48 h, respectively, which is 2-, 6-, and 12-fold longer than naked rMETase (Table 2B).

**Effect of PLP on PEG-rMETase Function *in Vivo*.** rMETase is a PLP-dependent enzyme, with one molecule of PLP conjugated to each of the four subunits of rMETase. PLP delivery *in vivo* by mini-osmotic pump implantation significantly increased plasma PLP concentration (Fig. 6). 80 U of naked or PEGylated rMETase were injected i.v. in animals with or without PLP supplementation from implanted mini-osmotic pumps. Blood samples were taken at different time points and measured for plasma methionine concentration. Plasma methionine depletion with PLP supplementation was prolonged significantly (Fig. 7) compared to methionine

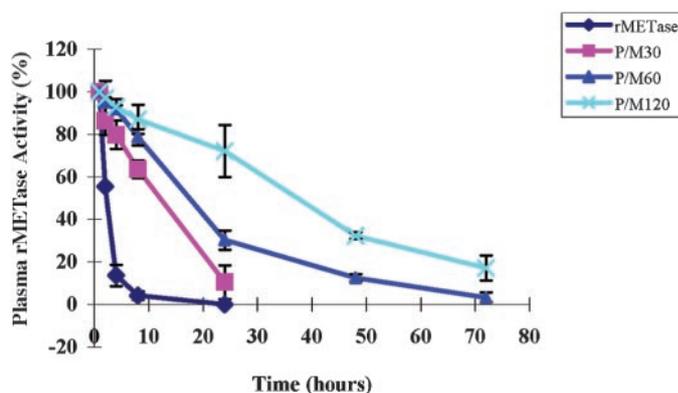


Fig. 4. Plasma rMETase enzyme activity after i.v. injection of naked and PEGylated rMETase in mice. The mice received i.v. injection of 80 units of naked rMETase or the indicated PEGylated rMETase. Blood samples were collected at different time points and measured for plasma enzyme activity as described in "Materials and Methods."

Table 2 Plasma half-life of PEGylated and naked rMETase with concomitant plasma methionine depletion with and without PLP

Naked or PEGylated rMETase (80 units) was injected i.v. in each mouse with or without PLP supplementation. Blood samples were collected at different time points and measured rMETase for enzyme activity and methionine concentration as described in "Materials and Methods." Plasma half-life was calculated from the plasma enzyme concentration-time profiles (Fig. 4). Maximum period of methionine depletion below 5  $\mu\text{M}$  was calculated from the plasma methionine levels in Figs. 5 and 7.

A. Plasma half-life of naked and PEGylated rMETase in mice		
	Plasma half-life in mice (h)	
Naked rMETase	2	
PEG/rMETase 30	12	
PEG/rMETase 60	18	
PEG/rMETase 120	38	
B. Maximum period of plasma methionine depletion below 5 $\mu\text{M}$ by naked and PEGylated rMETase with and without PLP supplementation		
	Maximum period of MET depletion below 5 $\mu\text{M}$ (h)	
	PLP <sup>-</sup>	PLP <sup>+</sup>
Naked rMETase	4	4
PEG/rMETase 30	8	48
PEG/rMETase 60	24	48
PEG/rMETase 120	48	72

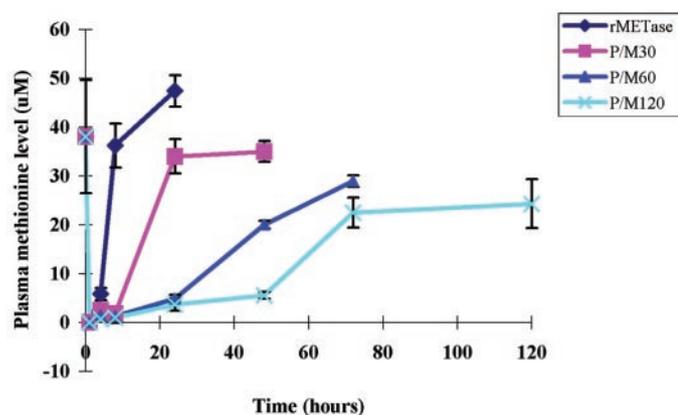


Fig. 5. Plasma methionine depletion after i.v. injection of naked and PEGylated rMETase in mice. The mice received i.v. injection with 80 units of naked rMETase or the indicated PEGylated rMETase. Blood samples were collected at different time points and measured for plasma methionine concentration as described in "Materials and Methods."

