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Purification and Characterization of the Hydantoin Racemase of *Pseudomonas* sp. Strain NS671 Expressed in *Escherichia coli*

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The hydantoin racemase gene of *Pseudomonas* sp. strain NS671 had been cloned and expressed in *Escherichia coli*. Hydantoin racemase was purified from the cell extract of the *E. coli* strain by phenyl-Sepharose, DEAE-Sephacel, and Sephadex G-200 chromatographies. The purified enzyme had an apparent molecular mass of 32 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. By gel filtration, a molecular mass of about 190 kDa was found, suggesting that the native enzyme is a hexamer. The optimal conditions for hydantoin racemase activity were pH 9.5 and a temperature of 45°C. The enzyme activity was slightly stimulated by the addition of not only Mn^{2+} or Co^{2+} but also metal-chelating agents, indicating that the enzyme is not a metalloenzyme. On the other hand, Cu^{2+} and Zn^{2+} strongly inhibited the enzyme activity. Kinetic studies showed substrate inhibition, and the V_{max} values for D- and L-5-(2-methylthioethyl)hydantoin were 35.2 and 79.0 μmol/min/mg of protein, respectively. The purified enzyme did not racemize 5-isopropylhydantoin, whereas the cells of *E. coli* expressing the enzyme are capable of racemizing it. After incubation of the purified enzyme with 5-isopropylhydantoin, the enzyme no longer showed 5-(2-methylthioethyl)hydantoin-racemizing activity. However, in the presence of 5-(2-methylthioethyl)hydantoin, the purified enzyme racemized 5-isopropylhydantoin completely, suggesting that 5-(2-methylthioethyl)hydantoin protects the enzyme from inactivation by 5-isopropylhydantoin. Thus, we examined the protective effect of various compounds and found that divalent-sulfur-containing compounds (R-S-R′ and R-SH) have this protective effect.

Pseudomonas sp. strain NS671 can convert racemic 5-substituted hydantoins to their corresponding L-amino acids asymmetrically. We have cloned the genes involved in this conversion from the 172-kb native plasmid (pHN671) of strain NS671 (17). The largest recombinant plasmid (pHPB14) contained five complete open reading frames, designated *hyuA*, *hyuB*, *hyuC*, ORF5, and *hyuE*. It was found that both *hyuA* and *hyuB* are the genes for DL-hydantoinase, *hyuC* is the gene for *N*-carbamyl L-amino acid amidohydrolase, and *hyuE* is the gene for hydantoin racemase, whereas no function for the ORF5 product has been found (17, 18).

Hydantoin racemase is of particular significance, because this enzyme enables the asymmetric conversion of racemic 5-substituted hydantoins to the corresponding L-amino acids in strain NS671. In this article, we describe the purification and characterization of the *hyuE* product hydantoin racemase of strain NS671 expressed in *Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions, and chemicals. Plasmid pKPN17 is a derivative of pUC18 (19) containing the *hyuE* gene of *Pseudomonas* sp. strain NS671 under the control of the *lac* promoter (18). *E. coli* JM103 (9) carrying pKPN17 was grown in Luria-Bertani medium (8) supplemented with 50 μg of ampicillin per ml at 30°C. Optically active 5-substituted hydantoins and *N*-carbamyl amino acids were prepared from the corresponding D- or L-amino acids (12) in our laboratory.

Determination of enzyme activity. Hydantoin racemase activity was analyzed during the course of the enzyme purification by measuring optical rotation at a wavelength of 589 nm. A mixture (2 ml) containing 2% L-5-(2-methylthioethyl)hydantoin, 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, and 1 mM EDTA was equilibrated at 30°C. The reaction was initiated by addition of a sample of enzyme (20 μl) to the mixture. After incubation at 30°C for 30 min, the solution was analyzed with a digital polarimeter (DIP-360; Japan Spectroscopic Co., Tokyo, Japan). One unit of enzyme activity was defined as the activity that catalyzes the formation of 1 μmol of the D-isomer per min under the conditions used.

Purification of the hydantoin racemase from *E. coli* carrying pKPN17. *E. coli* JM103 carrying pKPN17 was grown in 3 liters of a medium consisting of 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 5 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1% glycerol, and 2% yeast extract at 30°C with a 5-liter jar fermenter (Mituwa Rikagaku Kogyo, Osaka, Japan). IPTG (isopropyl-β-D-thiogalactopyranoside; 15 ml of a 200 mM solution) was added to the culture at an A_{550} of about 10 (3.1 mg [dry weight] per ml), and cultivation was continued for another 2.5 h. The cells were harvested by centrifugation (about 100 g [wet weight]) and stored at −20°C.

