S-Nitrosogluthathione is a substrate for rat alcohol dehydrogenase class III isoenzyme

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An enzyme isolated from rat liver cytosol (native molecular mass 78.3 kDa; polypeptide molecular mass 42.5 kDa) is capable of catalysing the NADH/NADPH-dependent degradation of *S*-nitrosogluthathione (GSNO). The activity utilizes 1 mol of co-enzyme per mol of GSNO processed. The isolated enzyme has, as well, several characteristics that are unique to alcohol dehydrogenase (ADH) class III isoenzyme: it is capable of catalysing the NAD⁺-dependent oxidations of octanol (insensitive to inhibition by 4-methylpyrazole), methylcrotyl alcohol (stimulated by added pentanoate) and 12-hydroxydodecanoic acid, and also the NADH/NADPH-dependent reduction of octanal. Methanol and ethanol oxidation activity is minimal. The enzyme has formaldehyde dehydrogenase activity in that it is capable of catalysing the NAD⁺/NADP⁺-dependent oxidation of *S*-hydroxymethylglutathione. Treatment with the arginine-specific reagent phenylglyoxal prevents the pentanoate stimulation of

methylcrotyl alcohol oxidation and markedly diminishes the enzymic activity towards octanol, 12-hydroxydodecanoic acid and *S*-hydroxymethylglutathione; the capacity to catalyse GSNO degradation is also checked. Additionally, limited peptide sequencing indicates 100 % correspondence with known ADH class III isoenzyme sequences. Kinetic studies demonstrate that GSNO is an exceptionally active substrate for this enzyme. *S*-Nitroso-*N*-acetylpenicillamine and *S*-nitrosated human serum albumin are not substrates; the activity towards *S*-nitrosated glutathione mono- and di-ethyl esters is minimal. Product analysis suggests that glutathione sulphinamide is the major stable product of enzymic GSNO processing, with minor yields of GSSG and NH₃; GSH, hydroxylamine, nitrite, nitrate and nitric oxide accumulations are minimal. Inclusion of GSH in the reaction mix decreases the yield of the supposed glutathione sulphinamide in favor of GSSG and hydroxylamine.

INTRODUCTION

It has been suggested that a number of the observed cellular responses to exogenously derived or endogenously generated nitric oxide may be due to intracellular thiol nitrosation [1,2]. Mechanisms illustrating how such a nitrosation process might occur, at both high and low ambient nitric oxide concentrations, have been devised and tested [3–5]. Further, there are several reports indicating that proteins can be isolated from nitric oxide-treated cells with modified functional characteristics shown as being due to the nitrosation of key cysteine residues [6–10]. If intracellular thiol nitrosation is indeed a consequence of nitric oxide exposure, then relatively abundant GSH might be expected to be a principal target for this reaction, producing *S*-nitrosogluthathione (GSNO). Also, it is feasible that the rapidly established nitroso-moiety exchange equilibria among available thiols, including those composing proteins, that has been characterized *in vitro* [11–14] applies in the intracellular milieu. It can then be expected that the available nitric oxide moiety will be redistributed among the cellular thiol species according to their relative abundance as well as the relative strength of each nitroso group–thiol interaction [1]. The GSH/GSNO couple would predominate in this equilibrium condition.

As a means to establish the role of GSNO and of nitroso group exchange among thiols in the cellular responses to nitric oxide,

we have developed a system by which GSNO can be introduced into the cytosol of rat hepatocytes in primary culture without the precondition of nitric oxide exposure. This approach takes advantage of the capacity of certain cytosolic, Mu class glutathione transferase isozymes to utilize the *N*-nitroso compound 1,3-dimethyl-2-cyano-1-nitrosoguanidine (cyanoDMNG) as substrate [15]. We have determined that the products of the enzymic reaction mediated by glutathione transferases isolated from rat liver are the denitrosated compound, 1,3-dimethyl-2-cyanoguanidine (cyanoDMG), and GSNO generated in a 1:1 ratio (Scheme 1). This same reaction stoichiometry has been verified upon applying cyanoDMNG and GSH to rat liver cytosol fraction incubates; the sensitivity of the cytosolic reaction to included glutathione transferase inhibitors has also been established [14,16].

When cyanoDMNG is applied to rat hepatocytes in primary culture, cyanoDMG appears in the culture medium at the same rate at which cyanoDMNG disappears [17]. Intracellular GSH is evidently essential to this processing, as the conversion does not occur in cultures of hepatocytes that have been completely depleted of cytosolic glutathione by pretreatment with diethyl maleate. In addition, the conversion is inhibited when the glutathione transferase inhibitor ethacrynic acid is applied to cultures coincidentally with the *N*-nitroso compound. These observations serve to support the notion that glutathione trans-

Abbreviations used: ADH, alcohol dehydrogenase; BCS, bathocuproinedisulphonic acid; cyanoDMG, 1,3-dimethyl-2-cyanoguanidine; cyanoDMNG, 1,3-dimethyl-2-cyano-1-nitrosoguanidine; GSNO, *S*-nitrosogluthathione; 12-HDA, 12-hydroxydodecanoic acid; HMGSH, *S*-hydroxymethylglutathione; HSA, human serum albumin; MCA, methylcrotyl alcohol; NO-HSA, *S*-nitrosated HSA; SNAP, *S*-nitroso-*N*-acetylpenicillamine.

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Scheme 1

ferases mediate the conversion of cyanoDMNG into cyanoDMG in intact rat hepatocytes.

However when a low concentration of cyanoDMNG (50 μM in the medium) is applied to hepatocyte cultures, the cells are capable of promptly converting up to twice the amount of cyanoDMNG into cyanoDMG than there is total intracellular glutathione available. At this treatment concentration the GSNO yield in cell lysates is below the levels detectable in the assay [17]. If glutathione transferases indeed mediate cyanoDMNG denitrosation, the GSNO product must be converted into GSH in order to sustain continued cyanoDMNG processing.

At a higher concentration of applied cyanoDMNG (200 μM), GSNO is readily detectable in hepatocyte lysates [17]. In this case the GSNO rapidly attains a plateau concentration (4 μM in the assay relative to an initial, total glutathione concentration of 30 μM in the lysate) and maintains this apparent steady state even though 90 μM the applied cyanoDMNG is converted into the denitrosated product over the 60 min of observation. This result suggests that the GSNO generated in the supposed glutathione transferase-mediated reaction may be actively catabolized.

