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Polyethylene Glycol Conjugation of Recombinant Methioninase for Cancer Therapy

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Recombinant methioninase (rMETase) is a homotetrameric pyridoxal 5′-phosphate enzyme of 172-kda molecular mass derived from *Pseudomonas putida* and cloned in *Escherichia coli.* rMETase has been found previously to be an effective, anti-tumor agent *in vitro* and *in vivo.* The enzyme targets the elevated minimal methionine requirement seen in all tumor types. In order 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) has been coupled to rMETase. Molar ratios of M-SPA-PEG-5000 (PEG) to rMETase from 10 to 40 were used for PEGylation of rMETase. PEGylation reactions were run at 20°C for 30 to 60 min in reaction buffer (20 mM sodium phosphate buffer, pH 8.3). The PEGylated molecules (PEG-rMETase) were purified from unreacted PEG with Amicon 30 K centriprep concentrators or by Sephacryl S-300 HR gel-filtration chromatography. Unreacted rMETase was removed by DEAE Sepharose FF anion-exchange chromatography. The resulting PEG-rMETase subunit, from a PEG/rMETase ratio of 30/1 in the synthetic reaction, had a molecular mass of approximately 53 kda determined by matrix-assisted laser desorption/ionization mass spectrometry, indicating the conjugation of two PEG molecules per subunit of rMETase and eight per tetramer. PEG-rMETase molecules obtained from reacting ratios of PEG /rMETase of 30/1 had enzyme activities of 70% of unmodified rMETase. 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 0.1 μM for approximately 8 h compared to 2 h for rMETase in rats. Efficacy studies of PEG-rMETase on human lung cancer and kidney cancer cells *in vitro* demonstrated a 50% inhibitory concentration (IC_{50}) of 0.04 and 0.06 units/ml, respectively. These IC_{50} values were almost identical to unmodified rMETase, thus indicating maintenance of antitumor efficacy in the PEGylated enzyme. PEG-rMETase had an IC_{50} for normal lung and kidney cells of 0.8 and 1.5 units/ml, respectively, similar to rMETase. The efficacy data indicated that PEG-rMETase maintained the high level tumor selectivity of rMETase. PEG-rMETase injected intravenously in mice demonstrated a tumor/blood retention ratio of approximately 1/6 compared to 1/10 of unmodified enzyme, indicating that PEG-rMETase distributes to the tumor at least as effectively as rMETase.

A tumor-selective target with high therapeutic potential is the elevated minimum methionine dependence of most and possibly all types of tumor cells relative to normal cells (1–12). The L-methionine α-deamino-γ-mercaptomethane lyase (methioninase, METase) gene from *Pseudomonas putida* has been previously cloned in *Escherichia coli* (12–14). A scale-up recombinant methioninase (rMETase) production protocol has been established with high yield (60%), high purity (98%), high stability, and low endotoxin for preclinical and clinical studies targeting the methionine dependence of human cancer (12).

Studies of the antitumor efficacy of rMETase *in vitro* and *in vivo* on human tumors xenografted in nude mice demonstrated that all types of human tumors tested were sensitive to rMETase (12). In contrast, normal cells were relatively insensitive to rMETase *in vitro* and, correspondingly, no toxicity was detected *in vivo* at the effective doses (12).

However, as with many other bacterial polypeptides and proteins, rMETase may be immunogenic in higher animals, which may limit the utility of rMETase especially with regard to multiple dosing. Anti-METase antibodies may accelerate methioninase clearance and consequently reduce its therapeutic effectiveness and may also reduce the enzyme potency by binding at the active site.

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Various approaches have been taken in attempts to solve the problem of antigenicity of polypeptides and proteins. Coupling of polyethylene glycol (PEG) to proteins is one of the most effective approaches to reducing protein antigenicity (17–23). Furthermore, PEG modification improves the pharmacological properties of proteins, usually extending plasma half-life and reducing both antigenicity and immunogenicity, and often increasing *in vivo* bioactivity (17–23). For example, polyethylene-glycol-conjugated recombinant human megakaryocyte growth and development factor, a polypeptide related to thrombopoietin, is approximately 10 times more potent *in vivo* than the unconjugated polypeptide and is active in humans (18).

L-asparaginase is used in the treatment of hematological malignancies. Adverse reactions such as an allergic reaction leading to anaphylactic shock have limited its clinical use (19). In addition, due to its rapid plasma clearance, large doses and repeated administration are required to achieve clinical effects (19). Polyethylene-glycol-conjugated L-asparaginase has reduced antigenicity and a very long half-life while maintaining high activity in hematological malignancies (19).