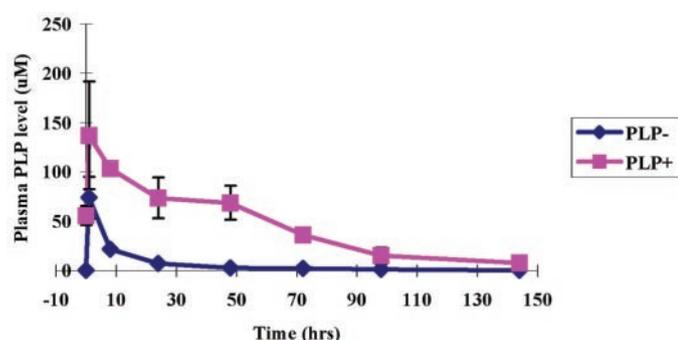


Fig. 6. Plasma PLP concentration with and without PLP pump implantation in mice. Mini-osmotic pumps filled with 250  $\mu$ l of 0.5 g/ml PLP were implanted s.c. in mice before i.v. injection of naked or PEGylated rMETase in PLP-containing buffer. Blood samples were collected at different time points and measured for plasma PLP concentration as described in "Materials and Methods." *PLP*<sup>-</sup> indicates the animals without PLP pumps. *PLP*<sup>+</sup> indicates the animals with PLP pumps.

depletion without PLP supplementation (Fig. 5), indicating PLP dependence for the *in vivo* methionine-depletion efficacy of PEG-rMETase. Plasma methionine levels depleted by the three PEGylated rMETase conjugates were maintained below 5  $\mu$ M for 48 h. Plasma methionine levels were depleted below 5  $\mu$ M for 72 h in case of PEG/rMETase 120 with PLP supplementation (Table 2B).

**High-Level PEGylation of rMETase Decreases PLP Dependence *in Vivo*.** To quantitate the effect of PEGylation on PLP dependence of rMETase, we calculated the ratios of the plasma methionine concentration remaining after treatment by PEG-rMETase without PLP supplementation (*PLP*<sup>-</sup>) (Fig. 5) versus that remaining after treatment with PEG-rMETase with PLP supplementation (*PLP*<sup>+</sup>) (Figs. 7 and 8). These ratios showed that PEG/rMETase 60 and PEG/rMETase 120 had lesser PLP dependence than PEG/rMETase-30 (Figs. 5, 7, 8), suggesting a protective effect of high-level PEGylation on retention of PLP by rMETase.

**Effect of PEGylated rMETase on Plasma Anti-rMETase-Specific Antibodies.** The antigenicity of naked and PEGylated rMETase was evaluated by measuring plasma anti-rMETase-specific IgG and IgM antibodies in normal BALB/c mice immunized with naked or PEGylated rMETase in the presence of FCA. As shown in Table 3, the plasma obtained from the mice immunized with PEG/rMETase-30, PEG/rMETase-60 and PEG/rMETase-120 produced IgG antibody titers of  $10^{-7}$ ,  $10^{-6}$ , and

$10^{-4}$ , respectively, as compared to the titer of  $10^{-8}$  produced from naked rMETase. For IgM antibody, an antibody titer of  $10^{-3}$  was detected in the mice immunized with three PEGylated rMETase conjugates, which was lower than the antibody titer of  $10^{-4}$  produced from naked rMETase.

## DISCUSSION

### Effect of rMETase Concentration in the PEGylation Reaction.

It was found that the higher the rMETase concentration in the PEGylation reaction, the greater the modification of rMETase. Therefore, we concentrated rMETase as high as possible to 120 mg/ml rMETase for the PEGylation reaction.