Hydantoin racemase was purified from the cells as follows. All purification steps were carried out at 0 to 4°C. The frozen cells were suspended in 500 ml of 20 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl and disrupted with a Gaulin homogenizer (15MR; APV Gaulin Inc., Everett, Mass.) by two passages at 8,000 lb/in^2. The cell debris was removed by centrifugation (17,000 × *g*, 30 min, 4°C), and the supernatant was fractionated by stepwise addition of solid ammonium sulfate. The precipitate obtained from 60 to 90% saturation was dissolved in 200 ml of 20 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl and 0.5 M ammonium sulfate and

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applied to a phenyl-Sepharose CL-4B (Pharmacia LKB, Uppsala, Sweden) column (4.4 by 13 cm) equilibrated previously with the same buffer. After the column was washed thoroughly, elution was carried out with a linear gradient of 0.5 to 0 M ammonium sulfate in 20 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl (total volume, 600 ml; fraction volume, 15 ml; flow rate, 70 ml/h). Active fractions, found at about 0.3 M ammonium sulfate, were pooled and concentrated by ammonium sulfate precipitation (90% saturation). The precipitate was dissolved in 100 ml of 20 mM Tris-HCl (pH 7.5) containing 50 mM NaCl and dialyzed against 4 liters of the same buffer overnight. The dialyzed material was applied to a DEAE-Sephacel (Pharmacia LKB) column (4.4 by 13 cm) equilibrated previously with 20 mM Tris-HCl (pH 7.5) containing 50 mM NaCl. The column was washed with the same buffer, and the enzyme was eluted with a linear gradient of 50 to 300 mM NaCl in 20 mM Tris-HCl (pH 7.5) (total volume, 600 ml; fraction volume, 15 ml). Active fractions, found at about 200 mM NaCl, were pooled and concentrated by ammonium sulfate precipitation (90% saturation). The precipitate was dissolved in 40 ml of 20 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl and 1 M ammonium sulfate. Further purification was carried out by gel filtration with a Sephadex G-200 superfine (Pharmacia LKB) column (3.2 by 85 cm) equilibrated previously with the same buffer. For molecular mass estimation, catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67.0 kDa), ovalbumin (43.0 kDa), chymotrypsinogen A (25.0 kDa), and RNase A (13.7 kDa) (gel filtration calibration kit; Pharmacia LKB) were used as standards. Solid ammonium sulfate was added to active fractions from the gel filtration chromatography (90% saturation), and the resultant protein suspension was stored at 4°C until use.

Protein assays. The molecular mass of a denatured hydantoin racemase was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (5). Protein standards were phosphorylase *b* (94.0 kDa), bovine serum albumin (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), and soybean trypsin inhibitor (20.1 kDa) (electrophoresis calibration kit; Pharmacia LKB). The gel contained 12% acrylamide and was stained with Coomassie brilliant blue after electrophoresis. The molecular mass of the native hydantoin racemase was determined by gel filtration with Sephadex G-200 superfine as described above. For N-terminal sequence analysis, peptides were electroblotted from the SDS-polyacrylamide gel onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Bedford, Mass.) and applied to a pulsed-liquid-phase sequencer (477A; Applied Biosystems) equipped with an on-line phenylthiohydantoin analyzer (120A; Applied Biosystems). The amount of protein was measured by the method described by Lowry et al. (7) with bovine serum albumin used as a standard.

Effects of pH, temperature, and metal ions on enzyme activity. Standard assay conditions were as follows. The reaction mixture contained 20 mM L-5-(2-methylthioethyl) hydantoin, 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, and 5 μg of enzyme per ml. The reaction was monitored at 30°C by measuring optical rotation at a wavelength of 589 nm, and initial velocity was determined. The effect of pH was examined under the standard assay conditions, except for the buffer. The buffers used were sodium acetate (pH 3.5 to 5.5), sodium phosphate (pH 5.5 to 8.0), Tris-HCl (pH 6.5 to 8.5), and glycine-NaOH (pH 8.5 to 11.0). The effect of temperature was examined under the standard assay conditions by varying the temperature (0 to 75°C).