We report here that polyethylene glycol conjugation of methioninase is feasible, results in readily purified molecules, maintains the potency of the enzyme, extends its serum half-life, and maintains its tumor-specific efficacy.

MATERIALS AND METHODS

Materials

The *N*-hydroxysuccinimidyl ester of methoxypolyethylene glycol propionic acid (M-SPA-PEG, MW 5000) was purchased from Shearwater Polymers Inc. (Huntsville, AL). Other chemicals were purchased from Sigma (St. Louis, MO). Cell culture medium was purchased from Fisher Scientific (Pittsburgh, PA).

Methods

Production of rMETase

High-expression clone of rMETase and fermentation conditions. The pAC-1 rMETase high-expression clone, constructed in our laboratory, was used for the production of rMETase. The fermentation procedure for host *E. coli* cells and the purification protocol for rMETase were as previously described (12). pAC-1 was constructed with the pT7-7 vector containing the T7 RNA polymerase promotor for high expression of the rMETase gene cloned from *Pseudomonas putida* as previously described (12). The host *E. coli* were grown in Terrific Broth in shaker flasks and harvested when the OD_{600} reached 20. The expression level of rMETase reached approximately 1 g/liter of fermentation broth under these conditions (12).

Purification of rMETase

Precolumn treatment. The bacterial pellet was disrupted with a cavitator-type homogenizer (Microfluidics Corp., Newton, MA, model No. HC 8000). Heat treatment of the homogenate was carried out at 50°C for 1 min. After centrifugation, the pH of the supernatant was adjusted to 7.2 by ultrafiltration (12).

DEAE Sepharose FF (pH 7.2) (Pharmacia, Uppsala, Sweden). Total protein (40–80 g) was loaded on a DEAE Sepharose FF column (100/30). rMETase was eluted with a linear gradient of 40 to 200 mM potassium chloride in 10 mM potassium phosphate buffer pH 7.2. The fractions containing rMETase were identified by activity assay (12).

DEAE Sepharose FF (pH 8.3). After dialysis, the pH of the sample was adjusted to 8.3, and 4–5 g of total protein (2–5 mg/ml) was applied on the column (50/30) (12). rMETase was eluted with a linear gradient of 80 to 200 mM potassium chloride in 10 mM potassium phosphate buffer (pH 8.3). The fractions containing rMETase were identified by activity assay (12).

Endotoxin removal by Acticlean Etox (Sterogen, Arcadia, CA). After dialysis in 0.15 M sodium chloride and 10 mM sodium phosphate buffer, pH 7.2, purified rMETase (10-20 mg protein/ml) was applied on an Acticlean Etox column (25/60). rMETase was eluted with elution buffer (0.12 M sodium chloride in 10 mM sodium phosphate, pH 7.2). The fractions containing rMETase were identified by activity assay (12).

PEGylation of rMETase

PEG linkers. The M-SPA-PEG 5000 was used for rMETase PEGylation.

PEGylation reaction. PEG reagents were dissolved in 20 mM sodium phosphate buffer (pH 8.3) at concentrations between 2 and 20 mM. The molar ratios of PEG to rMETase were varied from 10:1 to 60:1. For each condition, 0.3–0.5 g rMETase (15 mg/ml) was used. PEGylation reactions were carried out in reaction buffer (20 mM sodium phosphate buffer, pH 8.3), at 20°C for 30 to 60 min. The reactions were stopped with one-tenth volume stop buffer (1 M sodium phosphate buffer, pH 6.5) at 0°C (17). PEG-rMETase was then concentrated by a Centriprep-30 (Amicon Inc., Beverly, MA).

Purification of PEG-rMETases

Sephacryl S-300. Unreacted PEG was removed by size-exclusion chromatography Sephacryl S-300 HR column (HiPrep 26/60, Pharmacia), with a diameter of 26 nm and length of 60 cm (23). Ten milliliters of PEGylation products (15 mg/ml) were loaded on the column which was equilibrated and eluted with 0.15 M sodium chloride in 10 mM sodium phosphate (pH 7.4) at a flow rate of 1 ml/min. The fractions containing

