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Overexpression and Large-Scale Production of Recombinant L-Methionine-α-deamino-γ-mercaptomethane-lyase for Novel Anticancer Therapy

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The goal of the next generation of cancer chemotherapy is effective tumor-selectivity. A tumor-selective target with high therapeutic potential is the elevated methionine requirement of tumor cells relative to normal cells. We have termed the elevated requirement for methionine in tumors methionine dependence. To selectively target the methionine dependence of tumors for treatment on a large-scale preclinical and clinical basis, the L-methionine α-deamino-γ-mercaptomethane-lyase (methioninase, METase) gene from *Pseudomonas putida* has been cloned in *Escherichia coli* using the polymerase chain reaction (PCR). The METase gene was then ligated into the pT7-7 overexpression plasmid containing the T7 RNA polymerase promoter and recloned in *E. coli* strain BL21(DE3). The pAC-1 clone was isolated by its yellow-orange color which is due to high enrichment of the pyridoxal phosphate-containing recombinant methioninase (rMETase) and distinguished rMETase-overproducer from rMETase-negative colonies. A scale-up production protocol which contained a heat step, two DEAE Sepharose FF ion-exchange, and one ActiClean Etox endotoxin-affinity chromatography columns has been established. The pAC-1 clone produces rMETase at approximately 10% of the total soluble protein and up to 1 g/liter in shake-flask culture. The protocol can produce therapeutic rMETase at the multi-gram level per batch with high yield (>60%), high purity (>98%), high stability, and low endotoxin. Purified rMETase is stable to lyophilization. The $t_{1/2}$ of rMETase was 2 h when rMETase was administered by iv injection in mice. 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 including those from lung, colon, kidney, brain, prostate, and melanoma were sensitive to rMETase. In contrast normal cells were insensitive to rMETase *in vitro* and correspondingly, no toxicity was detected *in vivo* at the effective doses. In conclusion, the overexpression clone and large-scale production protocols for rMETase have enabled rMETase to be used as a tumor-selective therapeutic with broad indication and high promise for effective, low-toxicity human cancer therapy.

The chemotherapy of solid tumors, with a few exceptions, has had only limited efficacy (1). Thus, the majority of disseminated solid cancers are generally not responsive to current chemotherapy regimens. For the most part, existing drugs used for solid-tumor chemotherapy are cytotoxic drugs which are not tumor selective. Therefore, traditional cytotoxic drugs are generally not effective because of dose-limiting toxicity.

It is of critical importance to identify targets and agents which are tumor selective. Asparagine dependence is one such target for the enzyme asparaginase. However, the asparagine-dependence target may be limited only to hematological tumors (1). Approximately 25 years ago, our group and others identified a number of malignant cell lines that had an absolute requirement for methionine. The malignant lines would not grow on the methionine precursor homocysteine in place of methionine as opposed to normal cells (2–7). These data suggested that methionine could be a possible tumor-selective therapeutic target. We have demonstrated that actual patient tumors are also frequently methionine-dependent, indicating the clinical potential of the methionine-dependence target (8).

Studies on the mechanism of altered methionine metabolism in cancer have indicated that methionine-dependent tumor cells generally synthesize methionine at a normal rate from homocysteine (9), although there may be some exceptions in some cancer cell types where vitamin B_{12} metabolism is altered (10). There seems to be an abnormally high rate of methionine utilization

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in methionine-dependent tumor cells for methylation reactions that require more methionine than the cell can synthesize from homocysteine during methionine starvation (11–15). Some tumors are also altered in the methionine salvage pathway, but the impact of this alteration on methionine dependence is unclear (16).

When methionine-dependent tumor cells *in vitro* are deprived of methionine in a homocysteine-containing medium they reversibly arrest in the late-S/G_2 phase of the cell cycle (17, 21). The tumor-selective cell-cycle arrest allows methionine depletion to modulate the efficacy of currently used chemotherapeutic agents (18).

Dietary methionine starvation extended the life span of the tumor-bearing animals and lowered the metastatic rate of the methionine-dependent tumors (19, 20, 22). Methionine-free total parenteral nutrition (TPN) doubled the response rate of high-stage gastric patients treated with 5-fluorouracil and mitomycin C compared to patients treated with these drugs and fed methionine-containing TPN (23). This clinical trial demonstrated that methionine depletion has clinical activity. However, dietary methionine starvation is insufficient to completely deplete serum methionine and therefore does not completely arrest tumor growth.

A methionine-cleaving enzyme would lower methionine levels more than methionine starvation and thereby could have more therapeutic efficacy. For this purpose Kreis and Hession (24) attempted to purify a METase from *Clostridium sporogenes*. This enzyme had a molecular weight of approximately 150 kDa. The enzyme slowed the growth of the Walker 256 carcinoma in rats more than a methionine-free diet did. However, the enzyme preparation was highly unstable, its yield was only 2%, and it had a high K_m of 90 mM. The *C. sporogenes* enzyme therefore did not have the properties to be developed into a therapeutic.

Ito *et al.* purified a METase from *Pseudomonas putida* (25). This enzyme had a molecular weight of approximately 170 kDa and catalyzed the α, γ-elimination of methionine to α-ketobutyrate, methanethiol, and ammonia in the presence of pyridoxal 5′-phosphate. Simultaneously, Nakayama *et al.* isolated METase from *Pseudomonas ovalis* (26,27). This group's later results showed that METase from *P. putida* was composed of four identical subunits of molecular weight 43 kDa and this enzyme had a K_m of approximately 1 mM (28). Endotoxin was not removed from these rMETase preparations, precluding the METase from being used as a therapeutic.

A therapeutically useful METase requires not only sufficient activity and an efficient method of purification, it is also critical that the preparation be free of endotoxin for therapeutic use. We developed a simplified purification procedure that enables high-yield production of endotoxin-free METase from *P. putida* suitable for therapeutic use (29,30). The METase produced by our protocols has been shown to deplete serum methionine levels without toxicity both in mice and in patients (30,31). The *P. putida* endotoxin-free METase significantly retarded the growth of the Yoshida rat sarcoma and the H460 human non-small-cell-lung carcinoma in nude mice to a greater extent than standard drugs (32). The METase did not cause weight loss or other detectable toxicity for up to 10 days treatment. In order to procede to large scale preclinical and clinical studies of METase it was necessary to clone the METase gene to produce the protein on a large scale.

The gene encoding METase in *P. putida* was cloned and was expressed at relatively low levels in *Escherichia coli* by Inoue *et al.* (33). Recombinant methioninase (rMETase) was shown to be composed of 398 amino acid residues with a calculated molecular weight of 42,626, corresponding to the subunit of the homotetrameric enzyme of native METase (32,33).

Hori *et al.* also reported the cloning of a gene from *P. putida* that they termed L-methionine-γ-deamino-α-mercaptomethane-lyase (34). The peptide sequence deduced from the sequence of the gene has 398 amino acids with a molecular mass of 42,720 daltons. However, this gene and corresponding protein differ significantly in sequence from that reported by Inoue *et al.* and from native METase (33).

Toward the goal of developing METase into an effective antitumor therapeutic, we report here the cloning and overexpression of the *P. putida* METase gene in *E. coli*. A protocol for high-yield, large-scale purification of recombinant METase (rMETase) was developed and is also reported here. The rMETase thus produced had *in vitro* efficacy against all of a wide variety of human tumor cell lines but in contrast not against normal cell strains and had *in vivo* efficacy against human tumors in nude mice with negligible toxicity.

