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NADPH dependent activation of microsomal glutathione transferase 1

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Abstract

Microsomal glutathione transferase 1 (MGST1) can become activated up to 30-fold by several mechanisms in vitro (e.g. covalent modification by reactive electrophiles such as *N*-ethylmaleimide (NEM)). Activation has also been observed in vivo during oxidative stress. It has been noted that an NADPH generating system (g.s.) can activate MGST1 (up to 2-fold) in microsomal incubations [Y. Aniya, M.W. Anders, J. Biol. Chem. 264 (1989) 1998–2002], but the mechanism was unclear. We show here that NADPH g.s treatment impaired *N*-ethylmaleimide activation, indicating a shared target (identified as cysteine-49 in the latter case). Furthermore, NADPH activation was prevented by sulfhydryl compounds (glutathione and dithiothreitol). A well established candidate for activation would be oxidative stress, however we could exclude that oxidation mediated by cytochrome P450 2E1 (or flavine monooxygenase) was responsible for activation under a defined set of experimental conditions since superoxide or hydrogen peroxide alone did not activate the enzyme (in microsomes prepared by our routine procedure). Actually, the ability of MGST1 to become activated by hydrogen peroxide is critically dependent on the microsome preparation method (which influences hydrogen peroxide decomposition rate as shown here), explaining variable results in the literature. NADPH g.s. dependent activation of MGST1 could instead be explained, at least partly, by a direct effect observed also with purified enzyme (up to 1.4-fold activation). This activation was inhibited by sulfhydryl compounds and thus displays the same characteristics as that of the microsomal system. Whereas NADPH, and also ATP, activated purified MGST1, several nucleotide analogues did not, demonstrating specificity. It is thus an intriguing possibility that MGST1 function could be modulated by ligands (as well as reactive oxygen species) during oxidative stress when sulfhydryls are depleted.

Keywords: Microsomal glutathione transferase; NADPH; Hydrogen peroxide

Abbreviations: MGST 1, microsomal glutathione transferase 1; NEM, *N*-ethyl maleimide; CDNB, 1-chloro 2,4-dinitrobenzene; g.s., generating system; FMO, flavine monooxygenase

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1. Introduction

Microsomal glutathione transferase (MGST) is an abundant enzyme, found in most aerobic organisms, involved in the cellular protection from reactive electrophiles and oxidative stress [1]. The gene is induced during oxidative stress [2] but rarely by classical inducers of drug metabolism [3]. Instead, the enzyme can undergo activation by post-translational modifications including covalent modification by thiol-disulfide interchange [4,5], proteolysis [6], heat [7] and ligand binding [8]. Endogenous compounds that can activate MGST1 in vitro, and perhaps during oxidative stress in vivo, include hydrogen peroxide [9], *S*-nitrosoglutathione and peroxynitrite [10,11]. The fact that the enzyme is also activated by reactive electrophiles that target cysteine-49 is currently exploited as a means to detect unknown reactive intermediates in drug development [12,13]. In order to develop the most sensitive conditions possible we have expanded on previous observations that NADPH sometimes caused activation of MGST1 in microsomal incubations (necessary to generate reactive intermediates). The goal of the present study was thus to define conditions for optimal MGST1 activation, including the influence of the microsome preparation method, in order to reduce background and increase activation. A reasonable hypothesis that could explain background activation by NADPH (based on the fact that NADPH causes the generation of reactive oxygen species in microsomes) was investigated. As a result of these studies we define that microsome preparation methods are critical for reactive oxygen species dependent activation of rat liver MGST1 and that NADPH (as well as ATP) can cause a previously unrecognized direct activation of MGST1. These findings define methodological approaches for detecting reactive intermediates by use of MGST1 activation.