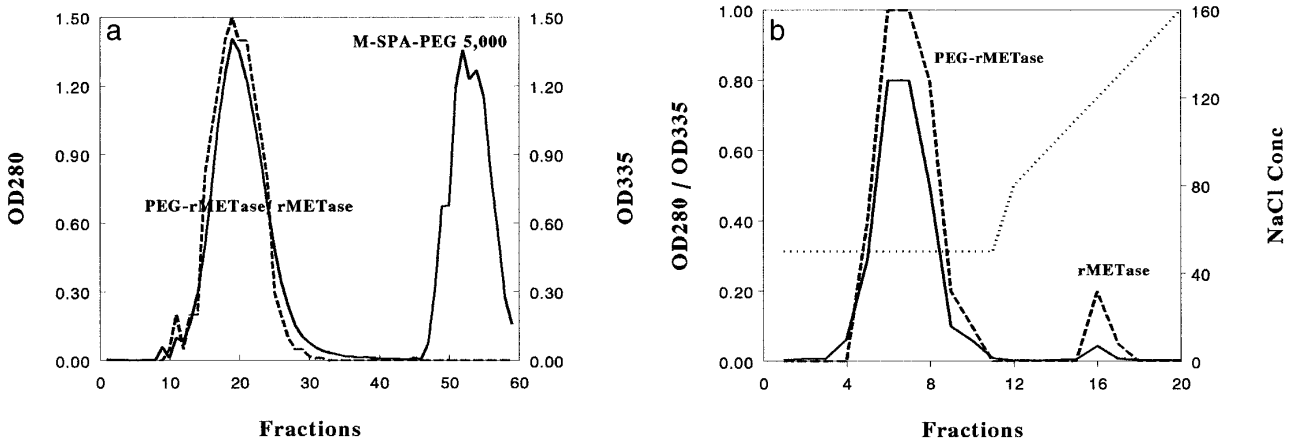

FIG. 1. Purification of PEG-rMETase. (a) Sephacryl S-300 HR chromatography. Unreacted M-SPA- PEG 5000 was removed by size-exclusion chromatography using Sephacryl S-300 HR (HiPrep 26/60, Pharmacia). Ten milliliters of PEGylation reaction product were loaded on the column which was eluted with 0.15 M sodium chloride in 10 mM sodium phosphate buffer (pH 7.4) at a flow rate of 1 ml/min. Five-milliliter fractions were collected. Peak 1 contained PEG- rMETase and rMETase. Peak 2 contained M-SPA-PEG 5000. Identify was confirmed by OD_{280}, activity assay, and electrophoresis on SDS–PAGE using standards indicated under Materials and Methods. (b) DEAE Sepharose FF chromatography. Un-PEGylated rMETase was removed by DEAE Sepharose FF chromatography (XK 16/15, Pharmacia). Twenty milliliters of peak 1 from the Sephacryl S-300 HR chromatography were loaded on the column. PEG-rMETase (peak 1) was eluted with 50 mM sodium chloride in 10 mM sodium phosphate, pH 7.4, while the rMETase (peak 2) was eluted with 120 mM sodium chloride in 10 mM sodium phosphate pH 7.4. The flow rate was 3 ml/min. Identify was confirmed by activity assay and electrophoresis on SDS–PAGE using standards indicated under Materials and Methods.

PEG-rMETase and rMETase were eluted at approximately 150–250 ml and identified by activity assay.

DEAE Sepharose FF. PEGylated rMETase was separated from un-PEGylated rMETase by anion-exchange chromatography using a DEAE Sepharose FF column (XK 16/15, Pharmacia) with a diameter of 16 mm and length of 15 cm (12, 22). The column was equilibrated with equilibration buffer [50 mM sodium chloride in 10 mM sodium phosphate (pH 7.4)] and eluted with a 0–60% linear gradient of 300 mM sodium chloride in 10 mM sodium phosphate (pH 7.4) at a 3 ml/min flow rate. The fractions containing PEG-rMETase were eluted with approximately 50–80 ml equilibration buffer and were indentified by activity assay and electrophoresis on SDS–PAGE.

Sterilization and stability of PEG-rMETase. The PEG-rMETases were sterilized by filtration with a 0.2 μM micrometer membrane filter. PEG-rMETase was stored at −80°C for 6 months without loss of activity.

Analysis of PEG-rMETase

Electrophoresis. PAGE was carried out in 7.5% polyacrylamide-precasted plates in 0.2 M Tris–glycine buffer, pH 8.3, both with 0.1% SDS (SDS–PAGE) and without SDS (native–PAGE) using Kaleidoscope prestained standards (Bio-Rad, Hercules, CA) as molecular weight markers which contains myosin (MW 192 k), β-galactosidase (MW 127 k), bovin serum albumin (MW 73 k), carbonic anhydrase (MW 43 k), soybean trypsin inhibitor (MW 32.3 k), lysozyme (MW 17 k), and aprotinin (MW 6.6 k) (12).