MATERIALS AND METHODS

Materials

Restriction endonuclease, T4 DNA ligase, and BL21(DE3) competent cells were purchased from Stratagene (San Diego, CA). The pT7-7 vector was obtained from Dr. Stan Tabor (Harvard Medical School, Boston, MA) (36). *The GeneAmp* PCR reagent kit was purchased from Roche (Branchburg, NJ). The DNA purification kit was purchased from Promega (Madison, WI). The pT7 Blue T-vector was purchased from Novagen (Madison, WI). The oligonucleotide probes for PCR amplification were synthesized by IDT Inc. (Coralville, IA). The other reagents were purchased from Sigma (St. Louis, MO). *P. putida* was purchased from ATCC (Rockville, MD).

Cloning of the *Pseudomonas putida* Methioninase Gene

The segment coding for the METase gene was amplified and isolated with the PCR technique from *P. put-*

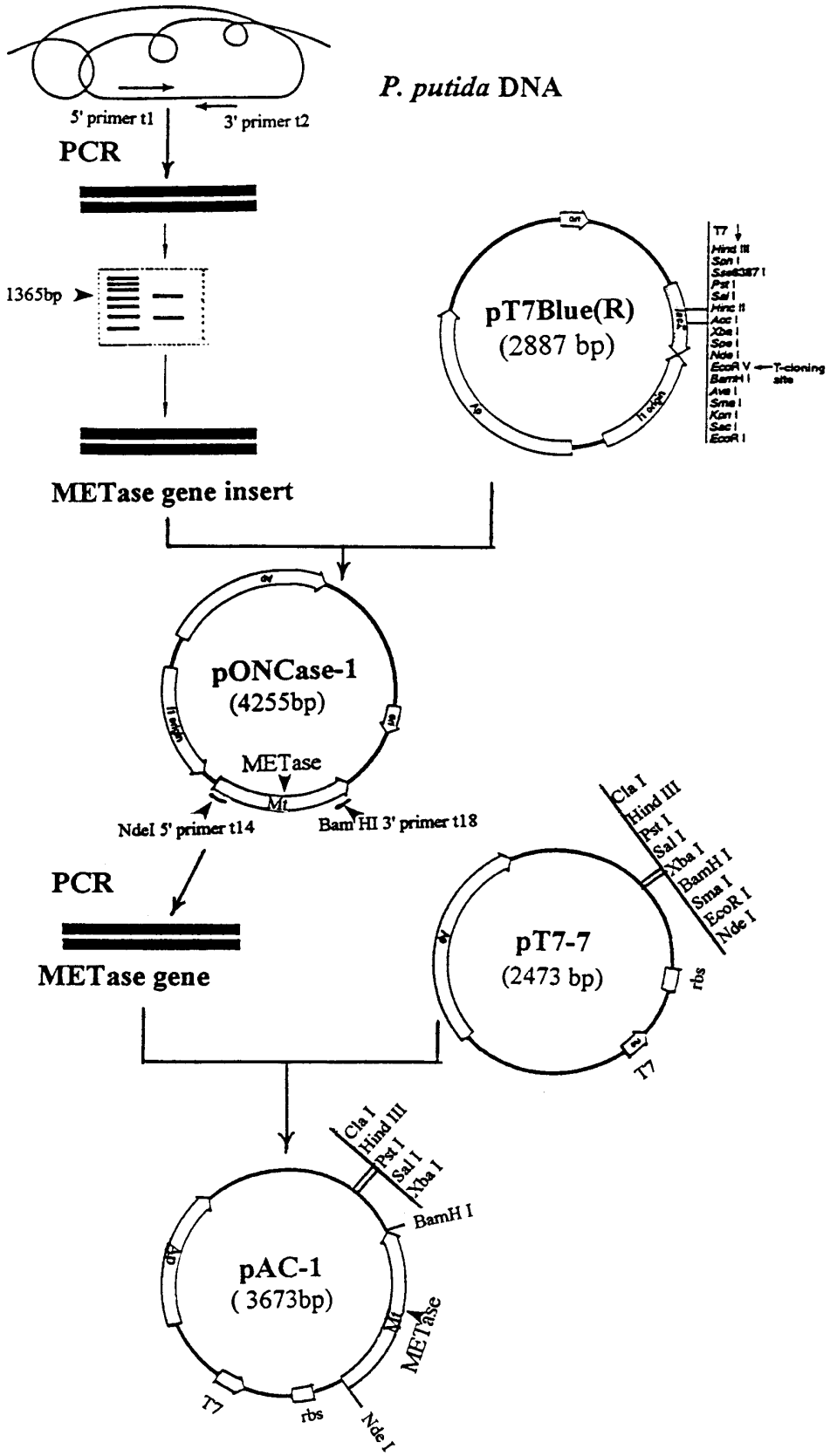

FIG. 1. Construction of the pAC-1 overexpression plasmid. A schematic diagram is shown to illustrate the various steps used to construct the expression vector used for the production of rMETase. The overexpression vector uses the very strong T7 RNA polymerase promoter to drive overexpression of the cloned rMETase gene. The host *E. coli* BL21(DE3) expresses the T7 RNA polymerase.

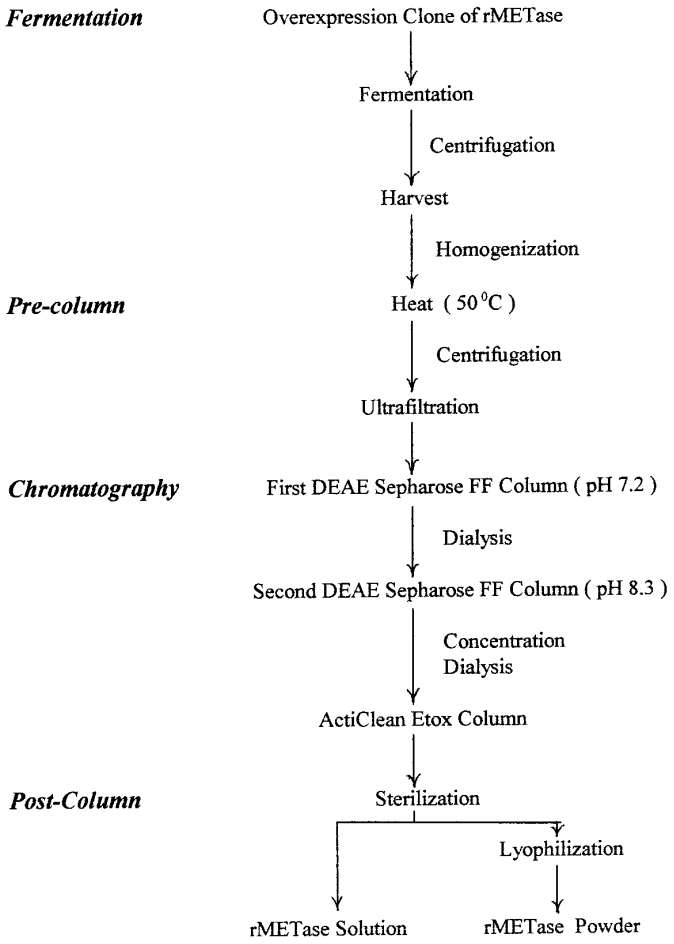

FIG. 2. rMETase production flow chart. Production of rMETase from overexpression clone pAC-1, including fermentation, purification, and formulation.

ida (30). Genomic DNA of *P. putida* AC-1, isolated by standard procedures, was used as a template for the PCR reaction. Oligonucleotide primers were synthesized on the basis of the nucleic sequence of the L-METase gene determined by Inoue *et al.* (33). The primers used were: t1, 5′-GCCGGTCTGTGGAATAAGCT-3′ (sense); and t2, 5′-CCAGGGTCGACTCCAGCGCC-3′ (antisense).