2. Materials and methods

2.1. Materials

GSH, glucose-6-phosphate dehydrogenase, glucose-6-phosphate, $NADP^+$, 4-methylpyrazole, DTT, ethylenediamine tetraacetic acid (EDTA), *N*-ethyl maleimide (NEM), methimazole were from Sigma. 1-Chloro-2,4-dinitrobenzene (CDNB) and hydrogen peroxide (30%) were obtained from Merck. ATP and NADPH were purchased from both Sigma Chemicals and Roche. Adenosine, inosine 5′-monophosphate (IP1), ADP and cyclic AMP were from Roche. The recombinant FMO3 in supersomes was from Gentist. All other chemicals were of reagent grade from common suppliers.

2.2. Methods

2.2.1. Pre-treatment of rats with acetone

One group of rats was starved for 48 h and injected with acetone (5 ml/kg) intragastrically once daily. These rats received no food but water ad libitum and were killed 18–24 h after the last injection. Control rats received food and water ad libitum until 24 h before sacrifice [27].

2.2.2. Isolation of rat liver microsomes

Method A: livers from male Sprague–Dawley rats (170–200 g) were homogenized in buffer (0.25 M sucrose, 10 mM Hepes, 1 mM EDTA, pH 7.4) with a Potter–Elvehjem homogenizer at 440 rpm. Microcosms were isolated by centrifugation of the homogenate at 10,000 × *g* for 15 min followed by ultracentrifugation of the resulting supernatant at 104,000 × *g* for 60 min. The microsomes were resuspended in potassium phosphate buffer (50 mM, pH 7.4) and pelleted at (30 min at 104,000 × *g*) and this wash was repeated. Microsomes were finally homogenised in the above buffer and diluted 10-fold in buffer (50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$) to give a final protein concentration of 2–4 mg/ml. The isolated microsomes were immediately used for experiments. Aliquots were stored at −70 °C for later protein determination by the method of Peterson [14].

Method B: The method of Aniya and Anders [9] was used. Liver microsomes were prepared from male Sprague–Dawley rats (approximately 250 g). The rats were starved overnight and killed by decapitation. The liver was removed after perfusion in situ with ice-cold 1.15% potassium chloride solution using a peristaltic pump for approximately 3 min. The liver was homogenized with 2 volumes of ice-cold 1.15% potassium chloride solution followed by 9000 × *g* centrifugation for 30 min at 4 °C. The filtered supernatant was centrifuged at 105,000 × *g* for 60 min at 4 °C. The micro-

somal pellet (resuspended in 60 ml per rat of 0.15 M Tris–HCl, pH 8.0) was recentrifuged at 105,000 × *g* for 60 min. This washing procedure was repeated once. Finally the microsomal pellet from each rat was divided into three tubes. Approximately 1 ml of suspension buffer (0.05 M potassium phosphate buffer, pH 7.4, containing 0.3 mM EDTA and 0.25 M sucrose) was added to each tube, which was kept on ice until the microsomes were resuspended. Protein concentration of liver microsomes prepared by this method was determined by the method of Lowry et al. [15].

2.2.3. Enzyme assay

Glutathione transferase activity was measured using GSH and CDNB as substrates essentially according to a modified method of Habig et al. [16]. The activity of the enzyme in liver microsomes was determined in 0.1 M potassium phosphate, pH 6.5, containing 0.1% Triton X-100 with 5 mM GSH and 0.5 mM CDNB at room temperature. The rate of product formation was monitored by measuring the change in absorbance at 340 nm ($\varepsilon = 9600\,M^{-1}\,cm^{-1}$) using a single-beam Philips PU8700 UV–Vis spectrophotometer (Philips Scientific & Analytical Equipment, Cambridge, UK). Enzyme activities were calculated after correction for the nonenzymatic reaction.

In some experiments (indicated in the text) the GST activity was measured by the original method of Habig et al. [16] with 1 mM CDNB and 5 mM GSH as substrates. The assay buffer was 0.1 M potassium phosphate buffer (pH 6.5) and the activity was measured at room temperature.

