Purification and Characterization of Glutathione-Independent Denitration Enzyme of Organic Nitrate Esters in Rabbit Hepatic Cytosol

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The enzyme responsible for glutathione (GSH)-independent denitration of organic nitrate esters was purified by gel chromatography, ion-exchange chromatography and affinity chromatography from rabbit hepatic cytosol. The enzyme showed a molecular mass of 175 kDa and consisted of three subunits of 59 kDa. The enzyme exerted its maximum activities at around pH 9, when isosorbide dinitrate (ISDN) was used as substrate. The enzyme possessed a low K_m value (10^{-6} M) for various organic nitrate esters. The present enzyme is likely to be involved in the denitration of organic nitrate esters in conjunction with known enzymes, GSH *S*-transferase (GST) and cytochrome P450.

Key words glutathione-independent denitration; organic nitrate ester; rabbit hepatic cytosol

Organic nitrate esters, such as nitroglycerin (NTG), isosorbide dinitrate (ISDN) and pentaerythritol tetranitrate (PETN), have been used widely in the treatment of angina pectoris. These compounds, in general, are rapidly denitrated to corresponding alcohols primarily in the liver but also in the intestine, kidney, lung, heart, blood and blood vessels.^{1–5)} Considerable evidence has now accumulated to suggest that vascular denitration of organic nitrate esters to an active species, presumably nitric oxide, is critical for vasodilating activity.^{6–9)} However, the biochemical pathways for the metabolic formation of nitric oxide from organic nitrate esters have not yet been completely defined.

Several metabolic denitration systems for organic nitrate esters have been proposed, including glutathione *S*-transferase (GST),^{10–12)} hemoproteins such as hemoglobin¹³⁾ and cytochrome P450,^{14–16)} serum proteins such as an albumin¹⁷⁾ and a membrane-bound enzyme in vascular smooth muscle cells.¹⁸⁾

In our previous study,¹⁹⁾ it has been shown by gel filtration chromatography that the denitration activity responsible for NTG and ISDN in rabbit hepatic cytosol was divided into two fractions (peak I and peak II). The denitration activity of peak I, which was potentiated by dithiothreitol (DTT), but not by glutathione (GSH), was not inhibited by *S*-hexyl GSH, an inhibitor of GST. By contrast, that of peak II, having GST activity, required GSH and was inhibited by *S*-hexyl GSH. These data strongly suggest that in addition to GST, GSH-independent pathway(s) can be responsible for the metabolic denitration of NTG and ISDN. Moreover, it has been suggested that GSH-independent enzyme exists in rabbit vascular cytosol. Thus, GSH-independent denitration enzyme might play a role in the pharmacological action of organic nitrate esters.

In the present study, we investigated the purification and characterization of GSH-independent enzymes responsible for denitration of organic nitrate esters in rabbit hepatic cytosol.

MATERIALS AND METHODS

Chemicals ¹⁴C-NTG, labelled at the C-1 and C-3 position (specific activity; 1600 MBq/mmol), with radiochemical purity greater than 97%, was synthesized from ¹⁴C-glycerol in our laboratory. ¹⁴C-2-Nitrooxypropyl 3-nitrooxypropyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate (NND) and ¹⁴C-(4*R*)-(–)-(2-nicotinoylamino)ethyl 3-nitrooxypropyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate (NAND), labelled at the C-4 position of the dihydropyridine nucleus, with radiochemical purity greater than 97%, were synthesized by Amersham (UK). Unlabelled NTG, ISDN and PETN were obtained from Nippon Kayaku Co., Ltd. (Tokyo, Japan), Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Shionogi & Co., Ltd. (Osaka, Japan), respectively. Unlabelled nicorandil, NND and NAND were synthesized in our laboratory. DTT was purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A.). Sephadex G-200, Fractogel EMD TMAE-650 and Matrex Gel Green A were obtained from Pharmacia LKB (Uppsala, Sweden), Merck (Darmstadt, Germany) and Amicon (Massachusetts, U.S.A.), respectively.

Animals and Enzyme Preparations Japanese White rabbits (Nippon Institute for Biological Science, Tokyo, Japan; body wt. 2–3 kg) were used. Rabbit hepatic cytosol was obtained as described previously.²⁰⁾

Purification of GSH-Independent Denitration Enzyme
Ammonium Sulphate Fractionation: Ammonium sulphate was added to the hepatic cytosol fraction at 4 °C and the precipitate at 40–80% saturation was collected by centrifugation at 9000 × *g* for 10 min. The precipitate was dissolved in 10 mM sodium (Na), potassium (K) phosphate buffer, pH 7.4.