**Effect of Extent of PEGylation on the Heterogeneity of the PEGylated rMETase Conjugate.** Heterogeneity of PEGylated rMETase was observed by both SDS-PAGE and MALDI. PEG, being a synthetic polymer, is polydispersed, which contributes to the heterogeneity of PEGylated conjugates. Ideally, a polydispersity value (*M<sub>w</sub>/M<sub>n</sub>*) ranging approximately from 1.01 for low molecular weight oligomers (3–5 kDa), to 1.2 for high molecular weight (20 kDa) may be expected for PEGylation of proteins and peptides (23). Besides polydispersity of PEG, it was found that the PEG/rMETase molar ratio influenced the heterogeneity of the resulting conjugate (Figs. 2 and 3; Table 1). Higher PEG/rMETase

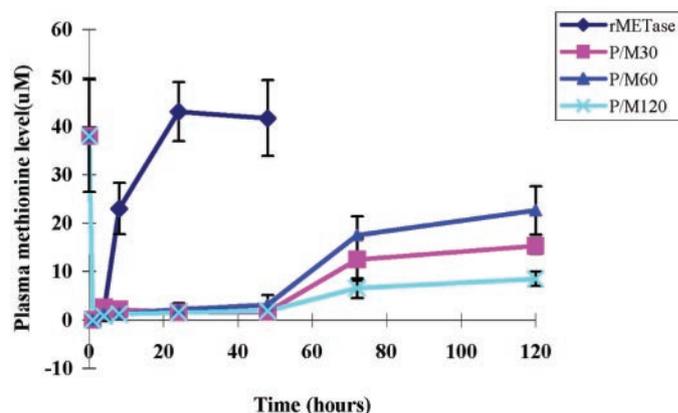


Fig. 7. Plasma methionine depletion after PLP pump implantation and i.v. injection of naked and pegylated rMETase. Eighty units of naked or indicated PEGylated rMETase were injected i.v. into each mouse after PLP pump implantation. Blood samples were collected at different time points and measured for plasma methionine concentration as described in "Materials and Methods."

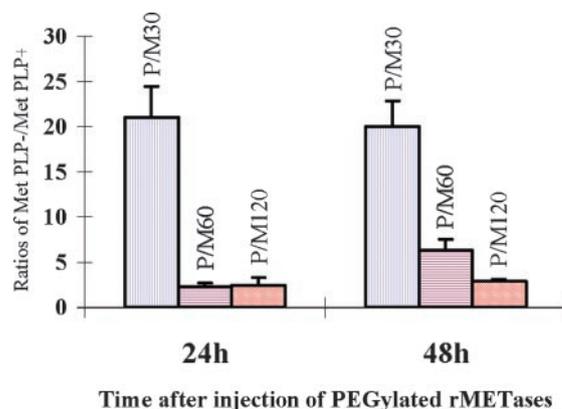


Fig. 8. Ratios of the plasma methionine level depleted by the indicated PEGylated rMETase without and with PLP supplementation. See Figs. 5 and 7 for original data.

Table 3 Plasma anti-rMETase-specific antibody titers induced by naked or PEGylated rMETase in mice

IgG and IgM anti-rMETase antibody titers of the plasma of the normal BALB/c mice immunized with naked or indicated PEGylated rMETase in the presence of FCA were determined using ELISA as described in "Materials and Methods." The numbers in parentheses represent the numbers of mice.

	IgG	IgM
Naked rMETase	$10^{-8}$ (5)	$10^{-4}$ (4), $10^{-5}$ (1)
PEG/rMETase 30	$10^{-7}$ (4), $10^{-6}$ (1)	$10^{-3}$ (5)
PEG/rMETase 60	$10^{-6}$ (5)	$10^{-3}$ (5)
PEG/rMETase 120	$10^{-4}$ (4), $10^{-5}$ (1)	$10^{-3}$ (5)

molar ratios resulted in less heterogeneity of the PEGylated rMETase conjugate.