We have discovered a NADH/NADPH-dependent activity in rat liver cytosol fraction that catabolizes GSNO [14]. This activity has been named GSNO terminase. An assay that monitors GSNO-dependent NADH utilization has facilitated the isolation of GSNO terminase activity. Purification procedures as well as the primary and secondary products generated in GSNO processing, including GSSG, which can be converted intracellularly into GSH, are described here. Limited peptide sequencing of fragments derived from the isolated enzyme has revealed correspondence with the known sequence of rat liver alcohol dehydrogenase (ADH) class III isozyme. Biochemical characterization of the purified enzyme with respect to the known activities of ADH class III isozyme as a medium-chain alcohol dehydrogenase and as a formaldehyde dehydrogenase, reported here, corroborate the identity. The present study establishes GSNO as among the most effective substrates for ADH class III isozyme.

MATERIALS AND METHODS

Materials

Reactive Red 120 Type 3000 agarose, Polybuffer Exchanger 94, Polybuffer 96, Sephacryl S-100-HR, Sephacryl S-200-HR, human serum albumin (HSA), *N*-acetyl-D,L-penicillamine, bathocuproinedisulphonic acid (BCS), 4-methylpyrazole, 12-hydroxy-dodecanoic acid (12-HDA), *n*-valeric acid (pentanoic acid), glutathione monoethyl ester (γ -Glu-Cys-Gly-OEt), superoxide dismutase (bovine erythrocyte) and an Ammonia Diagnostic Kit

were from Sigma (St. Louis, MO, U.S.A.). Glutathione diethyl ester was from Bachem Bioscience (King of Prussia, PA, U.S.A.). Spermine NONOate [18] was from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). Phenylglyoxal monohydrate, methylcrotyl alcohol (MCA; 3-methyl-2-buten-1-ol), *n*-butyl nitrite, 8-hydroxyquinoline and hydroxylamine hydrochloride were obtained from Aldrich (Milwaukee, WI, U.S.A.). Preparation of the S-nitrosated derivatives of glutathione, glutathione monoethyl ester and glutathione diethyl ester, immediately before the experimental requirement, followed the method of Park [11]. *S*-Nitroso-*N*-acetylpenicillamine (SNAP) was synthesized from *N*-acetyl-D,L-penicillamine by the method of Field et al. [19]. Glutathione sulphinic acid was synthesized and isolated according to the method described by Calam and Waley [20]. All other chemicals were of reagent grade and were purchased from commercial sources. Female Sprague-Dawley rats (150–200 g) were obtained from Charles River Laboratories (Wilmington, MA, U.S.A.).

Preparation of rat liver cytosol and isolation of GSNO terminase activity

The rat liver cytosol fraction from two rats was prepared as previously described [16], except that the homogenization mixture was 2 ml of buffer per g of liver. All subsequent enzyme isolation procedures were carried out at 5 °C. The crude cytosol was dialysed against changes of 10 mM K_2HPO_4 /0.5 mM dithiothreitol, pH 7.0, and a small amount of precipitate was removed by low-speed centrifugation. The sample was then applied to a Reactive Red 120 Type 3000 agarose column (1.6 cm $\times$ 38 cm) pre-equilibrated with 10 mM K_2HPO_4 , pH 7.0 (low-salt buffer). After 7 h, which included sample application followed by low-salt buffer, a 16 h linear gradient to 40 % high-salt buffer (2.0 M KCl and 10 mM K_2HPO_4 , pH 7.0) was initiated, followed by a 4 h wash with 100 % high-salt buffer; the flow rate was 20 ml per h throughout. The GSNO terminase activity in the collected 5 ml fractions was assessed as described below. The active fractions were pooled, and the sample volume was reduced to 10 ml using a Amicon stirred cell fitted with a YM10 membrane (Amicon, Beverly, MA, U.S.A.). The sample was dialysed with changes against 75 mM Tris base, pH 9.3, with acetic acid (equilibration buffer) and was then applied to a Polybuffer Exchanger 94 column (1.0 cm $\times$ 32 cm) pre-equilibrated with this same buffer. After a 5 ml chase with equilibration buffer, the column was eluted with 20-fold diluted Polybuffer 96, pH 6.0, with acetic acid, at a flow rate of 20 ml per h. The collected 5 ml fractions were assessed for GSNO terminase activity, the active fractions were pooled, and the volume was reduced to 10 ml as described above.

Finally, the sample was applied to a Sephacryl S-100-HR column (2.6 cm $\times$ 95 cm) pre-equilibrated with 100 mM Na_2HPO_4 , pH 7.4, and eluted with the same buffer at 20 ml per h. The collected 5 ml fractions were assessed for GSNO terminase activity, the active fractions were pooled, and the volume was reduced to 10 ml as described above. The isolated activity was stored in 2 ml aliquots at -80°C . The isolation procedure was generally completed in 72 h.

Protein concentrations were routinely assessed using a modification of the Bradford dye-binding procedure [21] relative to BSA standards.

For some experiments, isolated GSNO terminase activity was treated with phenylglyoxal; the reaction with 1.0 mM reagent, at 25°C , for 1 h was essentially as described by Holmquist et al. [22]. Similarly processed incubates but containing no phenylglyoxal provided the enzyme used as the non-treated controls in these experiments.

Molecular-mass determinations

SDS/PAGE (12.5 % polyacrylamide) was essentially as described by Laemmli [23]; the gels were calibrated using a broad-range protein molecular-mass standard kit from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Native protein molecular mass was determined by gel exclusion chromatography using a Sephacryl S-200-HR column (2.6 cm $\times$ 66 cm) calibrated using the Sigma protein standards kit MW-GF-200 (Sigma, St. Louis, MO, U.S.A.).

Peptide sequence analysis

The GSNO terminase protein was resolved by SDS/PAGE on 10 % Laemmli gels [23] and blotted on to PVDF Problott membranes according to the method of Matsudaira [24]. The Coomassie Brilliant Blue-stained bands were cut into approximately 1-mm-square pieces and treated overnight, in the dark at room temperature and under nitrogen, with 100 μl of BrCN solution [100 mg/ml in 70 % (v/v) formic acid] to cleave specifically the polypeptide at methionine residues. The supernatant was removed, and the PVDF pieces were eluted with 40 % (v/v) acetonitrile in water. The combined eluates containing the fragments were vacuum evaporated, washed with water, and separated on a Tris/Tricine gel [25] followed by electroblotting to Problott membranes and Coomassie Brilliant Blue staining. The bands representing the fragments of the enzyme were excised from the blot and loaded into the Blott Cartridge of an Applied Biosystems 477A protein sequencer equipped with a 120A phenylthiohydantoin analyser and a 610A data system; 21 cycles of the Edman degradation were performed using the BLOTT-2 program. The sequencer was modified for high-sensitivity analysis as proposed by Tempst and Riviere [26]. The sequences obtained were scanned against the NCBI databases using the BLAST network service.