2.2.4. NADPH-generating system and treatment of microsomes

Microsomes (prepared by method A) were diluted 10-fold by buffer 50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$ at 37 °C. The NADPH-generating system consisted of glucose-6-phosphate dehydrogenase (1 U/ml), $NADP^+$ (0.5 mM) and glucose-6-phosphate (5 mM) and was added at the start of the incubation. Control microsomes received buffer and enzyme only. GST activity towards 0.5 mM CDNB was measured by the modified assay after 15 min of incubation since 15 min incubation usually showed a plateau of the activation in our hands.

2.2.5. Treatment with NEM

NEM (0.1 M) was added to NADPH g.s. preincubated microsomal samples on ice to give a final concentration of 5 mM. Aliquots were withdrawn and GST activity toward CDNB determined by the modified assay as described.

2.2.6. Incubation of liver microsomes with GSH, DTT or the inhibitor of CYP2E1,4-methylpyrazole

4-Methylpyrazole (0.5, 1, and 2 mM), GSH (1 mM), or DTT (0.5 mM) (in 50 μl) were added to the microsomes just before the incubation at 37 °C. Then, glucose-6-phosphate dehydrogenase (1 U/ml), $NADP^+$ (0.5 mM) and glucose-6-phosphate (5 mM) were added and the solution was incubated for 15 min. Aliquots were withdrawn and GST activity toward CDNB determined by the modified assay as described.

2.2.7. Hydrogen peroxide treatment of microsomes prepared by method A

Hydrogen peroxide (0.1–10 mM) was dissolved in buffer (50 mM potassium phosphate, pH 7.4). The solution of hydrogen peroxide (50 μl) was added to microsomes and they were incubated at 37 °C for 15 min. Aliquots were withdrawn and GST activity toward CDNB determined by the modified assay as described.

2.2.8. Hydrogen peroxide treatment of microsomes prepared by method B and the effect of Triton X-100 on the activation

The activation was measured by the method of Aniya and Anders [17]. Just before catalytic activity measurement microsomes were resuspended in a Potter–Elvehjem homogenizer. The suspended microsomes were assayed for GST activity diluted in suspension buffer such that delta OD340 nm per min ranged from 0.07 to 0.1 (subtracting substrate blank). The suspended microsomal fraction from freshly prepared microsomes was used within 4 h. Hydrogen peroxide was diluted in 0.05 M potassium phosphate buffer (pH 7.4). Microsomes (25 μl of suspension, approximately 0.2 mg/ml protein) were incubated with 1.25 mM hydrogen peroxide (25 μl of 10 mM hydrogen peroxide) in 0.05 M potassium phosphate buffer (pH 7.4) in a final volume of 200 μl in a glass tube without shaking at room temperature for 30 min. Control microsomes received 25 μl of 0.05 M potassium phosphate buffer (pH 7.4) only.

GST activity was assayed by the original method of Habig et al. [16]. The methods to examine the effect of Triton X-100 on the activation by hydrogen peroxide were carried out as follows: Triton X-100 was diluted to 1% in 0.05 M potassium phosphate buffer (pH 7.4) and an incubation mixture at room temperature contained 1.3 ml of 0.05 M potassium phosphate buffer (pH 7.4), 0.2 ml of 1% Triton X-100, 10 mM hydrogen peroxide and 0.25 ml of the microsomal suspension described above. Activity was measured by the original method at 0 min, and at 5, 10, 20 and 30 min.

2.2.9. Purification of MGST1

Male Sprague–Dawley rats weighing approximately 250 g were starved overnight, killed by decapitation, and livers were homogenized in 0.25 M sucrose. Liver microsomes washed twice by 0.15 M Tris–HCl (pH 8.0) were prepared and purification of MGST1 was performed the same day by the method of Morgenstern et al. [18]. An additional CM-sepharose column chromatography step was carried out at pH 8.0.