The PCR reaction conditions were as follows: first denaturation at 95°C for 10 min; then 5 cycles of denaturation at 94°C for 30 s; annealing at 60°C for 30 s; and extension at 72°C for 2 min. This was followed by 25 cycles of denaturation at 94°C for 30 s; 60°C for 30 s; then extension at 72°C for 1.5 min; then a final extension at 72°C for 10 min.

The PCR-amplified products were two bands of which the 1365-bp band, identified by λDNA/*Eco*RI + *Hin*dIII markers, was collected. The purified 1365-bp band was sequenced and determined to be the METase gene. The METase gene sequence, termed *ONCase-1*, was ligated into the pT7Blue T-vector (Novagen) at the *Eco*RV T-cloning site. The resulting plasmid p*ONCase-1* was transformed into *E. coli* DH5-α using standard procedures.

DNA sequencing was performed by ACGT Inc (Northbrook, IL) using T7 DNA polymerase and the dideoxy nucleotide termination reaction. The primer walking method was used (35).

Construction of the Overexpression Clone of rMETase

A schematic diagram for construction of the rMETase overexpression clone is shown in Fig. 1. To construct

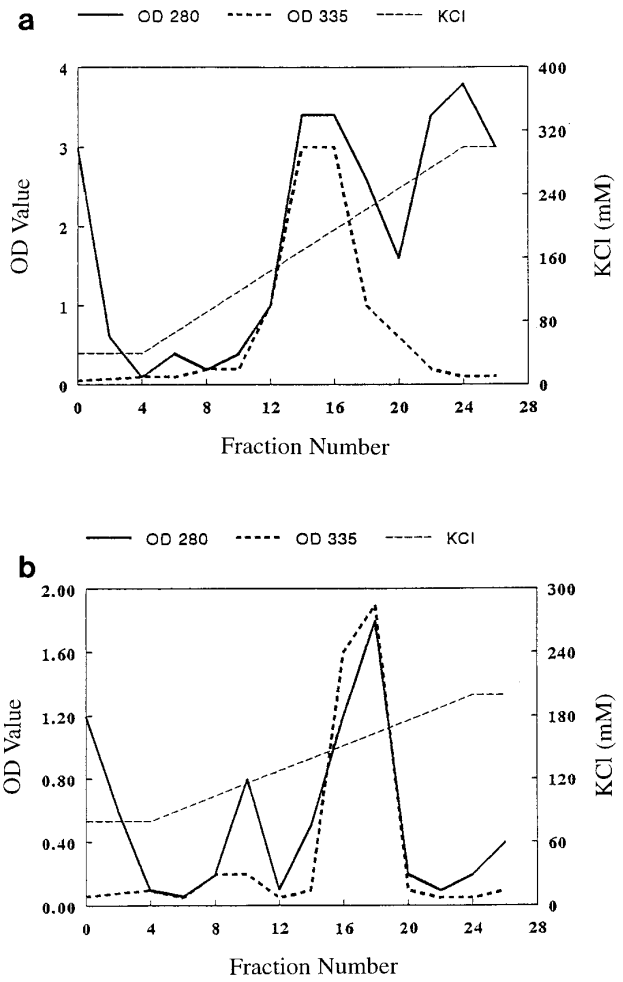

FIG. 3. DEAE Sepharose FF chromatography. (a) DEAE Sepharose FF 1. The sample, containing rMETase after precolumn treatment, was loaded and eluted on the first DEAE Sepharose FF column run at pH 7.2 as described under Materials and Methods. A gradient of 40–300 mM KCL was applied with 500-ml fractions collected. Protein was measured at OD_{280} and enzyme activity was measured at OD_{335} by spectrophotometery as described under Materials and Methods. (b) DEAE Sepharose FF 2. The sample containing rMETase, collected, pooled, and dialyzed after the first column was loaded and eluted on the second DEAE Sepharose FF column run at pH 8.3 as described under Materials and Methods. A gradient of 80–300 mM KCL was applied with 300-ml fractions collected. Protein was measured at OD_{280} and enzyme activity was measured at OD_{335} by spectrophotometery as described under Materials and Methods.

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GCCGGTCTGT GGAATAAGCT TATAACAAAC CACAAGAGGC GGTTGCCATG CACGGCTCCA      13
                                                    Met HisGlySer       4

ACAAGCTCCC AGGATTTGCC ACCCGCGCCA TTCACCATGG CTACGACCCC CAGGACCACG      73
AsnLysLeuPro GlyPheAla ThrArgAla IleHisHisGly TyrAspPro GlnAspHis       24

GCGGCGCACT GGTGCCACCG GTCTACCAGA CCGCGACGTT CACCTTCCCC ACCGTGGAAT     133
GlyGlyAlaLeu ValProPro ValTyrGln ThrAlaThrPhe ThrPhePro ThrValGlu      44

ACGGCGCTGC GTGCTTTGCC GGCGAGCAGG CCGGCCATTT CTACAGCCGC ATCTCCAACC     193
TyrGlyAlaAla CysPheAla GlyGluGln AlaGlyHisPhe TyrSerArg IleSerAsn      64

CCACCCTCAA CCTGCTGGAA GCACGCATGG CCTCGCTGGA AGGCGGCGAG GCCGGGCTGG     253
ProThrLeuAsn LeuLeuGlu AlaArgMet AlaSerLeuGlu GlyGlyGlu AlaGlyLeu      84

CGCTGGCCTC GGGCATGGGG GCGATCACGT CCACGCTATG GACACTGCTG CGCCCCGGTG     313
AlaLeuAlaSer GlyMetGly AlaIleThr SerThrLeuTrp ThrLeuLeu ArgProGly     104

ACGAGGTGCT GCTGGGCAAC ACCCTGTACG GCTGCACCTT TGCCTTCCTG CACCACGGCA     373
AspGluValLeu LeuGlyAsn ThrLeuTyr GlyCysThrPhe AlaPheLeu HisHisGly     124

TCGGCGAGTT CGGGGTCAAG CTGCGCCATG TGGACATGGC CGACCTGCAG GCACTGGAGG     433
IleGlyGluPhe GlyValLys LeuArgHis ValAspMetAla AspLeuGln AlaLeuGlu     144

CGGCCATGAC GCCGGCCACC CGGGTGATCT ATTTCGAGTC GCCGGCCAAC CCCAACATGC     493
AlaAlaMetThr ProAlaThr ArgValIle TyrPheGluSer ProAlaAsn ProAsnMet     164

ACATGGCCGA TATCGCCGGC GTGGCGAAGA TTGCACGCAA GCACGGCGCG ACCGTGGTGG     553
HisMetAlaAsp IleAlaGly ValAlaLys IleAlaArgLys HisGlyAla ThrValVal     184

TCGACAACAC CTACTGCACG CCGTACCTGC AACGGCCACT GGAGCTGGGC GCCGACCTGG     613
ValAspAsnThr TyrCysThr ProTyrLeu GlnArgProLeu GluLeuGly AlaAspLeu     204