Enzyme assays

The standard conditions for GSNO terminase activity evaluations were 100 mM Na_2HPO_4 /10 μM BCS, pH 7.4, 25°C ; the GSNO and coenzyme concentrations utilized are noted in the Figure legends and Table footnotes. The activity assay followed the rate of absorbance change at 340 nm (Perkin-Elmer Lambda 2 Spectrometer) due primarily to coenzyme utilization. The absorption coefficient used in activity calculations was $7.06\text{ mM}^{-1}\cdot\text{cm}^{-1}$, the sum of the coenzyme absorption coefficient at 340 nm of $6.22\text{ mM}^{-1}\cdot\text{cm}^{-1}$ and the GSNO absorption coefficient of $0.84\text{ mM}^{-1}\cdot\text{cm}^{-1}$ at this wavelength; justification for

this absorption coefficient is based on the observation that 1 mol of coenzyme is utilized for each mol of GSNO processed (see below).

Other enzyme kinetics assays closely approximated those previously described in the evaluation of ADH class III isoenzyme [22,27–29]. In all cases the assays were at 25°C and monitored the change in absorbance at 340 nm due to coenzyme (NAD^+ , NADP^+ , NADH or NADPH) utilization; the absorption coefficient for NADH/NADPH was taken to be $6.22\text{ mM}^{-1}\cdot\text{cm}^{-1}$. The various substrate and coenzyme concentrations and the buffer systems used in the several experiments are detailed in the appropriate Figure legends and Table footnotes.

One unit of activity is defined as the enzymic capacity to utilize 1 μmol of coenzyme per min at 25°C .

Preparation of nitrosated HSA

HSA was treated with dithiothreitol as previously described by Meyer et al. [12] and isolated by passage through EconoPac 10DG desalting columns (Bio-Rad Laboratories). The single exposed thiol per protein molecule was subject to nitrosation by two consecutive 15 min treatments with a 10-fold molar excess (relative to protein exposed thiol) of *n*-butyl nitrite, prepared as a 100 $\times$ stock of the required concentration in ethanol. The treated HSA was then isolated by two passages through fresh 10DG desalting columns. The protein concentration in the recovered sample was determined by the method of Bradford, and the adducted nitroso group concentration was determined using the mercuric chloride/azo dye assay previously described [14]. In the experiments reported here, 43 % of the HSA exposed thiols were nitrosated; the concentration of nitrosated HSA (NO-HSA) delivered to an incubation mix was calculated relative to this yield.

GSNO terminase reaction-product analysis

The nitrite and nitrate concentrations in aliquots drawn from reaction incubates were determined using the octanol-extraction, strong anion-exchange HPLC procedures previously described (SAX-HPLC [14]). Other incubate samples were delivered to equal volumes of 100 mM acetic acid, pH 3 (final pH 5.4), or 2 M H_3PO_4 (final pH 1.0). After 30 min some sample aliquots were assessed for NH_3 (see below). Other sample aliquots were derivatized (free thiol groups first carboxymethylated, then the primary amines dinitrophenylated [30]) using a modification of the method previously described (CM-DNP procedure [14]). In the present instance 400 μl of sample plus 400 μl of 50 mM Tris/HCl (pH 7.6)/100 μl iodoacetic acid (142 mg/ml) was treated with solid NaHCO_3 . After 1 h in the dark the solution was mixed with 600 μl of 3 % (v/v) 2,4-dinitro-1-fluorobenzene in ethanol and placed in the dark overnight. Derivatized GSNO, GSSG, GSH, glutathione sulphonic acid and the major product (see below) were resolved using strong anion-exchange HPLC as previously described (CM-DNP procedure/SAX-HPLC [14]). Product yields, other than that of the major product, were estimated by relating the integrated chromatogram peak areas to those generated by similarly processed standard solutions of known solute concentrations. The initial GSNO concentration in this analysis was an estimate based on the known concentration of GSH composing the GSNO synthesis mix and the observed concentration of contaminating GSSG.

In other experiments, non-dinitrophenylated major product was baseline resolved from other reaction mix components, retention time 4.5 min, using a tandem pair of Whatman Partisil 10 SAX 25 cm HPLC cartridge columns isocratically eluted with

7 mM H_3PO_4 , pH 2.25, at 2 ml/min; elution profiles were monitored at 214 nm and 340 nm.

The concentration of ammonia generated in incubate samples was determined using an enzymic assay (the NADPH-dependent conversion of 2-oxoglutarate plus NH_3 to glutamate catalysed by glutamate dehydrogenase) following the instructions and using the standard solution provided in the Sigma Ammonia Diagnostic Kit.

Hydroxylamine was assessed by adding 200 μl aliquots of incubate sample to 200 μl of 100 mM Na_2HPO_4 , pH 4.4, containing $\pm 100 \mu\text{M}$ hydroxylamine hydrochloride and 400 μl of 100 mM Na_2HPO_4 , pH 4.4; 400 μl of 1% (v/v) 8-hydroxyquinoline (prepared in aq. 50% ethanol) was added with mixing, and finally 400 μl of 1 M Na_2CO_3 was added. The mixture was heated, at 95 °C for 5 min, and cooled for 60 min, and the absorbance at 705 nm was determined [13,31]. Hydroxylamine yields were calculated relative to 100 μM hydroxylamine standards.

The quantity of nitric oxide generated by GSNO terminase action on GSNO substrate was determined using an oxyhaemoglobin-into-methaemoglobin conversion assay [32]. Human haemoglobin was isolated from peripheral whole blood and further purified by CM-Sephadex chromatography as previously described [14]. This haemoglobin was then treated with iodoacetamide to block the exposed thiol groups (thus preventing nitroso moiety exchange between these thiols and GSNO) as follows. The haemoglobin was passed through EconoPac 10DG columns pre-equilibrated with water, treated with a 5-fold concentration of iodoacetamide relative to total haem for 1 h, at 25 °C [33], and then passed through EconoPac 10DG columns pre-equilibrated with 100 mM Na_2HPO_4 /10 μM BCS, pH 7.4. Titration experiments with *p*-chloromercuribenzoate [14] indicated that 100% of the haemoglobin thiols were blocked relative to untreated haemoglobin controls. Spectral absorption characteristics of the modified haemoglobin preparations indicated that they were essentially 100% in the oxy form [34]. The concentration of haemoglobin was assessed after conversion into the cyano form, assuming an absorption coefficient at 540 nm of 11500 $\text{M}^{-1}\cdot\text{cm}^{-1}$ per haem moiety [35]. The degree of the oxyhaemoglobin-into-methaemoglobin conversion was evaluated by observing the change in absorbance at 577 versus 591 nm [32] and assuming a $\Delta\epsilon$ for the conversion at 577 nm of 10.1 $\text{mM}^{-1}\cdot\text{cm}^{-1}$. The latter parameter was established by determining the degree of absorbance change upon treating a 100 μM solution of modified oxyhaemoglobin with 100 μM spermine NONOate, known to generate spontaneously an excess of 100 μM nitric oxide [3].