2.2.10. The effect of NADPH, ATP, and other nucleotides on MGST1 activity

Nucleotides at 1 mM concentration were incubated with purified GSH free MGST1 at room temperature. The incubation mixture included 0.166 ml of 0.05 M potassium phosphate buffer (pH 7.4) and 0.05 ml of MGST1. An aliquote of 0.020 ml was taken at 0 min for GST assay and then 0.024 ml of 10 mM compound diluted in the same buffer was added. At 30 and 60 min, 0.020 ml was withdrawn and added to 0.17 ml of 0.1 M potassium phosphate, pH 7.0, containing 20% glycerol, 0.1 mM EDTA and 0.1% Triton X-100, 5 μl of GSH (final concentration 5 mM) and 5 μl of CDNB (final concentration 0.5 mM) for GST activity measurement at room temperature. The concentration of Triton X-100 is very important since a low concentration results in background activation of MGST1. In these experiments, the concentration of Triton X-100 in the incubation was kept close to 0.2%. For experiments in the presence of GSH, the original purified enzyme was used. To examine the effect of DTT, a sample was taken from the incubation mixture at 0 min, whereafter DTT and the other compounds were added. GSH free purified enzyme was stored at 4 °C and used within 1 week.

2.2.11. Treatment of purified MGST1 with recombinant FMO3

Purified MGST1 was applied to a 10 DG gel filtration column (Bio-Rad Laboratories) at room temperature for GSH removal. The GSH free enzyme was used the same day. Flavine monooxygenase (FMO) enzyme activity was checked by measurement of NADPH consumption using methimazole as substrate [19]. FMO3 supersomes were preincubated with the NADPH g.s. in 0.05 M potassium phosphate buffer (pH 7.4) at room temperature for 10 min. Subsequently GSH-free purified enzyme was added to the incubation mixture and GST activity was assayed at 0, 5, 10, 20 and 30 min using the original assay. Supersomes heated 3 min at 100 °C were used as a negative control. The NADPH g.s. consisted of 0.33 mM $NADP^+$, 8 mM glucose 6-phosphate, 6 mM $MgCl_2$ and 0.2 units of glucose 6-phosphate dehydrogenase [9]. All reagents added to the incubation mixture were dissolved in 0.05 M potassium phosphate (pH 7.4). The incubation mixture contained 12 μl of the supersomes expressing FMO3, 43 μl of an NADPH g.s. and 35 μl of 0.05 M potassium phosphate (pH 7.4). The solution was preincubated for 10 min at room temparure and then 30 μl of GSH-free purified MGST1 enzyme was added to the incubation mixture. At each time point, 20 μl was withdrawn and the activity measured in 0.1 M potassium phosphate, pH 6.5, with 5 mM GSH and 1.0 mM CDNB at room temperature. Total volume of incubation mixture plus assay solution was 0.15 ml.

2.2.12. Protein determination

Protein concentration was determined by the method of Peterson [14] or Lowry et al. [15] with bovine serum albumin as standard.

2.2.13. Measurement of hydrogen peroxide

Hydrogen peroxide levels in microsomal incubations were measured by the method of [20].

2.2.14. Numerical analyses

The data are expressed as mean ± S.E.M./S.D. Significance of differences was evaluated by Student's paired two tailed *t*-test for all data, except for 4-methylpyrazole results (Student's paired one tailed *t*-test).