TGGTGCATTC GGCCACCAAG TACCTGAGCG GCCATGGCGA CATCACTGCT GGCATTGTGG     673
ValValHisSer AlaThrLys TyrLeuSer GlyHisGlyAsp IleThrAla GlyIleVal     224

TGGGCAGCCA GGCACTGGTG GACCGTATAC GTCTGCAGGG CCTCAAGGAC ATGACCGGTG     733
ValGlySerGln AlaLeuVal AspArgIle ArgLeuGlnGly LeuLysAsp MetThrGly     244

CGGTGCTCTC GCCCCATGAC GCCGCACTGT TGATGCGCGG CATCAAGACC CTCAACCTGC     793
AlaValLeuSer ProHisAsp AlaAlaLeu LeuMetArgGly IleLysThr LeuAsnLeu     264

GCATGGACCG CCACTGCGCC AACGCTCAGG TGCTGGCCGA GTTCCTCGCC CGGCAGCCGC     853
ArgMetAspArg HisCysAla AsnAlaGln ValLeuAlaGlu PheLeuAla ArgGlnPro     284

AGGTGGAGCT GATCCATTAC CCGGGCCTGG CGAGCTTCCC GCAGTACACC CTGGCCCGCC     913
GlnValGluLeu IleHisTyr ProGlyLeu AlaSerPhePro GlnTyrThr LeuAlaArg     304

AGCAGATGAG CCAGCCGGGC GGCATGATCG CCTTCGAACT CAAGGGCGGC ATCGGTGCCG     973
GlnGlnMetSer GlnProGly GlyMetIle AlaPheGluLeu LysGlyGly IleGlyAla     324

GGCGGCGGTT CATGAACGCC CTGCAACTGT TCAGCCGCGC GGTGAGCCTG GGCGATGCCG    1033
GlyArgArgPhe MetAsnAla LeuGlnLeu PheSerArgAla ValSerLeu GlyAspAla     344

AGTCGCTGGC GCAGCACCCG GCAAGCATGA CTCATTCCAG CTATACCCCA GAGGAGCGTG    1093
GluSerLeuAla GlnHisPro AlaSerMet ThrHisSerSer TyrThrPro GluGluArg     364

CGCATTACGG CATCTCCGAG GGGCTGGTGC GGTTGTCGGT GGGGCTGGAA GACATCGACG    1153
AlaHisTyrGly IleSerGlu GlyLeuVal ArgLeuSerVal GlyLeuGlu AspIleAsp     384

ACCTGCTGGC CGATGTGCAA CAGGCACTCA AGGCGAGTGC CTGAACCCGT CACGGATGAG    1213
AspLeuLeuAla AspValGln GlnAlaLeu LysAlaSerAla                        398
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FIG. 4. Sequence of methioninase gene cloned from *P. putida* by PCR. Nucleotide sequence of the r-METase gene and the deduced amino acid sequence of pAC-1 are shown.

an overexpression vector, the p*ONCase-1* DNA sequence was used as the template with the following primers used for the PCR reaction: t14. 5′-GGAATTCCATATGCACGGCTCCAACAAGC-3′ (sense); and t18. 5′-AGTCATGGATCCTCAGGCACTCGCCTTGAGTGC-3′ (antisense). The PCR reaction conditions were as follows: first denaturation at 95°C for 10 min; then 5 cycles of denaturation at 94°C for 1 min; annealing at 56°C for 1.5 min; and extension at 72°C for 2 min. This was followed by 20 cycles of denaturation at 94°C

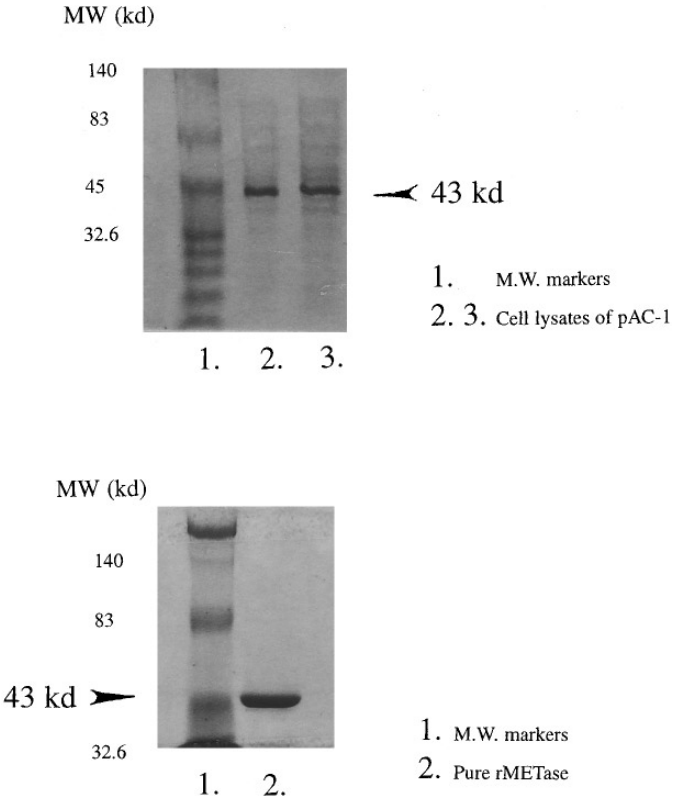

FIG. 5. SDS–PAGE of rMETase. The Kaleidoscope Prestained Standards were used as MW standard which contain MW 205, 140, 83, 45, 32, 18, 7.5 kDa markers. (a) SDS-PAGE of cell lysates of rMETase overexpression clone pAC-1. The pAC-1 clone was cultured as described under Materials and Methods. The cell pellet was sonicated and 20 μl of the supernatant was loaded on a 7.5% polyacrylamide SDS gel. The gel was run at 180 V for 30 min. Lane 1 contained protein molecular weight standards; lanes 2 and 3 contained lysates of overexpression clone pAC-1. The 43-kDa band contained the subunits of rMETase. (b) SDS–PAGE of purified rMETase. rMETase was purified from the AC-1 clone with the protocol described under Materials and Methods. 20 μg of rMETase were loaded on a 7.5% polyacrylamide SDS gel. The gel was run at 180 V for 30 min. Lane 1 contained protein molecular weight standards. Lane 2 contained native METase and lane 3 contained rMETase.

for 30 s; 56°C for 30 s; then extension at 72°C for 1.5 min; then final extension at 72°C for 10 min. The PCR-amplified product (1220 bp), which contained the METase gene with sites modified for the *Nde* I and *Bam*H I restriction enzymes, was collected and purified. This DNA sequence was termed *ONCase-3*.

ONCase-3 was digested with the *Nde* I and *Bam*H I restriction enzymes and ligated into the pT7-7 vector at the *Nde* I and *Bam*H I cloning sites using standard protocols. The resulting plasmid was then transformed into *E. coli* BL21(DE3) competent cells according to the instruction manual (Strategene) (37).

Positive clones were selected from ampicillin-containing plates. After storage at 4°C for 24 h the clones which expressed high levels of rMETase had a distinct yellow-orange color due to high enrichment of the pyridoxal phosphate-containing rMETase. The overexpression clones were first selected by their color and then by activity assay. All yellow-orange colonies were positive for METase and noncolored clones were METase negative. One overexpression clone was selected and termed the pAC-1 clone.