RESULTS

GSNO terminase activity isolation and peptide sequence analysis

A spectrophotometric kinetic assay that monitored changes in 340 nm absorbance due to GSNO-dependent NADH utilization facilitated the isolation of GSNO terminase activity from rat liver. Comparison of the activity levels detected in crude rat liver homogenates with those found in the low-speed and high-speed centrifugation supernatants generated in the preparation of cytosol fraction indicated that essentially all of the activity in the original homogenate was recovered in the cytosol fraction. The activity and protein yields in the subsequent steps of a typical GSNO terminase activity isolation procedure are noted in Table 1. The protein recovered from the final gel exclusion chromatography step was homogeneous as judged by SDS/PAGE; relative to molecular-mass markers, the detected polypeptide chain is 42.5 kDa. The estimated native molecular mass of the

protein, determined using gel exclusion chromatography and relative to marker standards, is 78.3 kDa (results not shown).

No N-terminal peptide sequence could be discerned in initial attempts to sequence the isolated enzyme by automated Edman degradation, suggesting that the N-terminus of the protein was blocked by post-translational modification. Edman degradation of isolated BrCN-generated fragments of the protein, however, was successful. Comparison of the derived peptide sequences with databases indicated sequence similarity with rat liver ADH class III isoenzyme (EC 1.1.1.1). An approx. 8 kDa fragment gave the sequence:

8 kDa GSNO terminase activity:

(M)RSALEAAHKGXGVSVV

ADH class III isoenzyme: M RSALEAAHKGWGVSVV

The residue at position 11 was not identified. The corresponding ADH class III isozyme peptide sequence [36] is illustrated below the 8 kDa fragment sequence. An approx. 13 kDa band produced two sequences, indicating the presence of two 13 kDa peptides. Because of differences in the concentration of the two fragments and the presence of the above 8 kDa peptide sequence as one of the sequences of the 13 kDa band (evidently resulting from incomplete cleavage at a downstream Met-Ser sequence), the remaining sequence was positively identified as:

13 kDa GSNO terminase activity: (M)GCKVAGASRIIG

ADH class III isoenzyme: M GCKVAGASRIIG

The corresponding ADH class III isozyme sequence is shown below that of the 13 kDa sequence. The N-terminal amino acid of rat liver ADH class III isozyme is known to be modified by acetylation [37].

Comparison of the GSNO terminase activity with the known activities of ADH class III isozyme

ADH class III isozyme is classified as a medium-chain alcohol dehydrogenase, capable, for example, of catalysing the NAD^+ -dependent oxidation of *n*-octanol as well as the reverse, NADH-dependent reduction of *n*-octanal. Short-chain compounds (e.g. methanol, ethanol, butan-1-ol, acetaldehyde and butanal) are ineffective as substrates [27]. The strong pH and coenzyme dependencies of the catalysed reactions, as illustrated in Figure 1(A), utilizing our enzyme preparation in the octanol and octanal reactions, is an established characteristic of rat liver ADH class III isoenzyme [27] and of the alcohol dehydrogenases in general [38].

It has recently been established that ADH class III isozyme and glutathione-dependent formaldehyde dehydrogenase are the same enzyme [29,39]. The latter activity utilizes NAD^+ or NADP^+ to catalyse the oxidation of *S*-hydroxymethylglutathione (HMGSH, formed spontaneously in the reversible interaction between glutathione and formaldehyde) to generate *S*-formylglutathione [40]. Class III isozyme is the only ADH known to be capable of this reaction. Figure 1(B) illustrates the activity of our enzyme preparation in this catalytic process. The observed pH dependencies of the reaction using the alternative coenzymes are qualitatively similar to those previously described for human ADH class III isozyme, $\chi\chi$ ADH ([40]; 94% amino acid sequence similarity with the rat enzyme [36]), the notable difference being that in our buffer system the NAD^+ -dependent reaction is maximal at pH 9.8. Detailed consideration of the reverse of this reaction, the NADH/NADPH-dependent reduction of *S*-formylglutathione, has been frustrated by the fact that the substrate is intrinsically unstable [41].

Table 1 Purification of rat liver GSNO terminase activity

Step	Protein (mg)	Total activity (units) ^a	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Isolated cytosol	576	121	0.21	1	100
Dialysed cytosol	539	122	0.23	1.1	100
Reactive Red 120 affinity resin	39 ^b	72	1.85	8.8	59
Polybuffer Exchanger 94 chromatofocusing	2.8	51	18.1	86	42
Sephacryl 100-HR gel filtration	0.80	33	41.3	197	27

^a Assay conditions: 100 mM Na₂HPO₄, 10 μ M BCS, pH 7.4, 25 °C, 0.5 mM GSNO and 0.15 mM NADH.

^b The Reactive Red affinity column represents a constriction point in this methodology in that the resin is saturable. In the present example, about 1% of the applied activity derived from 15 ml of crude cytosol (in this case 80% of the yield obtained from liver preparations from two 150 g Sprague–Dawley rats) appeared in the affinity-column void volume.

Figure 1 Specific activity of the isolated enzyme as a function of substrate, coenzyme and buffer pH

(A) 1.0 mM n-octanol/2.4 mM NAD⁺ (○) or /2.4 mM NADP⁺ (□); 1.0 mM n-octanol/0.262 mM NADH (●) or /0.262 mM NADPH (■). (B) 1.0 mM GSH/1.0 mM formaldehyde/2.4 mM NAD⁺ (○) or /2.4 mM NADP⁺ (□). (C) 1.0 mM GSNO/0.262 mM NADH (●) or /0.262 mM NADPH (■). The buffers utilized, adjusted to the requisite pH, were: A, 100 mM sodium acetate; B, 33 mM sodium phosphate monobasic; C, 33 mM sodium pyrophosphate; D, 100 mM glycine.