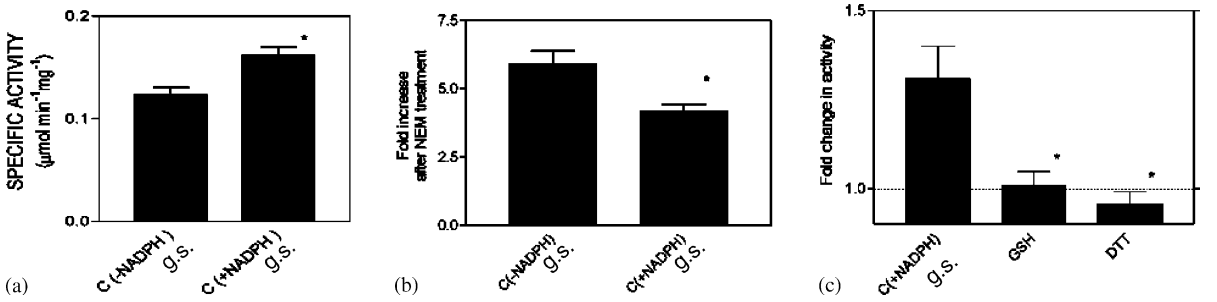

Fig. 1. (a) NADPH-dependent activation of MGST1 in rat liver microsomes. GSH transferase activity of rat liver microsomes after incubation for 15 min in the presence or absence of an NADPH generating system. C (−NADPH) (control without NADPH-generating system; C (+NADPH) (control with NADPH-generating system). $^{*}P < 0.0001$, $n = 23$. Experimental details are given under Section 2.2. (b) NEM dependent activation of microsomes pretreated with an NADPH generating system or reactive oxygen species. Fold increase in activity after NEM treatment. NEM (5 mM) was added to the samples, which were then stored on ice for approximately 10 min whereafter the activity was measured. C (−NADPH) (control without NADPH-generating system; C (+NADPH) (control with NADPH-generating system, $n = 4$). $^{*}P < 0.05$, $n = 4$. Experimental details are given under Section 2.2. (c) Inhibition of NADPH-dependent activation of MGST1 in rat liver microsomes. Inhibition of NADPH dependent activation of rat liver microsomal GSH transferase activity by subsequent addition of GSH and DTT. C (+NADPH) (control with NADPH-generating system, $n = 7$). $^{*}P < 0.011$, (GSH 1 mM, $n = 7$). $^{*}P < 0.0046$, (DTT 0.5 mM, $n = 7$). Experimental details are given under Section 2.2.

3. Results and discussion

3.1. NADPH activates MGST1 in rat liver microsomes

We could confirm earlier observations that NADPH can activate rat liver microsomal glutathione transferase 1 activity in freshly prepared rat liver microsomes (Fig. 1a). In fact, results in the literature were variable [9] and we also noted considerable variation between preparations with maximal activation reaching twofold. The NADPH treatment results in a diminished capacity for NEM-activation clearly demonstrating that MGST1 is a target (Fig. 1b). As sulfhydryl compounds prevented activation (Fig. 1c) we considered cysteine-49 as a possible molecular target. It is known that reactive oxygen species can activate MGST1 [9,21–23] and it is also known that NADPH generates reactive oxygen species in microsomal incubations, especially via cytochrome P450 2E1, CYP2E1 [24]. We therefore examined whether an inhibitor of CYP2E1,4-methylpyrazole, could inhibit activation (Fig. 2). Although some inhibition was observed the results did not show concentration dependence and should be interpreted as a partial inhibition of activation. Clearly a specific role for CYP2E1 is not indicated. Furthermore, incubation of rat liver microsomes with superoxide or hydrogen peroxide (10 μM–10 mM, not shown) did not yield activation. The latter result contradicted earlier observations [9] but agreed with those from other laboratories [11,25]. As microsome preparation methods differ between the different laboratories we investigated whether this is a decisive factor for reactive oxygen species activation. In Table 1 it is shown that the preparation method is indeed crucial for obtaining activation of microsomal

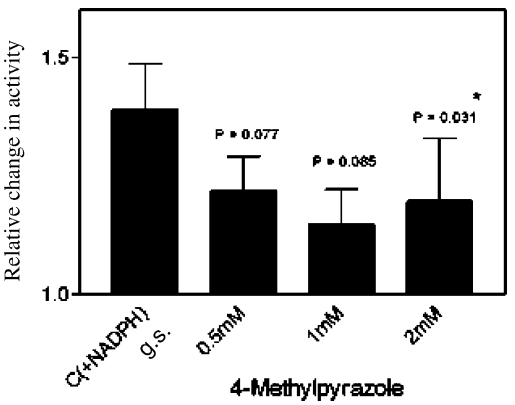

Fig. 2. Inhibition of NADPH-dependent activation of MGST1 in rat liver microsomes. Inhibition of NADPH dependent activation of rat liver microsomal GSH transferase activity by 4-methylpyrazole. C (+NADPH) (control with NADPH-generating system, $n = 9$). 4-Methylpyrazole (0.5 mM, $n = 5$); (1 mM, $n = 9$); (2 mM, $n = 4$). Experimental details are given under Section 2.2.