TABLE 1. Purification of hydantoin racemase

Step	Amt of total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
Crude extract	8,280	15,500	1.87	1.00	100
Ammonium sulfate	3,840	7,740	2.02	1.08	49.9
Phenyl-Sepharose	1,410	7,250	5.14	2.75	46.8
DEAE-Sephacel	289	2,940	10.2	5.45	19.0
Sephadex G-200	95.1	1,980	20.8	11.1	12.8

Thermostability was examined as follows. A mixture containing 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, and 10 μg of enzyme per ml was incubated at different temperatures (30 to 75°C) for 30 min in the presence or absence of 1 mM DL-5-(2-methylthioethyl)hydantoin. The mixture was cooled to 30°C, and then an equal volume of a solution containing 40 mM L-5-(2-methylthioethyl)hydantoin, 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, and 1 mM EDTA was added to start the assay. The effect of metal ions was examined under standard assay conditions by adding different metal ions instead of EDTA.

RESULTS

Purification of hydantoin racemase. The hydantoin racemase of *Pseudomonas* sp. strain NS671 was purified from a crude extract of IPTG-induced cells of *E. coli* carrying pKPN17. The results of a representative purification are shown in Table 1 and Fig. 1. The enzyme was purified 11-fold, with an overall yield of 13%. SDS-PAGE analysis revealed that the purified-enzyme preparation contained a major peptide with an apparent molecular mass of 32 kDa and a minor peptide of 28 kDa. These peptides were electroblotted from the SDS-polyacrylamide gel onto an Immobilon-P membrane and analyzed for their N-terminal sequences. The 32-kDa peptide had the sequence M-K-I-K-V-I-N-P-N-T, which agrees with the N-terminal sequence of the *hyuE* product predicted from the DNA sequence (18). On

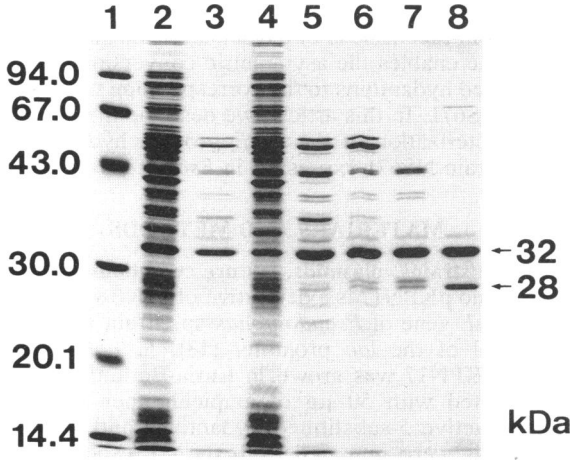

FIG. 1. SDS-PAGE analysis of hydantoin racemase preparations during purification. Lanes: 1, molecular size markers; 2, crude extract; 3, 60% ammonium sulfate supernatant; 4, 60% ammonium sulfate precipitate; 5, 60 to 90% ammonium sulfate precipitate; 6, phenyl-Sepharose step; 7, DEAE-Sephacel step; 8, Sephadex G-200 step.