The cells from the selected colony were grown in 5 ml LB medium at 37°C overnight. Two milliliters of bacteria was then transferred to a flask with 100 ml LB medium. This culture was grown at 37°C for 8 h. The bacteria were aliquoted into twenty 2-ml vials with 15% glycerol, and stored at −80°C as the Cell Bank.

Large-Scale Production of rMETase (Fig. 1)

A flowchart for large-scale production of rMETase is shown in Fig. 2.

Fermentation

Every production fermentation was started with one vial from the Cell Bank. Ten microliters of bacteria from the Cell Bank were seeded into 5 ml LB medium with 100 μg ampicillin and grown at 37°C at 400 rpm overnight. This culture was transferred to 800 ml Terrific Broth (TB) (38) in 6 L flasks and grown overnight at 37°C at 400 rpm at which time the OD_{600} was approximately 10. The 800-ml cultures were then transferred into 10 800-ml TB-medium cultures in 6 L flasks and grown at 37°C at 400 rpm for 16 h at which time the OD_{600} was approximately 10. The medium was changed with fresh TB and the incubation was continued for another 6 h. When the OD_{600} reached 20, the bacteria were harvested by centrifugation at 4000*g* at 4°C for 10 min.

Purification of rMETase

(1) Precolumn Treatment of the Sample

The bacterial pellet was suspended in extraction solution (20 mM potassium phosphate, pH 9.0, 10 μM pyridoxal phosphate and 0.01% β-mercaptoethanol) and disrupted with a cavitator-type homogenizer (Microfluidics Corp., Newton, MA; model HC 8000). Heat treatment of the homogenate was then carried out up to 50°C for 1 min. The suspension was centrifuged with an automatic refrigerated centrifuge (SORVALL, superspeed RC 2-B) at 4°C at 8000*g* for 30 min. The supernatant was then collected. This step was followed by ultrafiltration by a Millipore (Bedford, MA) Prep/Scale-TFF PLHK 100k, 2.5 ft^2 cartridge with buffer (10 mM potassium phosphate, pH 8.3). The pH was adjusted to 7.2 by ultrafiltration.

(2) Chromatographic Conditions

First column: DEAE Sepharose FF (pH 7.2). The first column was 100 mm diameter and 30 cm height, with a volume of 2400 ml of DEAE Sepharose FF (Phar-

TABLE 1

Purification of rMETase

Procedure (batch 11)	Volume (ml)	Activity (units)	Protein (g)	Specific activity (units/mg)	Yield (%)
Cell lysis	5,000	82,000	29.3	2.8	100
Heat & UF[a]	4,500	77,000	19.3	4.0	94
DEAE-FF(1)[b]	2,200	65,400	60	10.9	80
DEAE-FF(2)[c]	800	57,500	2.9	20.0	70
ActiClean[d]	182	52,000	2.6	20.1	63

Note. rMETase overexpression clone pAC-1 was grown in 10 800-ml TB-medium cultures in 6 L flasks at 37°C at 400 rpm for 16 h at which time the OD_{600} was approximately 10. The medium was changed with fresh TB and the incubation was continued for another 6 h. When the OD_{600} reached 20, the bacteria were harvested by centrifugation and then lysed by cavitation. rMETase was then purified by heating to 50°C for 1 min and ultrafiltration followed by chromatography on two DEAE Sepharose Fast Flow columns and one ActiClean endotoxin-affinity column as described under Materials and Methods.

[a] UF, ultrafiltration.

[b] DEAE-FF (1), DEAE Sepharose FF First column, pH 7.2

[c] DEAE-FF (2), DEAE Sepharose FF Second column, pH 8.3.

[d] Endotoxin level was less than 2 EU/mg after the ActiClean step.

macia, Uppsala, Sweden). The elution flow rate was 15–50 ml/min. Forty to eighty grams of total protein (10–20 mg/ml) was applied on the column. After loading, the column was prewashed with 40 mM potassium chloride in PPM buffer (10 mM potassium phosphate, pH 7.2, containing 10 μM pyridoxal phosphate and 0.01% β-mercaptoethanol) for approximately 10 vol, until the OD_{280} dropped below 0.1. The protein was then eluted with a linear gradient of 40 to 300 mM potassium chloride in PPM buffer. Elution fractions of 500 ml were collected. The fractions containing rMETase were identified by yellow color and activity assay (Fig. 3a).

Second column: DEAE Sepharose FF (pH 8.3). The height of the second column (XK 50/30) was 25 cm, with a volume of 500 ml. The elution flow rate was 6–8 ml/min. After 24 h dialysis in 80 mM potassium chloride and 10 mM potassium phosphate (pH 8.3), 5–10 g of total protein (2–5 mg/ml) were applied on the second column. After loading, the column was prewashed with 80 mM potassium chloride and 10 mM potassium phosphate (pH 8.3) for approximately 4 vol, until the OD_{280} dropped below 0.1. rMETase was eluted with a linear gradient of 80 to 300 mM potassium chloride in 10 mM potassium phosphate buffer (pH 8.3). Elution fractions of 300 ml were collected. The fractions containing rMETase were identified by yellow color and activity assay (Fig. 3b).

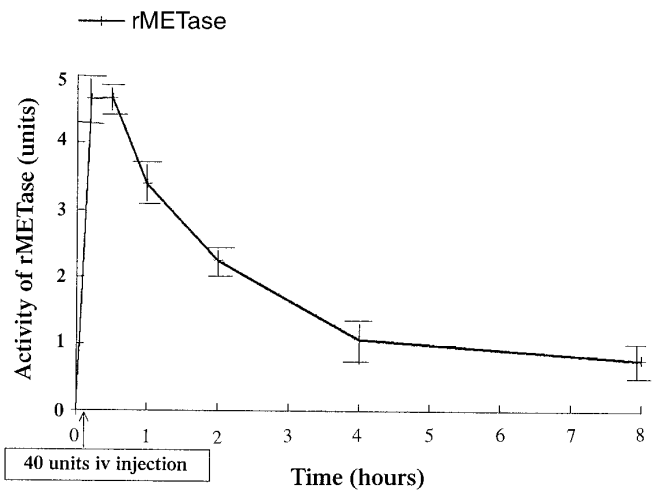

FIG. 6. Pharmacokinetics of rMETase in mice. Forty units of purified rMETase was injected into the tail vein of three BALB/c mice. The blood was collected before the injection, and at 10 min, 30 min, 1 hr, 2 hr, 4 hr, and 8 hr after injection. For each point, samples were collected from three mice for activity assay. The $t_{1/2}$ for rMETase was 2 h.

Third column: ActiClean Etox. To eliminate endotoxin, purified rMETase (10–20 mg protein/ml) in a volume of 200–300 ml was applied on an 800-ml Acticlean Etox (Sterogen, Arcadia, CA) column (25 × 60) with a bed height of 40 cm. The protein was eluted with elution buffer (0.12 M sodium chloride in 10 mM sodium phosphate, pH 7.2), at a flow rate of 1 ml/min. The enzyme fractions, identified by yellow color and activity assay, were collected.

The final eluant was concentrated with 30K Amicon (Lexington, MA) Centriprep concentrators by centrifugation at 4000*g* for 30 min at 4°C. Sterilization was performed with 0.2 μm Nylon filter (Nalgene).