Table 1
Preparation method and activation of MGST1 by hydrogen peroxide in rat liver microsomes

Treatment	Hydrogen peroxide (mM)	MGST1 activity (μmol/mg min)	
		Control	Hydrogen peroxide (% change)
Perfused with KCl	1.0	0.130[a]	0.166[a] (128)
	1.25	0.081 ± 0.002[b]	0.160 ± 0.010[b] (194)
Sucrose without perfusion	1.0	0.99 ± 0.008	0.098 ± 0.005 (99)

[a] Fresh microsomes prepared from 1.15% KCl perfused and homogenized livers. Twice washed microsomes by 0.15 M Tris–HCl (pH 8.0) were incubated with or without hydrogen peroxide at room temperature for 30 min. For assay, 0.1 M potassium phosphate (pH 6.5), 5 mM GSH and 1 mM CDNB were used. Absobance change at 340 nm from 1 to 3 min was used to calculate GST activity. The protein concentration was determined by the method of Lowry et al. [15]: (1) results from [17] and (2) mean ± S.D. Activation was measured in four rats independently and each average value of triplicate was analyzed statistically.

[b] In the sucrose experiments, livers were homogenized in 0.25 M sucrose in 10 mM Hepes, 1 mM EDTA (pH 7.4) and the freshly prepared microsomes were washed twice by 0.05 M potassium phosphate (pH 7.4). The microsomes were once suspended in 0.05 M potassium phosphate (pH 7.4) and diluted in 0.05 M Tris–HCl (pH 7.4) containing 10 mM $MgCl_2$. GST activity was determined in 0.1 M potassium phosphate (pH 6.5) containing 0.1% Triton X-100 with 5 mM GSH and 0.5 mM CDNB at room temperature. The fresh microsomes were incubated with 1 mM hydrogen peroxide for 15 min at 37 °C. The protein concentration was determined by the method of Peterson [14]. The values represents mean ± S.D. of triplicates. Three independent experiments reproduced similar results.

GST activity by hydrogen peroxide and the perfusion by KCl appears important. It was also noted that freshly resuspended microsomes prepared in this way kept activation capacity for only a few hours. The activation with 1.25 mM hydrogen peroxide is inhibited by inclusion of 0.1% Triton X-100 in the incubation with fresh liver microsomes (data not shown) demonstrating the sensitive nature of the activation system. As preliminary data (from Y.A.) indicated that the differences might result from altered radical scavenging properties dependent on membrane preparation we investigated the decomposition rate of hydrogen peroxide in microsomes prepared by the two methods. Indeed it was shown that hydrogen peroxide is decomposed faster in microsomes from non-perfused livers (Fig. 3). It is possible that antioxidant enzymes such as catalase contaminate microsomes prepared from unperfused livers to a larger extent. As direct activation by reactive oxygen species did not occur in microsomes prepared by the method (A) using sucrose [26] we can speculate that NADPH activation occurs, at least partly, by an alternate mechanism.

We were curious whether purified enzyme prepared from these microsomes had also lost the capability to become activated by hydrogen peroxide. Indeed our purified MGST1 required 10-fold higher concentrations of hydrogen peroxide to become activated (Fig. 4a) as compared to that observed previously in a laboratory that uses the KCl perfusion method to prepare microsomes before purification [17].