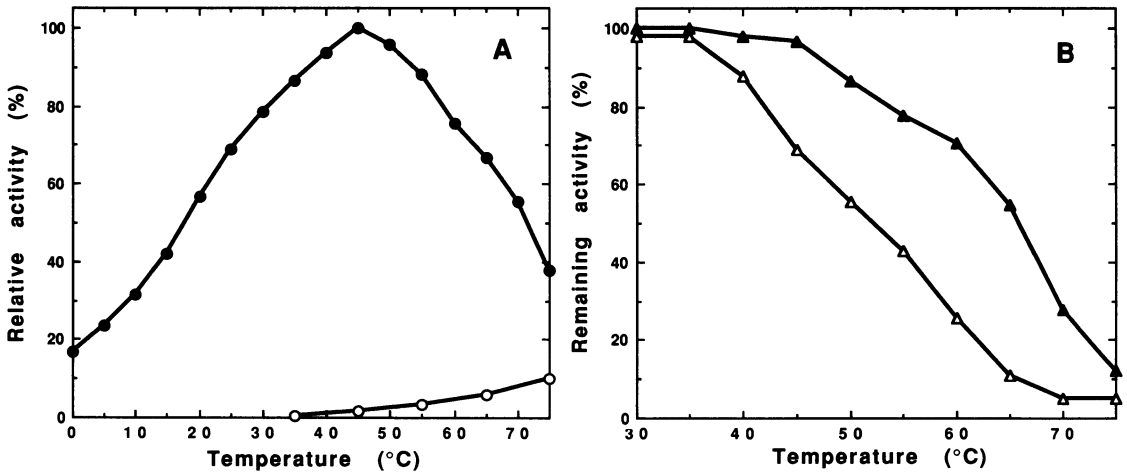

FIG. 2. Effect of temperature on hydantoin racemase activity. (A) Hydantoin racemase activity was assayed at the indicated temperatures. Symbols: ●, enzymatic racemization; ○, chemical racemization. Relative activity is expressed as percentages of the initial velocity of the enzymatic racemization at 45°C. (B) Hydantoin racemase was incubated at the indicated temperatures for 30 min in the presence (▲) or absence (△) of 1 mM DL-5-(2-methylthioethyl)hydantoin. The samples were cooled and then assayed for hydantoin racemase activity at 30°C.

the other hand, the 28-kDa peptide had the sequence S-K-S-D-V-F-X-L-G-L-T-K-N-D-L (in which X indicates an unidentified residue), indicating that this peptide is not the gene product of *hyuE*. The result of gel filtration chromatography with Sephadex G-200 indicated that the hydantoin racemase has a molecular mass of about 190 kDa, suggesting that the native enzyme is a hexamer.

We found that hydantoin racemase activity is stabilized by addition of ammonium sulfate to a solution containing the enzyme. Therefore, gel filtration chromatography with Sephadex G-200 was performed in the presence of 1 M ammonium sulfate, and a yield of 68% was obtained under these conditions. When ammonium sulfate was omitted, a yield of only 21% was obtained, although the enzyme activity was eluted as a single peak (data not shown). The purified enzyme was stable for at least 6 months when it was stored as a protein suspension in a solution containing ammonium sulfate (90% saturation) at 4°C.

Effects of pH and temperature. The optimal pH for hydantoin racemase activity was 9.5, and greater than 95% of the maximal activity was retained up to pH 11.0. However, chemical racemization occurred at alkaline pHs. At pH 11.0, the degree of chemical racemization was 5% of the degree of enzymatic racemization. The optimal temperature for the enzyme activity was 45°C (Fig. 2A), although chemical racemization also occurred at high temperatures. At 75°C, the degree of chemical racemization was 28% of the degree of enzymatic racemization. The thermal stability of the enzyme was examined, and it was found that the enzyme was stable in the presence of the substrate at a temperature of up to 45°C (Fig. 2B), which is the optimal temperature for the enzyme activity.

Effect of metals. The activity of the purified enzyme was assayed in the presence of different metal ions at 1 mM. It was found that the enzyme activity was slightly enhanced by the addition of Mn^{2+} or Co^{2+}. This enhancement was reproducible in different enzyme batches. The addition of Ca^{2+}, Mg^{2+}, or Ni^{2+} had no significant effect on the enzyme activity. On the other hand, Cu^{2+} and Zn^{2+} strongly inhibited the enzyme activity (Fig. 3). The concentration of Cu^{2+} required for 50% inhibition was about 0.3 μM, which is comparable to the enzyme monomer concentration in the assay mixture (about 0.2 μM).

The possible role of Mn^{2+} and Co^{2+} in hydantoin racemase was investigated by analyzing the effect of metal-chelating agents on the enzyme activity. EDTA and EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] at 1 mM also enhanced the enzyme activity slightly, indicating that the divalent metal ions are not required for catalytic activity. It seems that the low degree of stimulation by Mn^{2+} and Co^{2+} is caused by a competition with inhibitory metal ions such as Zn^{2+} or Cu^{2+}, which are ubiquitous (4).

Enzyme kinetics. The initial velocities of the hydantoin racemase reaction at different concentrations of D- or L-5-(2-methylthioethyl)hydantoin were measured, and substrate inhibition was observed with each substrate. Haldane presented the following equation for the substrate inhibition which occurs by the binding of two or more substrates to the active center of an enzyme (2):

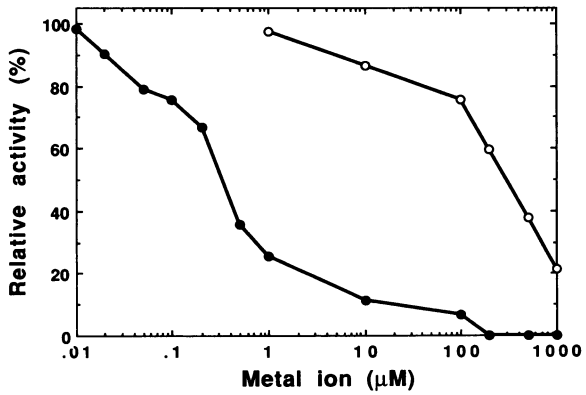

FIG. 3. Effect of metal ions on hydantoin racemase activity. Hydantoin racemase activity was assayed at different concentrations of Cu^{2+} (●) or Zn^{2+} (○). Relative activity is expressed as percentages of the initial velocity of the reaction without additives.