Potency activity assay. The assay was carried out in a 1-ml volume of 50 mM phosphate buffer pH 8.0, containing 10 μM pyridoxal phosphate and 10 mM methionine for 10 min at 37°C with varying amounts of enzyme. The α-ketobutyrate product of the enzyme reaction was detected with 3-methyl-2-benzothiazolinone hydrazone measured at OD_{335}. The specific activity was

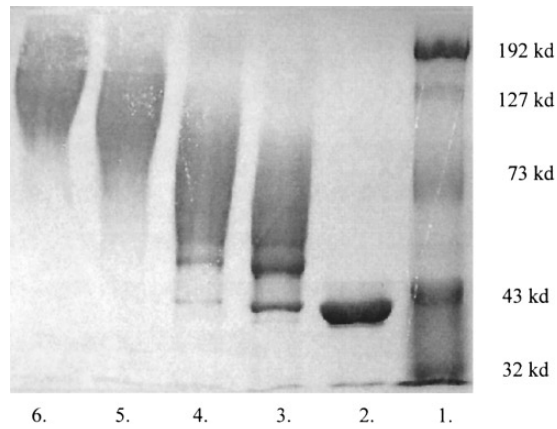

FIG. 2. SDS–PAGE. rMETase or PEG-rMETase (20 μg) was loaded on SDS–PAGE. Electrophoresis was run at 180 V for 30 min. The migration rates of PEG-rMETase molecules were much slower than those of rMETase due to the conjugation of PEG molecules and subsequent hydration. SDS–PAGE demonstrated that the subunits of rMETase were all linked with PEG at P/M ratios greater than 30/1. Lane 1, molecular weight markers; 2, rMETase; 3, PEG-rMETase (P/M = 10/1); 4, PEG-rMETase (P/M = 30/1); 5, PEG-rMETase (P/M = 60/1); 6, PEG-rMETase (P/M = 120/1). P/M, ratio of M-SPA-PEG 5000 to rMETase.

TABLE 1

Activity of PEG-rMETase

Enzyme	PEGylation molar ratio of PEG/rMETase	Specific activity (units/mg) ± SD	Relative activity compared to rMETase (% ± SD)
rMETase	—	20	100
PEG-rMETase	20	14.5 ± 1	75 ± 5
PEG-rMETase	30	12 ± 1	60 ± 5
PEG-rMETase	40	10 ± 1	50 ± 5
PEG-rMETase	60	8 ± 1	40 ± 5

Note. rMETase was PEGylated with M-SPA-PEG 5000 at the different molar ratios of PEG and rMETase under conditions described under Materials and Methods. The specific activity was measured and the relative activity to rMETase was calculated as described under Materials and Methods. The experiments were repeated twice and the standard deviations are given.

calculated as units per milligram protein, with 1 unit of enzyme defined as the amount that catalyzes the formation of 1 μmol of α-ketobutyrate per minute (12).

Endotoxin assay. The endotoxin level was measured with the *Limulus* amebocyte lysate test (Bio Whittaker, Walkersville, MD). The concentration of endotoxin was measured at a wavelength of 410 nm (12).

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was performed with a PerSeptive Biosystems Voyager-Elite mass spectrometer with delayed extraction. Samples were irradiated with a nitrogen laser (Laser Science, Inc.) operated at 337 nM. The laser beam was attenuated by a variable attenuator and focused on the sample target. Ions produced in the ion source were accelerated with a deflection voltage of 30,000 V. The ions were then differentiated according to the *m*/*z* using a time-of-flight mass analyzer (23).

Serum Half-life of PEG-rMETase

In vivo serum clearance rates for both rMETase and PEG-rMETase were analyzed in 8-week-old male Donryu rats with body weights of 180–200 g. Purified PEG-rMETase or rMETase (180 units) was injected into the tail vein. Blood was collected at the following time points : before injection, 5 min, 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, and 8 h after injection. Enzyme activity was measured as described above and the half-life of PEG-rMETase and that of rMETase were calculated.

Serum Methionine Measurement

Serum methionine measurement was performed by HPLC (Hitachi L-6200A Intelligent pump, Hitachi, Ltd, Tokyo, Japan) after derivitization of serum amino acids with a fluoraldehyde reagent, ophthaldialdehyde (OPA) (16). A methionine standard (Sigma) was derivitized by OPA. Serum samples (25 μl) were precipitated by acetonitrile (75 μl). Ten microliters of supernatant were mixed with 5 μl of OPA. After 1 min, 50 μl of 0.1 M sodium acetate (pH 7.0) was added, and a 20 μl sample was loaded on a reversed-phase Supelcosil LC-18-DB (particle size 5 μm, 25 cm $\times$ 4.8 mm) column at room temperature. Resolution of the amino acid derivatives was accomplished with solution A (tetrahydrofuran/methanol/0.1M sodium acetate, pH 7.2:5/95/900) and solution B (methanol). A gradient from 20–60% was run at a flow rate of 1.5 ml/min. The eluate was read by a fluorescence spectrophotometer (Hitachi, F1000) at 350–450 nm. The limit of detection was approximately 0.1 μM methionine.