Formulation of rMETase. rMETase in solution consisted of 0.12 M sodium chloride, 10 mM sodium phosphate buffer (pH 7.2), at a concentration of 10–20 mg/ml. This formulation was used *in vivo.* rMETase lyophilization rMETase, in solution, was frozen on dry ice and acetone and then lyophilized (Freeze mobil 24, Vertis) at −80°C, under a vaccum of 100 millibar for 72 h.

(3) Analysis of rMETase

HPLC. An Hitachi L-6200A Intelligent pump (Hitachi, Ltd, Tokyo, Japan) with a Supelco Progel-TSK

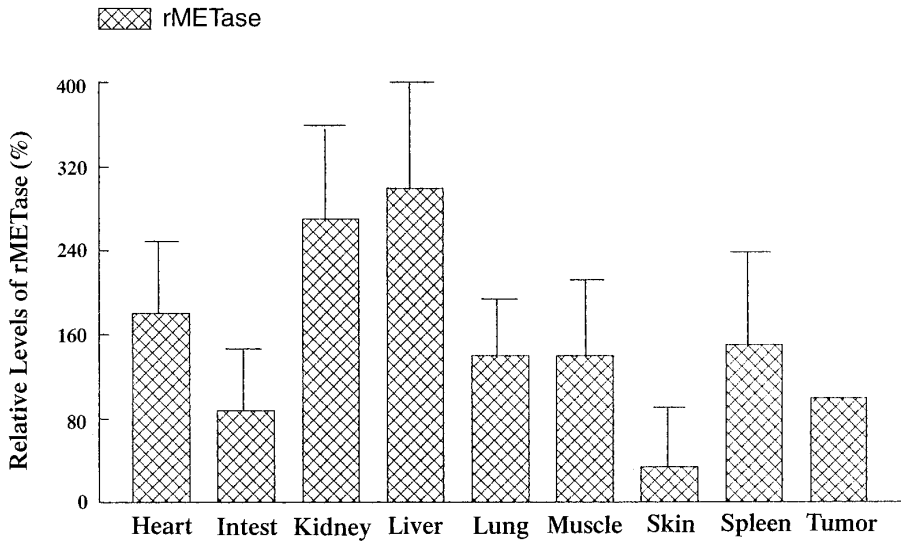

FIG. 7. Organ distribution of iv-administered rMETase in human tumor-bearing mice. One hundred units of rMETase was injected into the tail vein of three BALB/c nu/nu mice with subcutaneously growing human colon cancer HCT 15. After 1 h, the mice were sacrificed, and the liver, kidney, spleen, intestine, heart, lung, muscle, tumor, and skin were collected and weighed. The tissues were then sonicated, and the rMETase levels were determined by activity assay. The levels of rMETase were calculated as units per gram of tissue relative to the amount in the tumor which was considered 100%.

column (G3000 SW_{XL}, 30 cm × 7.8 mm) (Supelco, Bellefonte, PA) was used for all HPLC experiments. A sample of 20 μl (0.1–0.5 mg/ml) was loaded and eluted with elution solution (0.12 M sodium chloride in 10 mM sodium phosphate buffer, pH 7.2) at a flow rate of 1.0 ml/min. The protein-containing fractions were identified with a spectrophotometer (Hitachi U2000) at a wavelength of 280 nm. Bovine serum albumin (MW 66,000) and sweet potato β-amylase (MW 200,000) (Sigma, Louis, MO) were used as MW standards.

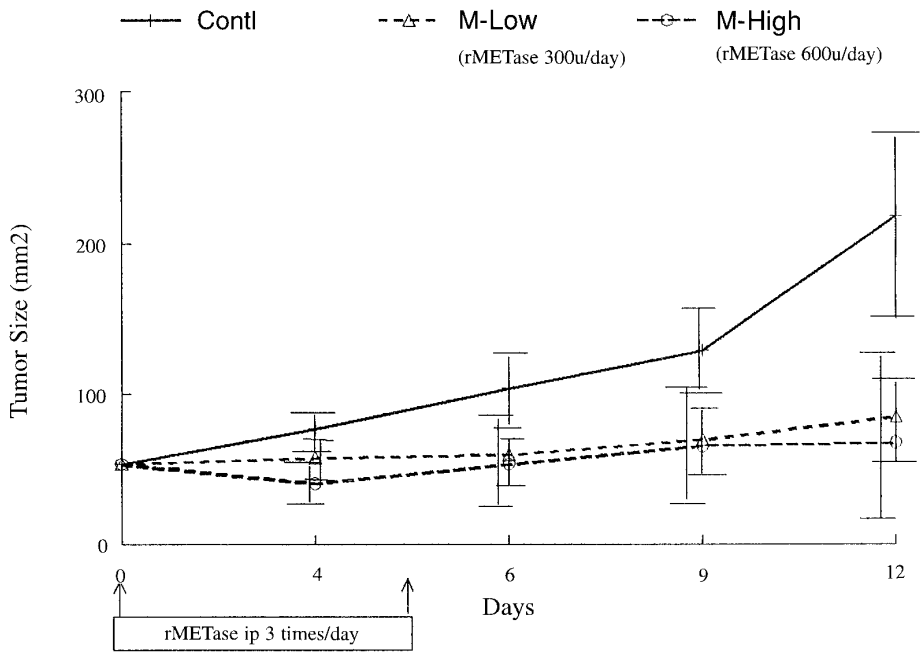

FIG. 8. Efficacy of rMETase on human colon tumor HCT 15 in nude mice. rMETase (100 and 200 units every 8 h) was administered by ip injection in nude mice with subcutaneously growing human colon tumor HCT 15. There were 4 mice with bilateral tumors in each group. Treatment started on Day 1 and continued until Day 5. The tumor size measured with calipers and body weight were determined every 2 days. The efficacy of rMETase was evaluated by tumor size, and the *P* value was calculated using the Student's *t* test.

TABLE 2

Evaluation of the Antitumor Efficacy and Toxicity of rMETase *in Vivo*

Tumor (s.c.)	Dose (units/day)	Schedule (day)	T/C[a] (%)	BWL (%)	WBC (%)	p[b]
HCT 15	300	Every 8 h	23.0	+5%	+8%	<0.01
HCT 15	600	Every 8 h	20.1	+3%	+6%	<0.01

Note. Human colon tumor HCT 15, growing subcutaneously in nude mice, was treated with rMETase administered ip every 8 h. There were four animals per group, each with bilateral tumors. Treatment started on Day 1 after the tumors could be measured and continued until Day 5. The tumor size was measured on Day 12. The T/C ratio was calculated from the tumor weights. rMETase was active at both doses tested with no demonstrable toxicity, indicated by lack of body weight loss or reduction of blood cell count. Abbreviations: BWL, average body weight loss per mouse; WBC, average white blood cell count compared to control group; T, median tumor weight of treated animals; C, median tumor weight of control animals; T/C, tumor growth inhibition rate.

[a] A T/C value of less than 42% is the minimum level for determining that treatment has activity.

[b] *P* value, tumor size comparison with control group as measured by the Student's *t* test.

Electrophoresis. Electrophoresis was carried out in 7.5% polyacrylamide-precasted plates in 0.2 M Tris–glycine buffer, pH 8.3, both with and without 0.1% SDS. Molecular weight standards used were Kaleidoscope Prestained Standards (Bio-Rad, Hercules, CA).