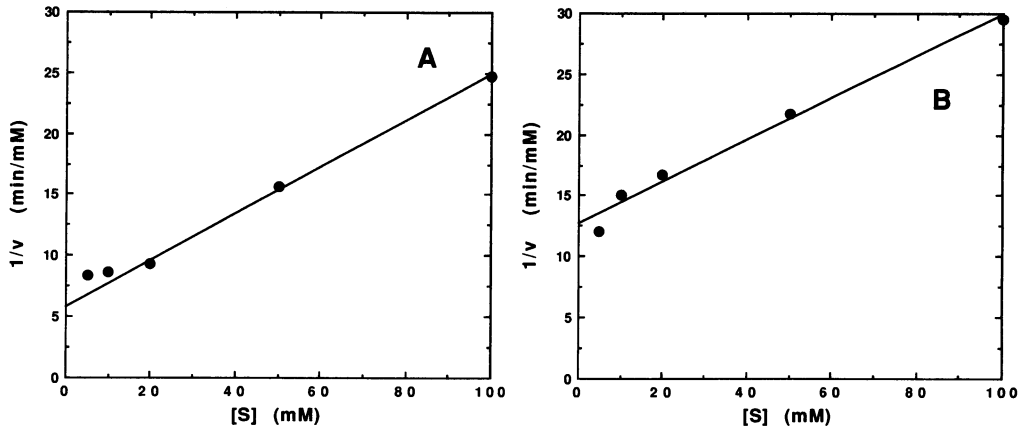

FIG. 4. Dependence of initial velocity of hydantoin racemase reaction on substrate concentration. The initial velocity of the hydantoin racemase reaction was measured at different concentrations of D- or L-5-(2-methylthioethyl)hydantoin (A and B, respectively) in 20 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl and 1 mM EDTA at 25°C. The data are plotted as the substrate concentrations versus the reciprocals of the initial velocities ($1/v$) of the hydantoin racemase reaction. The enzyme concentrations were 5 μg/ml for the D-isomer and 1 μg/ml for the L-isomer of the substrate.

$$1/v = 1/V_{max}(1 + K_s/[S] + [S]/K_s')$$

In this equation, K_s and K_s' are the dissociation constants of ES and ES_2, respectively. If $[S] \gg K_s$, plotting of $1/v$ versus [S] will result in a straight line. This linear relationship between the substrate concentration and the reciprocal of the initial velocity exists in the hydantoin racemase reactions (Fig. 4). The K_s' values for D- and L-5-(2-methylthioethyl) hydantoin were 29.8 and 73.7 mM, respectively, and the V_{max} values for the direction D- to L-isomer and the direction L- to D-isomer were 35.2 and 79.0 μmol/min/mg of protein, respectively (the hydantoin racemase content in the enzyme preparation was estimated to be 75% by scanning the SDS-polyacrylamide gel with a densitometer).

Substrate specificity. The ability of the purified enzyme to racemize different 5-substituted hydantoins was examined. The D- and L-isomers of 5-(2-methylthioethyl)hydantoin or 5-isobutylhydantoin were completely racemized (Fig. 5A and B). Racemization of the D-isomer of 5-methylhydantoin stopped halfway, whereas its L-isomer was thoroughly racemized (Fig. 5C). The enzyme had no detectable activity toward either isomer of 5-isopropylhydantoin (Fig. 5D). However, these inabilities of the enzyme are unexpected, because it is known that *E. coli* cells expressing the hydantoin racemase are capable of racemizing all of these 5-substituted hydantoins. In particular, 5-isopropylhydantoin is rapidly racemized by the *E. coli* cells (18).