Gel Chromatography: The ammonium sulfate fraction (40–80%) from hepatic cytosol was applied to a Sephadex G-200 column (2.6 cm × 60 cm) equilibrated with 10 mM phosphate buffer, pH 7.4. Fractions were collected and analyzed for denitration activity.

Ion-Exchange Chromatography: The active fraction

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from the gel chromatography was dialyzed for 40 h at 4 °C against 20 mM Tris-HCl buffer (pH 9.0), and applied to a Fractogel EMD TMAE-650 column (1.6 × 30 cm) that had been previously equilibrated in the same buffer. After washing the column with the buffer, the enzyme was eluted with a linear gradient established between 100 ml of the same buffer and 100 ml of the buffer containing 300 mM NaCl. Fractions containing high levels of denitration activity were pooled and concentrated approximately 4-fold by ultrafiltration on an Minicon-B (Amicon) membrane filter. The concentrated enzyme preparation was then dialyzed overnight against 10 mM Na-K phosphate buffer, pH 7.4.

Affinity Chromatography: The fraction obtained from the ion-exchange chromatography was applied to a Matrex-Gel Green A column chromatography. The enzyme was eluted with 30 mM Na-K phosphate buffer. Fractions were collected and analyzed for denitration activity.

Protein Determination Protein concentrations were determined by the method of Lowry *et al.*,²¹⁾ using bovine serum albumin as the standard.

Assay of Denitration Activity Unless otherwise indicated, incubations were conducted at 37 °C for 5 min in a 1.0 ml medium containing the protein from either fraction, 100 mM Na-K phosphate buffer, pH 7.4, 2 mM EDTA, and 100 μM substrate, in the presence of 5 mM DTT.

When ¹⁴C-labelled substrate was used, concentrations of ¹⁴C-unchanged drug and its metabolites were measured by TLC according to the method of Chong and Fung.¹⁷⁾ In case of unlabelled substrate, inorganic nitrite released from the substrate was assayed according to the method of Kabuto *et al.*¹⁾

Estimation of Molecular Weight Molecular weight was determined by Sephadex G-200 gel filtration. The Sephadex column (1.6 × 60 cm) was calibrated with 10 mM Na, K-phosphate buffer, pH 7.4, at 4 °C using blue dextran, myoglobin, catalase, γ-globulin and ovalbumin as standards.

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis (SDS/PAGE) SDS/PAGE was performed in the presence of SDS with the buffer system described by Laemmli.²²⁾ Samples were pretreated with the addition of 2-mercaptoethanol and SDS to final concentrations of 5% (v/v) and 1% (w/v), respectively, and then heated at 100 °C for 10 min. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250. Minimum subunit molecular masses were estimated by comparison with commercial molecular weight standards (phosphorylase, bovine serum albumin, ovalbumin, carbonic anhydrase,

soybean trypsin inhibitor, lysozyme).

RESULTS

Purification of GSH-Independent Denitration Enzyme

As previously shown,¹⁹⁾ Sephadex G-200 chromatography of ammonium sulphate precipitation (40–80%) of rabbit hepatic cytosol showed two peaks: GSH-independent activity (peak I) and GST (peak II). Peak I was further purified by column chromatography. Table 1 outlines a typical purification of GSH-independent denitration enzyme. The GSH-independent denitration enzyme was purified about 72-fold from the cytosol fraction of rabbit liver by ammonium sulphate fractionation, Sephadex G-200 gel filtration, Fractogel EMD TMAE-650 ion-exchange chromatography (Fig. 1) and Matrex Green A affinity chromatography. The overall yield of enzyme activity was 21%. The final enzyme preparation exhibited a single band on Coomassie-Blue-stained SDS/PAGE (Fig. 2). The position of peak I enzyme on the gel corresponded to a size of 59 kDa, whereas the native molecular mass of the enzyme on a Sephadex G-200 column was about 175 kDa (Fig. 3).