Activity assay. The assay was carried out in a 1-ml vol 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 reaction was stopped by adding 0.5 ml of 4.5% TCA. The suspension was centrifuged by eppendorf centrifuge at 13 krpm for 2 min. One-half milliliter of supernatant was added to 0.5 ml of 0.05% 3-methyl-2-benzothiazolinone hydrazone in 1 ml of 1 M sodium acetate, pH 5.2, and incubated at 50°C for 30 min. The amount of reaction product was determined by spectrophotometry at OD_{335}. The amount of protein was determined with the Lowry Reagent kit (Sigma) with bovine serum albumin as a standard. The specific activity was calculated as units/mg protein, with one unit of enzyme defined as the amount that catalyzes the formation of 1 μmol of α-ketobutyrate.

Endotoxin assay. The endotoxin level was measured by the Limulus Amebocyte Lysate (LAL) test (BioWhittaker, Walkersville, MD). A sample was mixed with the LAL and incubated at 37°C for 10 min. A substrate solution supplied with the kit was then mixed with the sample and incubated at 37°C for an additional 6 min. The reaction was stopped with stop reagent supplied with the kit. The absorbance of the sample was determined with a spectrophotometer (Hitachi, U 2000) at 410 nm. The concentration of endotoxin was calculated from a standard curve which was constructed from the endotoxin supplied in the kit at concentrations from 0.1 EU/ml to 1 EU/ml.

Half-Life Determination of rMETase *in Vivo*

The half-life of rMETase was analyzed in BALB/c mice. Forty units of purified rMETase were injected into the tail vein. The blood was collected before the injection, and at 10 min, 30 min, 1 hr, 2 hr, 4 hr, and 8 hr after injection. For each time point, samples were collected from three mice for activity assay. Standard deviations were calculated.

Organ Distribution of rMETase in Mice after iv Injection

One hundred units of rMETase were injected into the tail vein of three BALB/c nu/nu mice with subcuta-

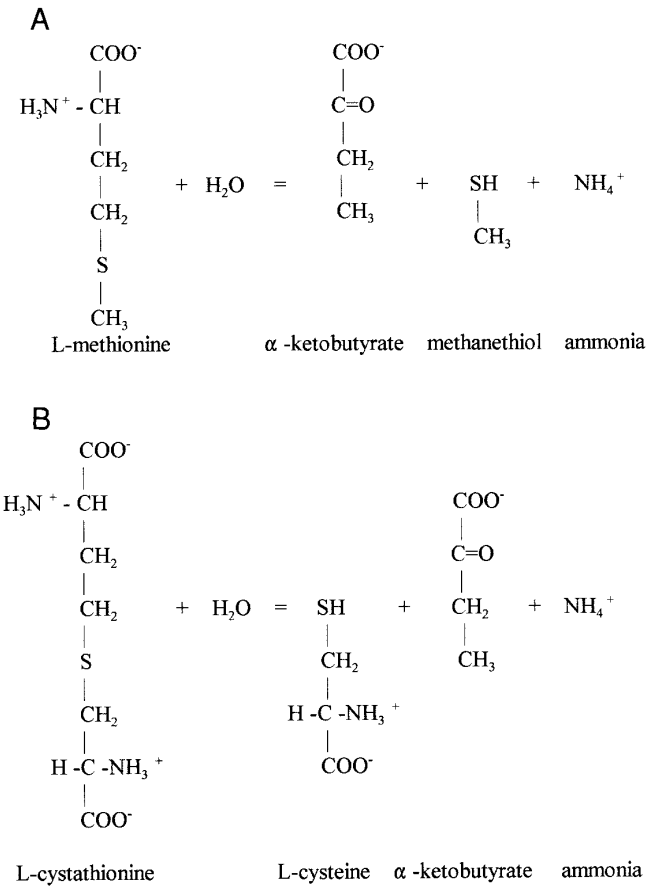

FIG. 9. Comparison of the L-methionine γ-lyase (A) and L-cystathionine γ-lyase (B) reactions.

neously growing human colon cancer HCT 15. After 1 h, the mice were sacrificed, and the liver, kidney, spleen, intestine, heart, lung, muscle, tumor, and skin were collected and weighed. The tissues were thoroughly washed before analysis such that any residual blood methioninase would have been eliminated. The tissues were then sonicated, and the rMETase levels were determined by activity assay. The relative levels of rMETase in tissues were calculated first as units per gram of tissue and then divided by the level in the tumor which was termed as 100%.

Growth Inhibition of Human Cancer Cells by rMETase *in Vitro*

Human lung, colon, kidney, brain, prostate, and melanoma cancer cells and various types of normal cells were incubated in methionine-containing RPMI 1640 medium supplemented with 10% FBS. Various concentrations (0.1–4 units/ml) of rMETase were added to the medium and incubated with the cells for 4 days at 37°C, 5% CO_2. The relative growth inhibition was calculated from cell counts.

Efficacy of rMETase on Human Cancer Xenograft in Nude Mice

Human colon cancer xenograft HCT 15, growing subcutaneously in nude mice in groups of four with bilateral tumors was used for the efficacy studies of rMETase. Treatment started after tumor size could be measured which was termed as Day-1. rMETase in 0.12 M NaCl, 10 mM sodium phosphate buffer, pH 7.2, was administered ip three times per day. Three hundred or 600 units rMETase per day were administered for 5 days (Day 1 to Day 5). Normal saline was used as the control.

Tumor size was measured with calipers and body weight determined every 3 days from Day 1 until Day 12. When the experiment was terminated at Day 12, blood, liver, lung, kidney, and tumor tissue were collected for pathological analysis. Tumor weight was calculated by (length $\times$ width^2) $\div$ 2. Tumor growth inhibition (T/C value) was calculated as [median tumor weight of the treated animal (T) $\div$ median tumor weight of the control animal (C) $\times$ 100] (39).

RESULTS

PCR Cloning and Sequencing of the METase Gene from *P. putida*

The segment coding for the METase gene from *P. putida* was amplified by PCR as described under Materials and Methods (Fig. 1). The two resulting bands included a 1365-bp band which was identified as the METase gene by DNA sequencing and termed *ONCase-1*. The nucleotide sequence of *ONCase-1* and the predicted amino acid sequence are shown in Fig. 4.

High Expression Clone of rMETase

The insert that was used to construct a high expression vector of rMETase was obtained by PCR amplification of *P. putida* DNA as described under Materials and Methods. The PCR-amplified product, termed *ONCase-3* (1220 bp), was collected, digested with the *Nde*I and *Bam*HI restriction enzymes and then ligated with the pT7-7 high expression vector at the *Nde*I and *Bam*HI cloning sites (Fig. 1). The resulting pONCase-3 plasmid was transformed into BL21(DE3) competent cells. Clones which expressed high levels of rMETase were selected by their distinct yellow-orange color seen when the agar-containing petri plates were incubated at 4°C for 24 h. The highest expression clone was selected by activity assay and termed pAC-1. pAC-1 expressed rMETase at approximately 10% of the total cell protein based on the specific activity of 20 units/mg for a 99% pure enzyme. Shaker flask cultures yielded up to 1 g rMETase per liter of medium. SDS–PAGE demonstrated a very prominent rMETase band in cell lysates, indicating rMETase overexpression (Fig. 5a).