The pH and coenzyme dependencies of the GSNO terminase activity of our enzyme preparation are shown in Figure 1(C); the catalysis requires reduced coenzyme. It is apparent that the specific activity for the GSNO terminase reaction is generally 10-fold greater than that of the octanal/octanol activities and the formaldehyde dehydrogenase activity across the pH range considered. The sensitivity to coenzyme species is pronounced; at

pH 7.4 NADH is approximately 8-fold more effective as the coenzyme participant than is NADPH. The characteristic prompt inactivation of ADH class III isoenzyme when incubated at or below pH 6 [27,40] has been detected in our assessment of the GSNO terminase activity. Also, the insensitivity of ADH class III isoenzyme to the classical ADH class I and class II isoenzyme inhibitor 4-methylpyrazole [27] maintains in the GSNO terminase reaction (up to 5 mM tested; results not shown). The GSNO terminase reaction is insensitive as well to up to 5 mM included DMSO (results not shown).

The specific activities of the purified GSNO terminase activity against a range of ADH class III isoenzyme substrates, assessed using the several conditions previously described in the literature [22,27–29], are collected in Table 2 (unmodified enzyme). Of note is the minimal activity of our preparation towards the methanol and ethanol substrates, a characteristic that distinguishes ADH class III isoenzyme from the class I and class II isoenzymes [27].

Utilizing human ADH class III isoenzyme, it has been established that certain fatty acids can stimulate the alcohol dehydrogenase activity [42]. The effect depends on the size of both the alcohol substrate and of the activator. It has been proposed that complementary binding of the suitably sized fatty acid–substrate pair would fully occupy the rather commodious [43] hydrophobic substrate-binding pocket and result in an enhanced catalytic rate [22]. The effect is perhaps best exemplified by the activation of MCA oxidation by pentanoate, where the stimulation can be several fold [22,28,42]. The activity of our rat enzyme preparation towards MCA is stimulated 4-fold when sodium pentanoate is included in the reaction mix (Table 2, unmodified enzyme).

Further, it has been determined that selective chemical modification of arginine-115 of the human ADH class III isozyme with phenylglyoxal also, but more modestly, stimulates MCA oxidation, but that this modification coincidentally eliminates the fatty acid stimulation effect [22]. The model is that modification of arginine-115 may block the charge interaction between the protein amino acid side chain and the activator carboxylic acid moiety and may also perturb the binding pocket fit [22]. Modification of our rat enzyme preparation with phenylglyoxal stimulates the MCA oxidation activity 2-fold and coincidentally prevents the stimulation by included sodium pentanoate (Table 2).

Finally, phenylglyoxal modification of human ADH class III isoenzyme markedly decreases the enzymic activity towards 12-HDA and of HMGSH [22], substrates that contain one and two carboxylic acid groups respectively. It has been proposed that arginine-115 is perhaps a charge interaction site for one of the carboxy groups (perhaps the carboxy of the glycine residue [43]) in the glutathione moiety composing HMGSH [28]. Site-directed mutagenesis of the arginine-115 residue in the human ADH class

Table 2 Activity (units/mg of enzyme protein) of unmodified and phenylglyoxal-treated isolated enzyme against various substrates

Assay conditions: **A**, 100 mM glycine/NaOH, pH 10.0, 25 °C, 2.4 mM NAD⁺, 7.4 μg of protein/ml; **B**, 50 mM sodium pyrophosphate, pH 8.5, 25 °C, 2.4 mM NAD⁺, 3.3% (v/v) methanol, ± 50 mM pentanoate (n-valeric acid), 5.9 μg of protein/ml; **C**, 100 mM glycine/NaOH, pH 10.0, 25 °C, 2.4 mM NAD⁺, 3.3% (v/v) methanol, 1.5 μg of protein/ml; **D**, 50 mM sodium pyrophosphate, pH 8.0, 25 °C, 2.4 mM NAD⁺, 1.5 μg of protein/ml; **E**, 100 mM Na₂HPO₄, 10 μM BCS, pH 7.4, 25 °C, 0.25 mM NADH, 0.7 μg of protein/ml. Values are means ± S.D., *n* = 3. Values in parentheses are the percentages of activity change relative to the unmodified enzyme; in the + pentanoate case, the value is relative to the unmodified enzyme in the absence of pentanoate. —, not done.

Substrate	Assay conditions	Activity (units/mg of enzyme protein)	
		Unmodified enzyme	Phenylglyoxal-treated enzyme
33 mM Methanol	A	below detectable levels	—
33 mM Ethanol	A	0.006 ± 0.001	—
1.0 mM MCA	B	0.045 ± 0.003 (100)	0.097 ± 0.004 (216)
1.0 mM MCA + 50 mM sodium pentanoate	B	0.187 ± 0.005 (416)	0.096 ± 0.001 (213)
1.0 mM n-Octanol	C	0.733 ± 0.029 (100)	0.476 ± 0.027 (65)
1.0 mM 12-HDA	C	1.01 ± 0.07 (100)	0.339 ± 0.032 (34)
1.0 mM Formaldehyde/1.0 mM GSH (HMGSH)	D	1.61 ± 0.04 (100)	0.265 ± 0.013 (16)
1.0 mM GSNO	E	42.0 ± 1.3 (100)	1.64 ± 0.18 (4)

Figure 2 GSNO catabolism by the isolated enzyme and the products generated as a function of time and of post-incubate sample processing

GSNO degradation in 100 mM Na₂HPO₄/10 μM BCS, pH 7.4, 25 °C, in incubates initially containing 1.0 mM GSNO and 2.0 mM NADH, and in the presence (solid symbols) or absence (open symbols) of 1 μg of isolated enzyme/ml. Indicated are several compounds quantified after: (A) a 30 min post-incubate treatment in pH 5.4, and (B) a 30 min post-incubate treatment in pH 1.0. The GSNO (●, ○), major product (■, □), GSSG (▼, ▽); expressed in glutathione units, and glutathione sulphinic acid (◆) yields were quantified after resolving precolumn-derivatized compounds by strong anion exchange, and NH₃ (x) quantified using a commercial ammonia kit. Zero time and 40 min evaluations were made in triplicate; indicated are the means and S.D. values (error bars not apparent are smaller than the data symbols). All other data points represent single quantitative determinations.

III isoenzyme to aspartic acid or alanine also results in marked curtailment of the fatty acid activation effect and of HMGSH oxidation [28]. The arginine residue at position 115 is conserved in rat ADH class III isoenzyme [36]. Phenylglyoxal modification of our rat enzyme significantly reduces the observed activity towards 12-HDA and HMGSH (Table 2).

Taken together these evaluations support our contention that the isolated GSNO terminase activity is indeed rat ADH class III isozyme.

Phenylglyoxal modification resulted in an almost complete abrogation of the GSNO terminase activity towards GSNO

(Table 2), an observation suggesting a charge interaction between an arginine side chain in the active site of the native enzyme and a carboxylic acid moiety in GSNO. In this context we note that the S-nitrosated derivative of the monoethyl (glycyl) ester of glutathione is about 5% as active as a substrate for the unmodified enzyme relative to GSNO (using assay conditions E., Table 2), and that the enzymic activity towards the S-nitrosated derivative of glutathione diethyl ester is below detectable levels (results not shown).