Inactivation of hydantoin racemase by 5-substituted hydantoins. The hydantoin racemase reaction with D-5-methylhydantoin stopped halfway (Fig. 5C). Another addition of the enzyme (5 μg/ml) to the reaction mixture at 25 min of incubation resulted again in a rapid progress of the reaction, and once again the reaction stopped before reaching completion (data not shown). Therefore, it is thought that the enzyme added was inactivated quickly by D-5-methylhydantoin. To make certain of this, we performed the following experiments. The purified enzyme was preincubated with a 5-substituted hydantoin for 5 min at 30°C, and residual enzyme activity was examined with 5-(2-methylthioethyl)hydantoin as a substrate. Preincubation with the D-isomer of 5-methylhydantoin resulted in significant loss of 5-(2-methylthioethyl)hydantoin-racemizing activity, whereas this enzyme activity remained after preincubation with the L-isomer of 5-methylhydantoin. Preincubation with the D- or L-isomer of 5-isopropylhydantoin resulted in disappearance of the enzyme activity. These results indicate that 5-isopropylhydantoin (the D- and/or L-isomer) and the D-isomer of 5-methylhydantoin inactivate the hydantoin racemase.

Protection of hydantoin racemase from inactivation by 5-isopropylhydantoin. We carried out a reaction in which 5-isopropylhydantoin and 5-(2-methylthioethyl)hydantoin were mixed before addition of the enzyme and unexpectedly found that both 5-substituted hydantoins are racemized completely (Fig. 6). Therefore, it is thought that 5-(2-methylthioethyl)hydantoin protects the enzyme from inactivation by 5-isopropylhydantoin. On the other hand, 5-isobutylhydantoin and 5-methylhydantoin (the L-isomer) did not have this protective effect.

To test which component of 5-(2-methylthioethyl)hydantoin is responsible for the protective effect, we carried out a reaction with a mixture containing methionine or hydantoin instead of 5-(2-methylthioethyl)hydantoin. The results indicate that methionine is more effective than 5-(2-methylthioethyl)hydantoin, whereas hydantoin has no effect. Thus, we examined the protective effects of various compounds on the enzyme (Table 2). D-Methionine was as effective as L-methionine. Therefore, the protective effect is independent of the configuration of methionine. Aside from methionine, L-cysteine had a significant protective effect among the amino acids tested (glycine, L-alanine, L-lysine, L-arginine, L-glutamic acid, L-glutamine, L-aspartic acid, L-asparagine, L-threonine, L-valine, L-serine, L-proline, L-histidine, L-phenylalanine, L-leucine, and L-isoleucine had no effect). Methionine analogs containing divalent sulfur (R-S-R′) were all effective, whereas methionine sulfoxide (R-SO-R′), methionine sulfone ($R\text{-}SO_2\text{-}R'$), and cysteic acid ($R\text{-}SO_3H$) were not. Seleno-DL-methionine had a small protective effect. From these results, it is thought that divalent-sulfur-containing compounds (R-S-R′ and R-SH) have a protective effect. Thus, we tried to test some heterocyclic compounds containing divalent sulfur and found that D-biotin is effective (thiamine and ampicillin were not effective). Reducing agents, β-mercaptoethanol, dithiothreitol, and glutathione were also effective (glutathione in the oxidized form was not effective).