Organ Distribution of rMETase and PEG-rMETase in Mice after iv Injection

rMETase and PEG-rMETase (60 units) were injected into the tail vein of three BALB/c *nu*/*nu* mice with subcutaneously growing human colon cancer HCT 15. After 1 h, the mice were sacrificed, and the tumor, liver, kidney, spleen, intestine, heart, lung, muscle, and skin were collected and weighed. The tissues were thoroughly washed before analysis such that any residual blood methioninase was eliminated. The tissues were then sonicated, and the PEG-rMETase or rMETase levels were determined by activity assay. The levels of PEG-rMETase or rMETase in tissues were calculated as units per milligram protein of tissue.

Growth Inhibitory Effect of PEG-rMETase on Human Cancer Cells in Vitro

Human normal and tumor cells were incubated in methionine-containing RPMI 1640 medium supplemented with 10% fetal bovine serum with PEG-rMETase or rMETase (0.005–4 units/ml) for 72 h at 37°C/5% CO_2. After the 72-h incubation period, the cells were counted with a hemocytometer and relative growth rates were calculated.

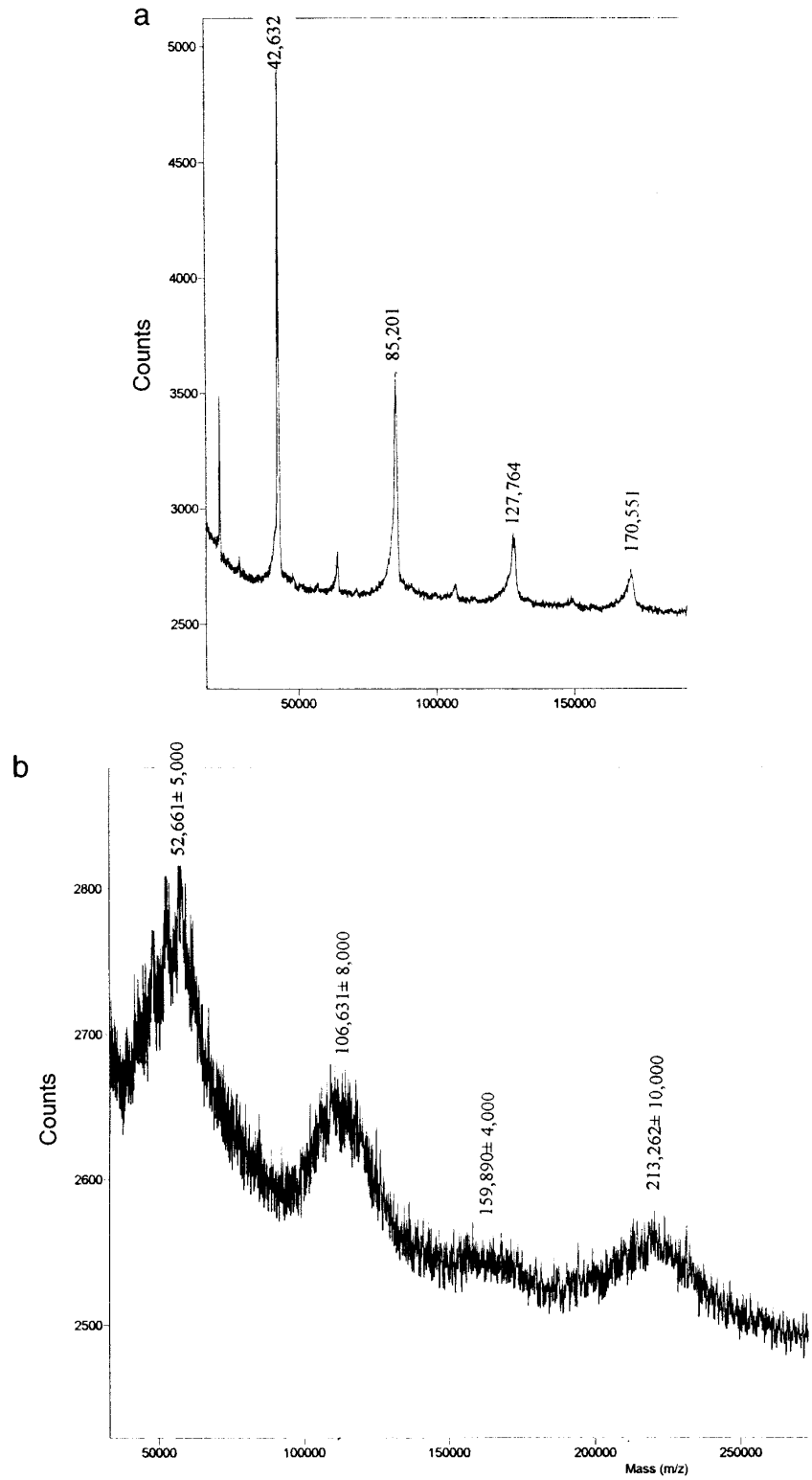

FIG. 3. MALDI mass spectrometry. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was performed on a PerSeptive Biosystems Voyager-Elite mass spectrometer with delayed extraction. Samples were irradiated with a nitrogen laser (Laser Science, Inc.) operated at 337 nM as described under Materials and Methods. (a) MALDI mass spectrometry of rMETase. MALDI mass spectrometry results indicated that there are four distinct sharp signals corresponding to the integer numbers of the r-METase subunit (42,632, monomer; 85,201, dimer; 127,764, trimer; 170,551, tetramer). (b)MALDI mass spectrometry of PEG-rMETase. MALDI mass spectrometry results indicated