**Comparison of the Pharmacokinetics of MEGC-PEG-rMETase and M-SPA-PEG.** Pharmacokinetic data of the PEGylated rMETase conjugates indicated that the plasma circulating time depended on the PEGylation degree (Fig. 4). Compared to the plasma half-life of 160 min for the rMETase PEGylated with M-SPA-PEG (17), rMETase PEGylated with MEGC-PEG demonstrated a longer circulating time in blood. The half-life of PEG/rMETase-30, PEG/rMETase-60 and PEG/rMETase-120 was prolonged to 12 h, 18 h and 38 h, respectively (Table 2). This improved pharmacokinetic property may reflect the higher PEGylation efficiency of MEGC-PEG than compared to M-SPA-PEG for rMETase.

**In Vivo Effect of PLP on PEG-rMETase.** We used 5  $\mu$ M plasma methionine concentration as an end point for methionine depletion since it was reported that plasma methionine depletion below 5  $\mu$ M was an effective therapeutic level of rMETase antitumor efficacy using mouse models of human cancer (24). Without PLP supplementation this level of methionine depletion could be achieved for 8 h by PEG/rMETase-30; 24 h by PEG/rMETase-60; and 48 h by PEG/rMETase-120. With supplementation, depletion to less than 5  $\mu$ M methionine could be achieved for 48 h for PEG/rMETase-30 and PEG/rMETase-60; and 72 h by PEG/rMETase-120. These data suggest that PLP supplementation may be important for the antitumor efficacy of PEG-rMETase.

The protective effect of PEGylation on the apparent *in vivo* retention of PLP by rMETase was an unexpected result as was the rapid loss *in vivo* of PLP by naked rMETase and low-degree PEGylated rMETase. PLP *in vitro* is relatively tightly bound to rMETase (25), but *in vivo* PLP appears to readily dissociate from rMETase. The recognition of the PLP effect which resulted in evidence of *in vivo* stabilization and retention of enzyme and prolonged efficacy in terms of reduction in plasma methionine levels is a most remarkable and potentially important therapeutic issue. The PLP effect further enhances the potential for utilization of PEG-rMETase as a clinical agent and suggests that it may have an important role in exploiting other PEGylated enzymes. The mechanism of dissociation of PLP from rMETase and apparent inhibition of dissociation by high-level PEGylation will be investigated in future studies.

Plasma anti-rMETase-specific antibody determination showed that PEGylated rMETase could reduce the antigenicity of rMETase in mice. Plasma IgG antibody is a critical antibody subtype which is related to hypersensitivity reactions and antibody neutralization of foreign proteins *in vivo*. PEG-rMETase demonstrated a significant decrease in antigenicity. For example, plasma anti-rMETase IgG antibody titer was reduced to  $10^{-4}$  by PEG/rMETase 120 as compared to  $10^{-8}$  for naked rMETase. Reduction in plasma IgG antibody depended on the number of PEG-derivatized amino

groups, indicating the decreased antigenicity of PEGylated rMETase is a consequence of masking the protein antigenic sites by the polymer modification. Future studies will involve IgE studies, since this class of antibody may mediate hypersensitivity/allergic reactions.

In conclusion, a high degree of modification of rMETase could be generated with MEGC-PEG with long-term retention of *in vivo* efficacy of methionine depletion. These PEGylated rMETase conjugates demonstrated increased pharmacokinetic efficacy by increasing plasma half-life and partially decreasing the PLP dependence of rMETase *in vivo*. Moreover, reduced antigenicity of rMETase was also achieved by PEGylated rMETase. Thus, molecular modification of PEGylation confers critical new properties to rMETase for its development as a novel cancer therapeutic.

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## *In Vivo* Efficacy of Recombinant Methioninase Is Enhanced by the Combination of Polyethylene Glycol Conjugation and Pyridoxal 5'-Phosphate Supplementation

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