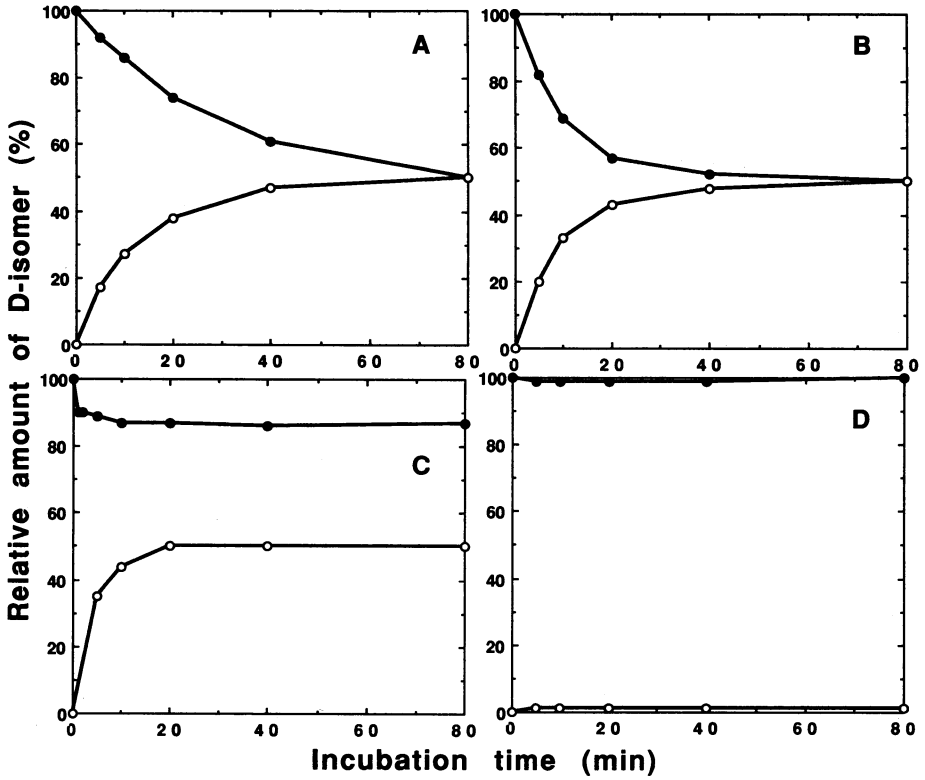

FIG. 5. Substrate specificities of the purified hydantoin racemase. Purified enzyme (5 μg/ml) was incubated with the D-isomer (●) or the L-isomer (○) of a 5-substituted hydantoin (10 mM) in 20 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl and 1 mM EDTA at 30°C, and optical rotations (at 589 nm) of the solution were measured at the points of time indicated. (A) 5-(2-Methylthioethyl)hydantoin; (B) 5-isobutylhydantoin; (C) 5-methylhydantoin; (D) 5-isopropylhydantoin.

The strength of the protective effect of L-methionine was compared with that of β-mercaptoethanol by reducing their concentrations from 10 to 0.1 mM. The protective effect was observed at 0.2 mM or above for L-methionine and at 0.5 mM or above for β-mercaptoethanol, indicating that L-methionine is almost equivalent to β-mercaptoethanol in its protective effect. Therefore, it is thought that the protective effect of the divalent-sulfur-containing compounds is not due to their reducing power. L-Methionine had no effect on the velocities of the hydantoin racemase reactions with 5-(2-methylthioethyl)hydantoin and 5-isobutylhydantoin. This suggests that the effect of the divalent-sulfur-containing compounds is not stimulative. The hydantoin racemase reaction with the D-isomer of 5-methylhydantoin, which inactivates the enzyme, also reached completion in the presence of L-methionine. Therefore, it is thought that the mechanism of inactivation with D-5-methylhydantoin is the same as that with 5-isopropylhydantoin.

DISCUSSION

The cloning and expression of *hyuE* in *E. coli* enabled us to purify the gene product hydantoin racemase with relative ease. The purified-enzyme preparation contained a major peptide with an apparent molecular mass of 32 kDa and a minor peptide of 28 kDa. From N-terminal sequence analysis, it was found that the 32-kDa peptide is the *hyuE* product but that the 28-kDa peptide is not. A search of the NBRF (National Biomedical Research Foundation; release 31) and SWISS (European Molecular Biology Laboratory; release

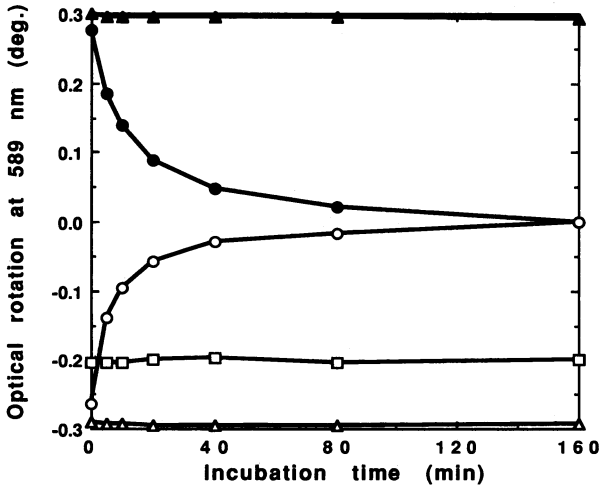

FIG. 6. Protection of hydantoin racemase activity by 5-(2-methylthioethyl)hydantoin. 5-Isopropylhydantoin (20 mM) in 20 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl and 1 mM EDTA was mixed with an equal volume of 20 mM 5-(2-methylthioethyl)hydantoin (circles), 5-isobutylhydantoin (triangles), or 5-methylhydantoin (squares) in the same buffer. The reactions were initiated by addition of the enzyme (5 μg/ml) to the mixture. Incubations were carried out at 30°C, and optical rotations of the solution were measured at the points of time indicated. Closed symbols, D-5-isopropylhydantoin and the D-isomer of the 5-substituted hydantoin tested; open symbols, L-5-isopropylhydantoin and the L-isomer of the 5-substituted hydantoin tested.