Purification of rMETase from Overexpression Clone pAC-1

The purification of rMETase was carried out as described under Materials and Methods (Fig. 2). The heat step denatured the heat-sensitive proteins which were removed by centrifugation, thereby increasing the amount of rMETase that could be loaded on the subsequent chromatography column by 30%. Ion exchange chromatography included two DEAE Sepharose FF columns, run at pH 7.2 and 8.3, respectively. The capacity of DEAE Sepharose FF is large with a rapid flow rate. The pH 7.2 Sepharose FF step resulted in a two- to threefold purification. The pH 8.3 Sepharose FF step also resulted in a two- to threefold purification. With this protocol, the yield was more than 63% and the specific activity was 20–30 units/mg. As seen in Table 1, the simple procedure gave a yield of 85–94% for each step. There was an approximate doubling of specific activity for each step until endotoxin removal. The ActiClean endotoxin affinity step reduced endotoxin levels from more than 1000 EU/mg to less than 2 EU/mg as measured by LAL. The representive run shown in Table 1 yielded approximately 2.6 g rMETase. The protocol and equipment described can readily yield 10 g rMETase.

Analysis of Purified rMETase

SDS–PAGE analysis of rMETase demonstrated a single band of 43 kDa identical to native METase (Fig. 5b). HPLC analysis demonstrated a major peak with a retention time (RT) of 8.5 min and a purity calculated at greater than 99%. The RT of the standard of bovine serum albumin was 8.88 min and the RT of the standard sweet potato β-amylase was 7.82 min.

Lyophilization of rMETase

Purified rMETase was lyophilized as described under Materials and Methods. The specific activity of rMETase in lyophilized powder and solution were comparable. rMETase could be stably stored as a lyophilized powder.

Half-life of rMETase *in Vivo*

When rMETase was administered by iv injection in mice, the $t_{1/2}$ of rMETase was 2 h (Fig. 6). The peak level occurred within approximately 10 min.

Tissue Distribution of rMETase in Mice after iv Administration

After iv administration to nude mice the tissue distribution of rMETase was in the following decreasing order: kidney, muscle, heart, liver, spleen, lung, tumor, intestine, and skin (Fig. 7). Significant rMETase levels accumulated in the tumor which contains the cells most sensitive to methionine depletion.

Growth Inhibition of Human Cancer Cells by rMETase *in Vitro*

rMETase was incubated with cancer cells including those from the lung, colon, kidney, brain, prostate, and melanoma *in vitro* for 4 days. All of the human cancer cell lines tested were highly sensitive to rMETase and died, while in contrast, normal cells were insensitive to rMETase (data not shown).

Efficacy of rMETase *in Vivo*

The efficacy of rMETase on human colon cancer cell HCT 15 in nude mice was studied as described under Materials and Methods. The results demonstrated that rMETase inhibited tumor growth both at low dose (100 units, every 8 h, ip) and high dose (200 units, every 8 h, ip) compared to control ($P < 0.01$ for both doses). The differences in the curves are statistically significant ($P < 0.01$) at the Day 6, Day 9, and Day 12 time points. The P values were calculated by the Student's t test. The T/C values were approximately 23 and 20%, at the low and high doses, respectively (Fig. 8, Table 2), thereby indicating high antitumor efficacy of rMETase. The body weight and blood cell count showed no toxicity, indicating that increased levels of rMETase could be administered before dose-limiting toxicity was reached.

DISCUSSION

Human tumor cells both *in vitro* and *in vivo* have an elevated requirement for methionine compared to normal cells (2–14). We have previously shown that methionine dependence is a promising chemotherapeutic target, due in part to the facts that methionine dependence is found in all tumor types and in part to the tumor-selective late-S/G_2 cell-cycle block induced by methionine depletion (17,18). Indeed, Goseki *et al.* recently have reported that methionine-free total parenteral nutrition (TPN) solution had synergistic efficacy with the chemotherapic agents 5-fluorouracil, vincristine, and mitomycin C in advanced gastric cancer patients, including increased survival (19,23). These results suggest that methionine depletion is active clinically.

Kreis *et al.* (24) first attempted to use a METase as an antitumor agent. Their enzyme, derived from *C. sporogenes,* was highly unstable and had a K_m of 90 mM, making it impractacle as a therapeutic agent. The *P. putida* enzyme in contrast has a K_m of 1 mM (28). We previously reported the isolation of high-purity low-endotoxin nonrecombinant METase derived from *P. putida* (29). The purified nonrecombinant methioninase depleted the serum level of methionine to 1% or less of the original level in mice and patients (30,31). We also reported that the nonrecombinant *P. putida* METase inhibited human tumor growth in nude mice without toxicity *in vivo* (32). However, the yield of nonrecombinant METase from *P. putida* was highly limiting and precluded large scale preclinical and clinical studies. It was clear that recombinant methioninase was necessary for large scale studies.

Inoue *et al.* first cloned the METase gene from *P. putida* and published the sequence of 1,194 nucleotides encoding a protein of 398 amino acid residues (33). The lac promoter in the expression clone pYH103, used by Inoue *et al.,* requires induction with IPTG. However, even with induction, the pYH103 clone produces rMETase at 3.7% of the total soluble protein (0.71 units/mg protein).

Hori *et al.* subsequently reported a clone derived from *P. putida* that they termed the METase gene (34). However, 74 amino acid residues (18.5% of total) of the sequence of Hori *et al.* are different from that of Inoue *et al.* (33) and our clone. Reasons for the large differences in Hori's clone compared with Inoue *et al.* and ours, include the possibility that there were mutations in Hori's PCR products due to the 50°C annealing temperature in their PCR which may not have been sufficiently stringent. In contrast, the annealing temperature in our PCR for amplifying the methioninase gene from the genomic DNA of *P. putida* was 60°C. The differences could also be due to the degeneration of Hori's primers which resulted in the PCR product not being methioninase but another protein which is homologous to methioninase.

The sequence of the METase gene cloned from *P. Putida* described here matches the METase gene cloned by Inoue *et al.* (33). We subcloned the METase gene into the pT7-7 overexpression plasmid which contains the strong T7 RNA polymerase promoter. This vector does not need an inducer making the production more economical and practical. The translation start site for the T7-7 plasmid also enhances the expression

of the rMETase gene. The overexpression clone, pAC-1, described here, produces rMETase at 10% or more of the total soluble protein (3.0 units/mg protein). We have also developed protocols for scale-up production of rMETase with high yield (more than 60%), high purity (more than 98%), low endotoxin (less than 2 EU/mg), and high stability (Table 2). This protocol is suitable for scaled-up manufacturing of rMETase as a cancer therapeutic.

METase has significant sequence homology with cystathionine γ-lyase, cystathionine β-lyase, and cystathionine γ-synthetase. These sequences are aligned and compared by Inoue *et al.* (33). These enzymes are classified into the "γ " family of pyridoxal enzymes by Alexander *et al.* (40). Comparison of the reactions of METase and cystathionine γ-lyase are shown in Fig. 9.

The rMETase produced with the protocols described here had a half-life of approximately 2 h when it was administrated by iv injection. Antitumor efficacy studies *in vitro* showed that all types of human tumor cell lines tested, including colon, lung, renal, brain, and melanoma, were highly sensitive to rMETase, in contrast to normal cells which were resistant to rMETase (data not shown). The growth of human colon (Fig. 8) and lung carcinoma (data not shown) were significantly inhibited by rMETase in nude mice without body weight loss, indicating apparent lack of toxicity.

In conclusion, the pAC-1 clone, which contains the T7 RNA polymerase promoter, overexpresses rMETase at a high level. The overexpression, efficient large-scale high-yield production, low toxicity, high efficacy, and broad target of rMETase described here indicates the feasibility of rMETase for use as a novel, tumor-selective therapeutic of high potential.

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