Characterization of the GSNO terminase reaction

An evaluation of the time course of GSNO disappearance from an incubate containing GSNO terminase activity and NADH and of some of the products generated is summarized in Figure 2. In the first instance (A), samples were withdrawn from the incubate and delivered to an equal volume of 100 mM acetic acid (pH 3); the final pH 5.4 inactivates the enzyme. In the second instance (B), aliquots were delivered to an equal volume of 2 M H₃PO₄, final pH 1.0. After 30 min the samples were derivatized (CM-DNP procedure) or not and the several solutes were quantified. Within the resolution of the experiment, the post-incubate treatments had little effect on the recovery of intact GSNO.

The major product generated consequent to the GSNO terminase catalytic reaction (Figure 2A) is the same 2,4-dinitro-phenyl-derivatized unknown compound detected in our earlier evaluation of NADPH-dependent GSNO catabolism in rat liver cytosol fraction (identical HPLC retention time and similar yield [14]). If it is assumed that this derivative has the same absorption coefficient at the HPLC monitor wavelength of 365 nm as that established for derivatized GSH and GSNO, then, at the 40 min time point, approx. 70% of the GSNO processed had been converted into this product. GSSG also accumulated with time, representing about 15% (in terms of glutathione units) of the input GSNO concentration at the 40 min time point. No HPLC peak corresponding to derivatized GSH was detected at any time point. NH₃ was also observed to accumulate in the enzyme incubates during GSNO processing, representing about 15% of the substrate catabolized (Figure 2).

Neither nitrite, nitrate nor hydroxylamine were generated in significant yields during GSNO processing (Table 3, Control

Table 3 GSNO terminase-mediated GSNO catabolism and product yields (μM) in control incubates and in incubates containing 5 mM GSHAssay conditions: 100 mM Na_2HPO_4 , 10 μM BCS, pH 7.4, 25 °C, 1.0 μg of protein/ml, initially 1.0 mM GSNO, 2.0 mM NADH and $\pm$ 5 mM GSH. Values are means $\pm$ S.D., $n = 3$. nd, not done.

	Concentration (μM)					
	Control incubates			Incubates containing 5 mM GSH		
	Time (min) ...	0	40	0	40	
Enzyme ...	—	—	+	—	—	+
GSNO		950 $\pm$ 47	890 $\pm$ 13	0	985 $\pm$ 37	865 $\pm$ 8
Major product (supposed glutathione sulphinamide)		0	32 $\pm$ 9	570 $\pm$ 33	0	49 $\pm$ 9
GSSG		50 $\pm$ 1	89 $\pm$ 17	206 $\pm$ 8	209 $\pm$ 29	576 $\pm$ 23
NH_2OH		4 $\pm$ 2	6 $\pm$ 4	1 $\pm$ 5	0 $\pm$ 1	2 $\pm$ 7
NH_3		0 $\pm$ 13	13 $\pm$ 18	152 $\pm$ 15	0 $\pm$ 11	23 $\pm$ 20
NO_2^-		42 $\pm$ 9	43 $\pm$ 5	61 $\pm$ 5	nd	57 $\pm$ 18
NO_3^-		7 $\pm$ 1	5 $\pm$ 0	11 $\pm$ 1	nd	5 $\pm$ 1

incubates). Utilizing the oxyhaemoglobin-into-methaemoglobin conversion assay to evaluate nitric oxide release, it was determined that less than 5% of the GSNO processed in the NADH-dependent enzymic reaction generated nitric oxide as a product (results not shown).

Exposure of post-incubate aliquots to pH 1 solution for 30 min resulted in an approx. 85% loss of the major product with the concomitant increase in detected NH_3 and the appearance of a corresponding yield of glutathione sulphinic acid (Figure 2 B). Based on these results the argument will be made that the major product is glutathione sulphinamide (see the Discussion).

When 5 mM GSH was added to a post-reaction GSNO terminase mixture and incubation was continued for 30 min, there was no significant effect on the detected yield of the major product. However, when 5 mM GSH was included in the incubate during the enzymic processing of GSNO, the yield of the major product was diminished by about 50% and hydroxylamine and enhanced yields of GSSG were detected (Table 3). The yield of hydroxylamine generated approximated the quantity of major product lost from the accounting but the GSSG generated was very much greater, about 4–5-fold in terms of glutathione units, than would be expected even after spontaneous GSH oxidation was factored in. The minimal nitrite and nitrate yields were little affected when 5 mM GSH was included in the incubate, and NH_3 production was essentially the same as that observed in the control incubates (Table 3). Inclusion of superoxide dismutase at 500 units/ml in an enzymic incubate containing 5 mM GSH (conditions otherwise as noted in Table 3) had no detectable effect on the GSSG yield (results not shown).

Coenzyme utilization in the GSNO terminase reaction was evaluated by considering the change in reaction incubate absorbance at 340 nm with time (Figure 3). Both NADH and the nitroso compound contribute to the 340 nm absorbance and, consequently, absorbance changes due to the enzymic reaction will be influenced by both substrate consumption and coenzyme utilization. In the GSNO case the substrate contribution will be small: the ratio of the NADH/GSNO absorption coefficients is 7.4. The contribution of the detected reaction products to the 340 nm absorbance is also expected to be small. Using a tandem pair of SAX HPLC columns, the baseline resolution of non-derivatized major product was achieved, and evaluation of the HPLC absorbance detector elution response at both 214 nm and 340 nm indicated that this compound absorbs minimally at 340 nm, a characteristic shared by GSSG.

Figure 3 NADH utilization (A_{340} change) by the isolated enzyme with time as a function of coenzyme concentration, and of potential substrate $\pm$ added GSH

Absorbance changes at 340 nm (25 °C) detected in samples prepared in 100 mM Na_2HPO_4 /10 μM BCS (pH 7.4)/200 μM NADH, and, unless otherwise noted, 0.2 μg of isolated enzyme/ml, and the indicated solutes. (A) 100 μM GSNO, no protein ($\circ$); 50 μM GSNO ($\blacktriangle$); 100 μM GSNO ($\bullet$); 150 μM GSNO ($\blacklozenge$). (B) 100 μM GSNO ($\bullet$); 100 μM GSNO and 100 μM GSH ($\blacksquare$). (C) 100 μM SNAP ($\bullet$); 100 μM SNAP and 100 μM GSH ($\blacksquare$); 100 μM SNAP and 50 μM GSH ($\blacktriangle$); 50 μM SNAP and 100 μM GSH ($\blacktriangledown$). (D) 100 μM NO-HSA ($\bullet$); 100 μM NO-HSA and 100 μM GSH ($\blacksquare$); 100 μM NO-HSA and 50 μM GSH ($\blacktriangle$); 50 μM NO-HSA and 100 μM GSH ($\blacktriangledown$). The reactions were initiated at zero time by the addition of the nitroso compound. Also indicated in each panel is a scaling bar representing the expected absorbance decrease when 100 μM NADH is converted into NAD^+ .