TABLE 2. Protection of hydantoin racemase from inactivation by 5-isopropylhydantoin[a]

Compound tested	Relative activity (%)[b]
None	0
L-Methionine	100
D-Methionine	99
L-Cysteine	84
N-Carbamyl-L-methionine	100
N-Acetyl-DL-methionine	98
2-Keto-4-(methylthio)butyric acid	98
DL-2-Hydroxy-4-(methylthio)butyric acid	100
L-Methionine methyl ester	79
DL-Methionine hydroxamic acid	73
S-Methyl-L-cysteine	95
L-Ethionine	96
DL-Methionine sulfoxide	3
DL-Methionine sulfone	0
L-Cysteic acid	0
Seleno-DL-methionine	13
D-Biotin	98
β-Mercaptoethanol	95
Dithiothreitol	105
Glutathione (reduced form)	94

[a] The reactions were initiated by addition of the purified enzyme (10 μg) to 10 ml of reaction mixtures containing 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 10 mM L-5-isopropylhydantoin, and a compound tested (10 mM). Initial velocities of the reactions were determined at 30°C by measuring optical rotations at a wavelength of 589 nm.

[b] Relative activities are expressed as percentages of the initial velocity obtained with L-methionine.

20) protein data bases revealed an identity between the N-terminal sequences of the 28-kDa peptide and a uridine phosphorylase (EC 2.4.2.3) from *E. coli* (16), although the seventh amino acid residue, His, of the uridine phosphorylase could not be identified in the 28-kDa peptide. The uridine phosphorylase was purified from a crude extract of *E. coli*. The precipitate obtained from a 60 to 90% saturation of ammonium sulfate contained uridine phosphorylase, and this enzyme was eluted from a DEAE-Sephadex column with a buffer containing about 150 mM NaCl (6). Moreover, uridine phosphorylase is a hexamer with 27.5-kDa identical subunits (1). Thus, the behavior of uridine phosphorylase in purification resembles that of hydantoin racemase. Therefore, it seems that the uridine phosphorylase of *E. coli* was copurified with hydantoin racemase.

The purified hydantoin racemase could not racemize 5-isopropylhydantoin. However, in the presence of a divalent-sulfur-containing compound (R-S-R′ or R-SH), 5-isopropylhydantoin was easily racemized by the purified enzyme. The initial velocity of the hydantoin racemase reaction with L-5-isopropylhydantoin in the presence of L-methionine was 16-fold higher than that with L-5-(2-methylthioethyl)hydantoin, suggesting that 5-isopropylhydantoin is a good substrate for the enzyme. Therefore, 5-isopropylhydantoin may be a suicide substrate which binds to the active center of the enzyme irreversibly (3, 15) in the absence of the divalent-sulfur-containing compound. If this was the case, the divalent-sulfur-containing compound would be an allosteric effector which interacts with the enzyme through the sulfur atom so that the catalysis proceeds.

A number of enzymes are known to show an absolute requirement for a thiol compound. The following enzymes are examples. The hydroxymethylglutaryl-coenzyme A lyase (EC 4.1.3.4) from bovine liver exhibits an absolute requirement for a thiol compound (dithiothreitol, cysteine, or glutathione), and preincubation with the thiol compound is necessary for an optimal initial reaction rate (13). The cellodextrin phosphorylase (EC 2.4.1.49) from *Clostridium thermocellum* is inactive in the absence of thiol compounds such as cysteine or dithiothreitol, but non-sulfur-reducing compounds such as $NaBH_4$ or ascorbic acid are not effective (10). The arginyltransferase (EC 2.3.2.8) from rabbit liver requires the presence of a thiol compound, and dithiothreitol is effective at 1/10 the concentration required for β-mercaptoethanol (11). The pyruvate synthase (EC 1.2.7.1) from *Clostridium acidi-urici* requires a high concentration of a thiol compound for maximum activity and is also activated by the thiol compound (14). In the enzymes mentioned above, the effect of the thiol compounds was attributed to their reducing power, which stabilizes the enzymes in a reduced form. On the other hand, we have shown that the divalent-sulfur-containing compounds (R-S-R′ and R-SH) regulate enzyme activity without reduction.

In a bacterial cell, hydantoin racemase activity is thought to be protected by a divalent-sulfur-containing compound(s) present in the cell. At this point, it is still impossible to speculate on what significance the divalent-sulfur-containing compound(s) has in regulating the enzyme activity.

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