Substrate Specificity NTG, ISDN, PETN, nicorandil, NND and NAND were examined as substrate for peak I enzyme (Table 2). Among organic nitrate esters tested, NAND, which has a nitrate ester in the propanoyl side chain at the dihydropyridine ring, showed the highest activity under the incubation condition used. NTG and ISDN had relatively low activity and nicorandil had no or very low activity. The apparent K_m and V_{max} for several organic nitrate esters were estimated (Table 3). The K_m values for the denitrated metabolites formation ranged from 1.08 to 9.57 μM. The V_{max}/K_m value for 1,2-glyceryldinitrate formation from NTG was twice higher than that for 1,3-glyceryldinitrate formation. But there was a little regioselectivity in the formation for two mono-denitrated metabolites of NND, M-1 and M-2.

pH Optimum of Peak I Enzyme The pH dependence of GSH-independent denitration for ISDN was examined in sodium acetate buffer (pH 3.5–5.0), sodium-potassium phosphate buffer (pH 5.0–8.0), Tris-HCl buffer (pH 7.4–9.0) and glycine-KOH buffer (pH 9.0–11.0). As shown in Fig. 4, the enzyme exerted its maximum activity at pH 9.0.

Stability of Peak I Enzyme The purified peak I enzyme was stored at 4 °C or heated at 70 °C, and the remaining activity was determined. Storage at 4 °C for 2, 5 and 10 d resulted in 6, 21 and 87% loss of the enzyme activity, respectively. Treatment at 70 °C for 5 min abolished the activities completely.

Table 1. Purification of Peak I Enzyme from Rabbit Liver

	Protein (mg)	Activity (nmol/min)	Recovery (%)	Specific activity (nmol/min/mg)	Purification (fold)
Cytosol	6906	2002	100	0.29	1.0
40–80% (NH ₄) ₂ SO ₄	3240	1568	78	0.48	1.7
Sephadex G-200	251	1489	74	5.9	20
Fractogel TMAE	69	827	41	12	41
Green A	12	252	13	21	72

Fig. 1. Fractogel EMD TMAE-650 Column Chromatography

The pooled Sephadex G-200 fraction (peak I) was applied to the Fractogel EMD TMAE-650 column as described under Materials and Methods. Fractions were analyzed for A_{280} (---) and denitration activity (—●—) towards ISDN.

Fig. 2. SDS/PAGE Analysis of the Purification of Peak I Enzyme

A 12.5% polyacrylamide gel was stained with Coomassie Blue. Lanes: 1, molecular weight standards; phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa; 2, rabbit hepatic cytosol; 3, Sephadex G-200 fraction; 4, Fractogel EMD TMAE-650 fraction; 5, Green A Fraction.

Fig. 3. Molecular Weight Estimation of Peak I Enzyme by Sephadex G-200 Gel Filtration

Peak I enzyme is indicated in the figure (○). Molecular weight standard proteins (●).

Table 2. Substrate Specificity of Peak I Enzyme

Substrate	Relative activity
NTG	100
ISDN	103
PETN	141
Nicorandil	< 17
NND	157
NAND	191

Each substrate (5 μ M) was added to a reaction mixture containing 5 mM DTT, enzyme and 0.1 M Na, K-phosphate buffer, pH 7.4. The denitration activities were determined by measuring the nitrite ions from substrates as described under Materials and Methods.

Table 3. Substrate Specificity of Peak I Enzyme in Rabbit Hepatic Cytosol

Substrate	Metabolite	K_m (μ M)	V_{max} (nmol/min/mg)	V_{max}/K_m (ml/min/mg)
NTG	Total metabolites ^{a)}	8.09	7.58	0.94
	1,2-Dinitrate	7.11	4.61	0.65
	1,3-Dinitrate	9.57	3.05	0.32
NND	Total metabolites ^{b)}	1.12	5.46	4.88
	M-1 ^{c)}	1.08	2.05	1.89
	M-2 ^{d)}	1.41	3.56	2.52
NAND	M-1 ^{e)}	2.78	8.00	2.88

Various concentrations of substrates were added to a reaction mixture containing 5 mM DTT, enzyme and 0.1 M Na, K-phosphate buffer, pH 7.4. The denitration activities were determined by measuring the denitrated metabolites as described under Materials and Methods. Michaelis constants and maximal velocities were estimated by Lineweaver-Burk double-reciprocal plots. *a)* 1,2-Dinitrate and 1,3-dinitrate. *b)* M-1 and M-2. *c)* 2-Hydroxypropyl 3-nitrooxypropyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate. *d)* 2-Nitrooxypropyl 3-hydroxypropyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate. *e)* (4*R*)-(—)-(2-Nicotinoylamino) ethyl 3-hydroxypropyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate.