PEG-rMETase contained mainly four broad peaks, corresponding to molecular weights 52,661 ± 5000, monomer; 106,631 ± 8000, dimer; 159,890 ± 4000, trimer; 213,262 ± 10,000, tetramer, respectively. These molecular weights are consistant with two M- SPA-PEG 5000 molecules linked to each subunit. The broadness of the signals is due to molecular heterogeneity of SPA-PEG 5000.

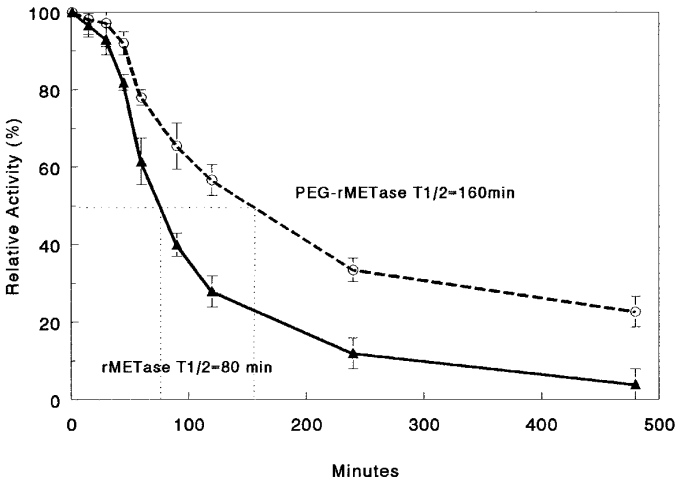

FIG. 4. Pharmacokinetics of PEG-rMETase. One hundred eighty units of purified PEG-rMETase from the PEGylation reaction ratio of PEG/rMDETase 30/1 or purified rMETase was injected into the tail vein of Donryu rats. The blood was collected at the following time points: before injection, 5 min, 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, and 8 h after injection. Serum enzyme activities were measured as described under Materials and Methods.

RESULTS AND DISCUSSION

Purification of PEG-rMETase

After the conjugation reaction of rMETase and M-SPA- PEG 5000, Sephacryl S-300 HR was used to remove the excess free PEG. The PEG-rMETase and rMETase were eluted in the exclusion volume (Fig. 1a). Free M-SPA-PEG 5000 eluted in later fractions.

The un-PEGylated rMETase was separated from rMETase by DEAE Sepharose FF column chromatography. PEG-rMETase eluted early before the gradient was applied due to the reduced charge, while rMETase eluted after application of the gradient (Fig. 1b). The results suggest that rMETase was efficiently conjugated with PEG. Identify was confirmed with electrophoresis on both SDS–PAGE and native–PAGE (Fig. 2). PEG-rMETase had a much larger molecular mass than rMETase, which is 172 kda on native–PAGE. The subunits of PEG-rMETase were also larger than the subunits of rMETase, which have a molecular mass of 43 kda.