Absorption coefficients have not been applied to the plots shown in Figure 3; the scaling bar included in each panel represents the expected change when 100 μM NADH is converted into NAD^+ , based on an absorption coefficient of

Figure 4 GSNO terminase kinetic analysis presented as a Lineweaver-Burk plot

The dependence of the initial velocity (v) of the GSNO terminase activity on the initial GSNO concentration (S) presented as a Lineweaver-Burk plot; $1.0 \mu\text{g}$ of isolated enzyme/ml (12.5 nM based on a molecular mass of $80\,000 \text{ Da}$), 0.25 mM NADH, 100 mM Na_2HPO_4 , $10 \mu\text{M}$ BCS, pH 7.4 , 25°C . Initial-rate calculations were based on the observed sample absorbance changes at 340 nm and the application of the absorption coefficient $7.06 \text{ mM}^{-1}\cdot\text{cm}^{-1}$. Shown are data collected in the absence of glutathione (●) and in the presence of 5 mM (△), 10 mM (○) and 20 mM glutathione (□). Each data point represents the average of two initial-rate evaluations.

$6.22 \text{ mM}^{-1}\cdot\text{cm}^{-1}$. The change in absorbance at 340 nm as the enzymic processing of GSNO approached apparent completion appeared to be directly proportional to the input substrate concentration (Figure 3A). Also, based on the expected absorbance change due to NADH utilization, it is evident that 1 mol of coenzyme was oxidized for each mol of GSNO processed. This observation justifies our use of the sum of the GSNO and NADH absorption coefficients ($7.06 \text{ mM}^{-1}\cdot\text{cm}^{-1}$) to calculate reaction progress in other experiments. Inclusion of GSH at a concentration equimolar to the input GSNO had no detectable effect on the rate or degree of absorbance at 340 nm change due to enzymic processing (Figure 3B).

SNAP and NO-HSA were tested as substrates for the GSNO terminase activity in the experiments summarized in Figures 3(C) and 3(D) respectively. Based on the observation that neither of these nitroso compounds, introduced at a concentration of $100 \mu\text{M}$ in nitroso group, stimulated a significant decrease in 340 nm absorbance in the presence of isolated enzyme and NADH, it is concluded that neither is able to serve as a substrate for the enzymic activity. However, when $100 \mu\text{M}$ GSH was included in the reaction mix, a 340 nm absorbance decrease, approximating $100 \mu\text{M}$ NADH utilized, was observed. It has been established that rapid NO-exchange reactions occur in systems containing thiol and *S*-nitrosothiol [11–13], and it is likely that this exchange is occurring in the SNAP/GSH and NO-HSA/GSH mixtures to generate GSNO, which is subsequently used by the enzyme as substrate. Support for this supposition derives from the observation that decreasing the input SNAP or NO-HSA by 50% results in one-half of the above noted absorbance change (Figures 3C and 3D); correspondingly, decreasing the input GSH by 50% has the same effect.

Table 4 Kinetic constants of isolated enzyme towards GSNO

Experimental conditions: 100 mM Na_2HPO_4 , $10 \mu\text{M}$ BCS, pH 7.4 , 25°C , $1 \mu\text{g}$ of protein/ml. The kinetic parameters for the GSNO substrate were determined using 0.25 mM NADH; the V_{max} for 12.5 nM enzyme (based on a molecular mass of $80\,000 \text{ Da}$) was $0.033 \text{ mM}\cdot\text{min}^{-1}$. The K_i for GSH was estimated from a secondary plot of $1/v$ against GSH and as a function of substrate concentration. The K_m values for NADH and NADPH were determined using 1.0 mM GSNO.

Constant	Value
K_m^{GSNO}	0.028 mM
k_{cat}	2640 min^{-1}
$k_{\text{cat}}/K_m^{\text{GSNO}}$	$94\,300 \text{ mM}^{-1}\cdot\text{min}^{-1}$
K_i^{GSH}	$\approx 30 \text{ mM}$
K_m^{NADH}	0.024 mM
K_m^{NADPH}	0.56 mM

A kinetic analysis of the NADH-dependent GSNO terminase reaction is summarized in Figure 4. It is apparent that over the range of substrate concentrations accessible to our assay (10 – $100 \mu\text{M}$) the catalysed reaction conforms to Michaelis-Menten kinetics. The kinetic parameters derived from this analysis are noted in Table 4. The Lineweaver-Burk plots of initial-rate evaluations for reactions that included GSH (Figure 4) suggest that GSH is a non-competitive inhibitor of GSNO terminase activity; a secondary plot of these data (not shown) indicate a K_i for glutathione of approx. 30 mM . It has been noted that high GSH concentrations inhibit the HMGS oxidation reaction mediated by human ADH class III isozyme [44].

DISCUSSION

In the process of purifying to apparent homogeneity an NADH- or NADPH-dependent activity from rat liver cytosol that catabolizes GSNO, we discovered that we had isolated an enzyme that has a range of characteristics that are unique to ADH class III isoenzyme. We have demonstrated that the isolated protein is capable of coenzyme-dependent catalysis of *n*-octanol, MCA, 12-HDA and HMGS oxidation and *n*-octanol reduction, but has minimal activity with methanol or ethanol substrates. The catalytic process shows pronounced pH sensitivity and coenzyme preferences and is not inhibited by 4-methylpyrazole. The marked stimulation of enzymic MCA oxidation by included pentanoate detected in our experiments mimics the well studied fatty acid activation characteristic of human ADH class III isoenzyme. In addition, treatment of our enzyme preparation with phenylglyoxal, a reagent that, in the highly similar human enzyme, specifically modifies an active-site arginine residue, quenches the fatty acid activation effect and inhibits the catalysis of *n*-octanol, 12-HDA and HMGS oxidation just as it does in the human ADH class III isoenzyme case. The estimated polypeptide chain and native protein molecular masses of our preparation (42.5 and 78.3 kDa respectively) correspond to the analogous values determined for rat liver ADH class III isoenzyme [27,36]. Finally, although our amino acid sequencing data are limited to 16 and 12 residues from two BrCN-generated polypeptide fragments respectively, the sequences obtained nevertheless show 100% correspondence with sequences in rat liver ADH class III isoenzyme. We conclude that the protein we have isolated is indeed ADH class III isoenzyme and that GSNO represents an alternative substrate for this enzyme.