Fig. 4. Denitration Activity as a Function of pH

Sodium acetate buffer (□), Na, K-phosphate buffer (△), Tris-HCl buffer (○) and glycine-KOH buffer (◇) were used for assay.

DISCUSSION

To date, at least four pathways that can catalyze denitration of organic nitrate esters have been reported: 1) cytosolic and microsomal GST,¹⁰⁻¹² 2) hemoproteins such as cytochrome P450¹⁴⁻¹⁶ and hemoglobin,¹³ 3) serum thiol protein such as albumin¹⁷ and 4) microsomal enzyme in vascular smooth muscle cells.¹⁸ It is clear from our previous^{19,20,23} and present studies that peak I enzyme is different from GST. Moreover, the second pathway, denitration by hemoprotein, should be ruled out

for peak I denitration, since carbon monoxide had no effect on the denitration activity of peak I (100% vs. control). Based on the findings that SH-blocking reagent caused a decrease of peak I denitration activity, it is conceivable that free thiol(s) on peak I enzyme(s) is critical for expression of the denitration activity.¹⁹⁾ With regard to this point, the mechanism of the denitration by peak I enzyme is similar to that of third or fourth pathway. However, the peak I enzyme is thought not to be due to plasma proteins, because of its high activity compared to that of plasma (data not shown). The enzyme that Seth and Fung¹⁸⁾ have identified in vascular microsomes is activated by thiol compounds and shows a molecular mass of 200 kDa consisting of four subunits. On the other hand, the molecular weight of peak I estimated from elution pattern from the Sephadex G-200 was 175000 and was an oligomer consisting of three subunits of 59 kDa. Thus, peak I enzyme has many points of similarity to the enzyme reported by Seth and Fung,¹⁸⁾ except for its subcellular distribution. However, the peak I enzyme is thought not to be due to contamination of the microsomes, since the denitration activity toward ISDN in rabbit hepatic microsomes was negligible (data not shown). Therefore, the denitration catalyzed by peak I enzyme differs from previously reported denitration pathways.

Lau and Benet¹²⁾ reported that the $V_{\max}/K_m$ ratio for 1,2-dinitrate and 1,3-dinitrate formation from NTG in GSH-dependent denitration, which is considered to be catalyzed by GST, of rabbit hepatic cytosol was 0.20 and 0.07 ml/min per mg protein of the cytosol, respectively. The $V_{\max}/K_m$ for NTG in GST might be about 10–20 times greater than that in peak I enzyme considering that peak I enzyme was purified about 72-fold from the cytosol. Therefore, the contribution of peak I enzyme to *in vivo* NTG metabolism should be negligible compared to GST. However, we demonstrate that, in the other organic nitrates, peak I denitration pathway cannot be ignored. Because, in our preliminary study using partially purified peak II, GST, from rabbit hepatic cytosol, the denitration activity toward NTG was highest among various organic nitrates, while the denitration activity of peak I toward NTG was relatively low compared to other organic nitrates. In addition, the K_m value for GST was higher than that for peak I (10^{-6} M), and the concentration of the free fraction of the organic nitrate esters *in vivo* are considered to be less than 10^{-6} M.²⁴⁾ It is likely, therefore, that peak I enzyme plays a role in the metabolic denitration of organic nitrates *in vivo*, although what is not clear is the relative importance of peak I and the other biotransformation systems in the metabolic denitration of nitrate esters *in vivo*.

We have demonstrated that DTT, which is used as a cofactor in the assay of the denitration activity, maintains the free thiol group on the peak I enzyme at its reduced state.¹⁹⁾ However, the origin and nature of the physiological reduction system, replaced by DTT, is not yet clear. In our preliminary study, dihydrolipoamide potentiated peak I denitration activity as well as DTT. To identify the physiologically relevant reducing agent for this reaction, further study is needed.

In our previous report,¹⁹⁾ GSH-independent denitration

enzyme that is similar to in hepatic cytosol exists in vascular cytosol. Several previous reports have demonstrated that thiol is intimately involved in the metabolic denitration and pharmacological effects of organic nitrate esters.^{6,25)} Moreover, it has been proposed that intracellular sulfhydryl groups necessary as cofactor for denitration are depleted during sustained nitrate exposure, thereby leading to the development of nitrate tolerance.^{26,27)} Thus, the denitration activity of peak I, which was activated by the thiol compound DTT, might play a role in the pharmacological effects of nitrate esters and the development of nitrate tolerance. However, clarification of this possibility requires further study.

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