Purity of PEG-rMETase

Electrophoretic analyses by SDS–PAGE demonstrated PEGylation of rMETase at PEG/rMETase (P/M) ratios from 10–120 (Fig. 2). The migration rates of PEG-rMETase molecules were much slower than those of rMETase due to the conjugation of PEG molecules and subsequent hydration. SDS–PAGE demonstrated that the subunits of rMETase were all linked with PEG at P/M ratios greater than 30. There was a slight band at 43 kda on SDS–PAGE of PEG-rMETase synthesized at P/M ratios of 10–30 (Fig. 2), which might be due to PEG molecules released from some subunits during denaturation with SDS and heating to 94°C.

Enzyme Activity of PEG-rMETase

The enzyme activity of PEG-rMETase depended on the P/M ratio. The PEG-rMETase synthesized at P/M ratios of 20 to 30 had activities of approximately 60–70% that of rMETase. At higher P/M ratios of 60 to 120, the resulting PEG-rMETase had activities of approximately 40–20% that of rMETase (Table 1).

Extent of Derivitization of rMETase with PEG

MALDI mass spectrometry results confirmed that rMETase (molecular mass 172 kda) is composed of four identical subunits of MW 43 kda (Fig. 3a). MALDI results for PEG-rMETase indicated that PEG-rMETase contained mainly four broad peaks, corresponding to molecular weights 52,661 ± 5000 and 106,631 ± 8000 for the monomer and dimer, respectively. These molecular weights are consistent with each subunit linked to two M-SPA-PEG 5000 molecules. The broadness of the signals is due to molecular heterogeneity of M-SPA-PEG 5000 (Fig. 3b).

Half-life of PEG-rMETase in Vivo

The $t_{1/2}$ in serum of PEG-rMETase after iv injection in rats was approximately 160 min compared to approximately 80 min of rMETase (Fig. 4). The results demonstrated that the serum half-life of PEG-rMETase increased to approximately twice that of rMETase when given by iv injection.

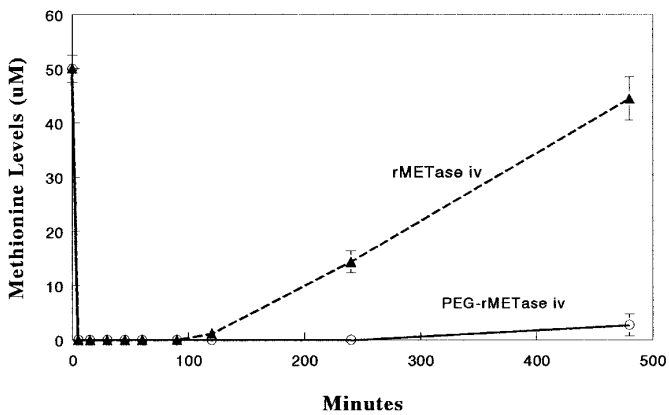

FIG. 5. Depletion of serum methionine by PEG-rMETase. One hundred eighty units of purified PEG-rMETase from the PEGylation reaction ratio of PEG/ rMDETase 30/ 1 or purified rMETase was injected into the tail vein of Donryu rats. The blood was collected at the time points indicated as described in Fig. 4. Serum methionine levels were measured as described under Materials and Methods.