The reversible reaction catalysed by ADH class III isoenzyme as it operates as a glutathione-dependent formaldehyde dehydro-

Scheme 2 Scheme for ADH class III isozyme operating as a formaldehyde dehydrogenase and the chemical structure of GSNO

(A) The reversible NAD^+ -dependent oxidation of *S*-hydroxymethylglutathione (formed by the spontaneous and reversible interaction between glutathione and formaldehyde) to *S*-formylglutathione catalysed by ADH class III isozyme [45]. (B) *S*-nitrosoglutathione.

Scheme 3 Scheme for the ADH class III isozyme catabolism of GSNO included GSH

genase is illustrated in Scheme 2(A) [45]. The similarity between *S*-formylglutathione, the substrate for the reductive reaction, and GSNO (Scheme 2B) is apparent. In analogy to this scheme for the ADH class III isozyme-mediated reduction of *S*-formylglutathione [45] and to the model for ADH-catalysed reduction of aldehydes [46], it is envisioned that the catalytic action of ADH class III isozyme operating on GSNO may involve hydride transfer from the coenzyme to the substrate and the associated absorption of a proton from the solvent. This enzymic process may generate the immediate product illustrated in Scheme 3. A similar product, called a 'semimercaptal', has been considered to arise consequent to the reaction of glutathione with nitroso-

arenes, the latter formed through the metabolic N-oxygenation of aromatic amines or the reduction of nitro aromatics [47–49]. The semimercaptal evidently has a transient existence, however, and spontaneously 'rearranges' to a glutathione sulphinamide (shown to be a loss and subsequent addition of hydroxy moiety [48]). Glutathione sulphinamides are somewhat labile in mild acid and hydrolyse to glutathione sulphinic acid and an amine [47]. Our evidence that glutathione sulphinamide is the major product detected consequent to the GSNO terminase reaction derives from the observation that incubation of our post-reaction mixtures at pH 1, 30 min, room temperature, results in significant loss of the major product and the concomitant appearance of glutathione sulphinic acid and ammonia (Figure 2), as illustrated in Scheme 3.

When a 5-fold excess of GSH, relative to the GSNO substrate, is added to the GSNO terminase reaction mix, the yield of the putative glutathione sulphinamide product is decreased by 50 % and a corresponding yield of hydroxylamine is detected (Table 3). It seems likely that GSH is interacting with the supposed immediate product of the enzymic reaction (Scheme 3), since adding the glutathione to the reaction mix after the GSNO processing is complete has no effect on the major product (glutathione sulphinamide) yield. However, the nature of this interaction remains to be elucidated. The hydroxylamine yield suggests a bifurcated pathway, as illustrated in Scheme 3, but the detected 4–5-fold greater yield of GSSG (in terms of glutathione units) than would be expected from a simple 1:1 stoichiometry indicates a somewhat more complicated reaction.

Finally, we detect some release of NH_3 during the enzymic processing of GSNO (15 % relative to the substrate consumed in the cited experiments) that is little affected by added GSH. This result may signal an alternative fate of the presumed immediate product of the enzymic reaction, via a pathway that remains to be characterized.

With regard to our kinetic analysis, the estimated K_m for GSNO in the NADH-dependent reaction catalysed by the rat liver preparation, 0.028 mM at pH 7.4, is similar to the substrate K_m of 0.008 mM, pH 8.0, observed in the human ADH class III catalysed oxidation of HMGSH [40]. These K_m values are very much lower than those obtained upon considering the ADH class III isoenzyme-mediated oxidation of the various medium and long chain alcohols [27,42]. In addition, the k_{cat} estimated for the GSNO terminase reaction (2640 min^{-1} , NADH, pH 7.4) is greater than that determined for any other ADH class III isoenzyme (rat or human) substrate (generally evaluated at the pH optima [27,42]). These parameters yield a catalytic efficiency value for the GSNO substrate ($k_{\text{cat}}/K_m = 94300 \text{ mM}^{-1} \cdot \text{min}^{-1}$) that exceeds that of all other ADH class III isoenzyme substrates thus far considered, even though the present kinetic analysis was made at pH 7.4, far from the apparent pH optimum for the GSNO terminase reaction (Figure 1C). We thus conclude that GSNO is among the most active substrates for ADH class III isoenzyme.

ADH class III isoenzyme is a ubiquitous enzyme found in a host of animal and plant species [50,51]. mRNA representing this enzyme has been detected in the full range of rat tissues considered [52]. The isoenzyme has been judged to serve a 'housekeeping' function, engaged in the critical cytoprotective function of formaldehyde oxidation [52]. The importance of this enzyme may well be increased with the finding that GSNO serves as an alternative substrate.

As noted, our experiments utilizing the cyanoDMNG/rat hepatocyte system suggest that GSNO is catabolized and that the glutathione moiety is recycled. The GSNO terminase activity, which we have identified as ADH class III isoenzyme, would

seem to be a good candidate for the enzyme that mediates this processing. The isoenzyme is well represented in rat liver cytosol [27], has a high specific activity for the GSNO substrate, and generates a product that, in the presence of excess GSH (perhaps in the range of 10 mM in hepatocytes [53]), is converted into GSSG, which in turn will be reduced to GSH by the action of glutathione reductase. We were unable to detect any of the product, which we now suppose is glutathione sulphinamide, in the lysates of hepatocytes that had metabolized cyanoDMNG [17]. Within the framework of the model being considered, it is possible that this product is produced but is subsequently metabolized in the hepatocytes, or perhaps the pathway to GSSG and hydroxylamine predominates in the cellular milieu.

Several studies have suggested that intracellular glutathione serves to protect cells from the toxicity of applied nitric oxide [3,54–56]. We conjecture that glutathione may sequester the NO moiety consequent to the thiol nitrosation process and may also, reversibly, relieve key enzymes of thiol moiety nitrosations that might otherwise prove deleterious to function. The GSNO terminase activity of ADH class III isoenzyme may then dispatch the intracellular GSNO generated. A recent investigation has revealed that functioning glutathione reductase is required for the glutathione-mediated protective effect in cells exposed to nitric oxide [57]. We submit that glutathione reductase operates on the GSSG product of the GSNO terminase activity and thus contributes to glutathione homeostasis.

It is possible as well that the GSNO terminase activity of ADH class III isoenzyme serves, in association with the NO exchange equilibrium, to consummate signal-transduction processes that rely on the S-nitrosation of proteins.

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