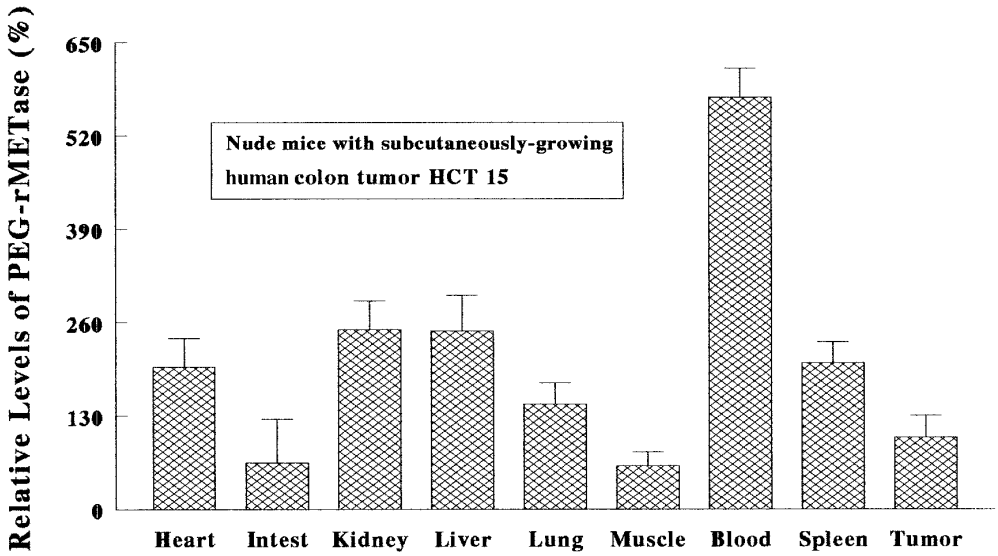

FIG. 6. Organ distribution of iv-administered PEGrMETase and rMETase in human tumor-bearing mice. Sixty units of rMETase or PEG-rMETase were injected into the tail vein of three BALB/c *nu/nu* mice with subcutaneously growing human colon cancer HCT 15. After 1 h, the mice were sacrificed, and the tumor, liver, kidney, spleen, intestine, heart, lung, muscle, and skin were collected and weighed. The tissues were then sonicated, and the rMETase levels were determined by activity assay. The relative levels of rMETase were calculated as units per milligram protein of tissue.

Depletion of Methionine in the Serum

The depletion of methionine in rat serum by iv injection of 180 units PEG-rMETase reached maximum depletion from 50 to 0.1 μM within 5 min after the start of the infusion. Methionine depletion to approximately 0.1 μM was maintained for at least 8 h after a single iv injection of PEG-rMETase, which was much more effective than rMETase, which could deplete methionine after one injection but could only maintain depletion at 0.1 μM for 2 h (Fig. 5). These levels of depleted methionine correspond to therapeutic levels for cancer cells *in vitro* (M. Xu., Y. Tan, and R. M. Hoffman, *et al.*, unpublished data).

Organ Distribution of PEG-rMETase in Mice after iv Administration

After iv administration to nude mice, the tissue distribution of PEG-rMETase was found to be in the following decreasing order: blood, kidney, liver, spleen, heart, lung, tumor, intestine, and muscle (Fig. 6). Significant PEG-rMETase levels accumulated in the tumor. One hour after iv injection of 60 units of PEG-rMETase, levels were approximately 0.026 units/mg protein in human colon tumor HCT 15 grown subcutaneously in nude mice compared to 0.017 units/mg protein for rMETase. These results demonstrated that PEG-rMETase could accumulate in the tumor at least as well as rMETase.

Growth Inhibitory Effect of PEG-rMETase on Human Cancer and Normal Cells in Vitro

Human lung cancer cell line H460 and human kidney cancer cell line SN12C had PEG-rMETase IC_{50}s (concentrations which resulted in 50% inhibition of cell growth) of 0.04 and 0.06 units/ml, respectively, *in vitro.*

TABLE 2

IC_{50} of PEG-rMETase vs rMETase for Human Normal and Tumor Cells *in Vitro*

Normal cell strains			Tumor cell lines		
	IC_{50} ± SD			IC_{50} ± SD	
Cell type	rMETase	PEG-rMETase	Cancer type	rMETase	PEG-rMETase
Lung	0.8 ± 0.05	0.8 ± 0.05	Lung cancer H460	0.04 ± 0.005	0.04 ± 0.008
Kidney	1.5 ± 0.1	1.5 ± 0.1	Kidney cancer SN12C	0.06 ± 0.005	0.06 ± 0.005

Note. Human cells were incubated in methionine-containing RPMI 1640 medium supplemented with 10% FBS with rMETase or PEG-rMETase (0.125–4 units/ml) for 72 h at 37°C/5% CO_2. The IC_{50} was calculated as the concentration of rMETase which inhibited 50% cell growth. The experiments were repeated twice and the standard deviations are given. The differences in the IC_{50} of tumor cell lines compared with normal cell strains were significant ($P < 0.01$).

In striking contrast, normal human lung bronchial cell strain NHBE and normal human kidney cells HRCE had PEG-rMETase IC_{50}s of 1.5 and 0.8, respectively, *in vitro* (Table 2). The results demonstrated that PEG-rMETase had high tumor-selective efficacy similar to rMETase.

In conclusion, rMETase can be PEGylated under mild reaction conditions with conservation of enzyme activity and tumor selective efficacy. The increased half-life of PEG-rMETase extended the time of serum methionine depletion in the mice which should give enhanced *in vivo* anti-tumor efficacy and decrease the therapeutic dose of enzyme needed. In a study of the active systemic anaphylaxis assay (ASA) of PEG-METase in Guinea pigs, the results demonstrated that Guinea pigs had no detectable ASA reaction to PEG-METase compared to METase which caused ASA shock and death of the animals as did the positive control protein BSA (Y. Tan and R. M. Hoffman *et al.*, unpublished data). Similar studies will be carried out for PEG-rMETase which should give similar results. The conjugation of two PEG molecules per enzyme subunit was sufficient to confer these advantages. PEG-rMETase has holds promise as an anti-tumor agent.

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