We next considered whether flavine monooxygenase (FMO) could activate MGST1 by catalysing oxidation of cysteine-49. To examine a possible role for FMO we incubated purified MGST1 with a commercial source of FMO containing supersomes in the presence of NADPH but did not observe significant activation above control levels (when only an NADPH g.s. was included)(Fig. 4b). This observation agrees with that of Onderwater et al. [27].

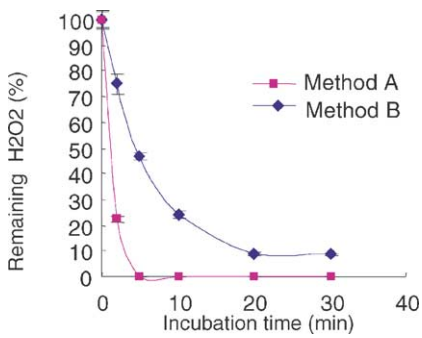

Fig. 3. Hydrogen peroxide decomposition in microsomes prepared by method A (without perfusion) and B (including liver perfusion). Microsomes were prepared and treated in a similar manner as described in Table 1. Aliquots were withdrawn at the time points indicated and hydrogen peroxide determined [20]. Means of triplicate values ± S.D. are given and similar results were obtained in three independent experiments. Decomposition of hydrogen peroxide in buffers alone was negligable.

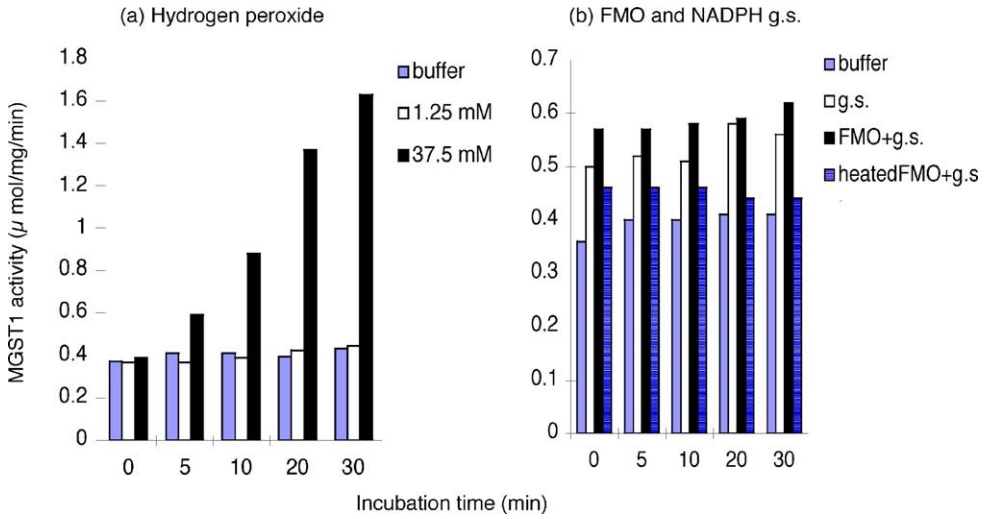

Fig. 4. Activation of purified MGST1 by hydrogen peroxide but not by flavine monooxygenase. (a) Hydrogen peroxide activation of MGST1 activity with GSH-free purified enzyme. Hydrogen peroxide in 0.05 M potassium phosphate (pH 7.4) was added to purified enzyme at room temperature. GST activity was assayed in 0.1 M potassium phosphate, pH 6.5, with 5 mM GSH and 1 mM CDNB at room temperature. The values are means of duplicate experiments. (b) Incubation of FMO and NADPH g.s. with purified MGST1 activity in the absence of GSH.

3.2. NADPH activates purified MGST1

We were surprised however that purified enzyme becomes activated during incubation with NADPH alone (Fig. 5a). The activation is inhibited by GSH and DTT (Fig. 5b and c) and thus displays similar characteristics as the NADPH activation observed in microsomes. Actually, the DTT treated MGST1 showed

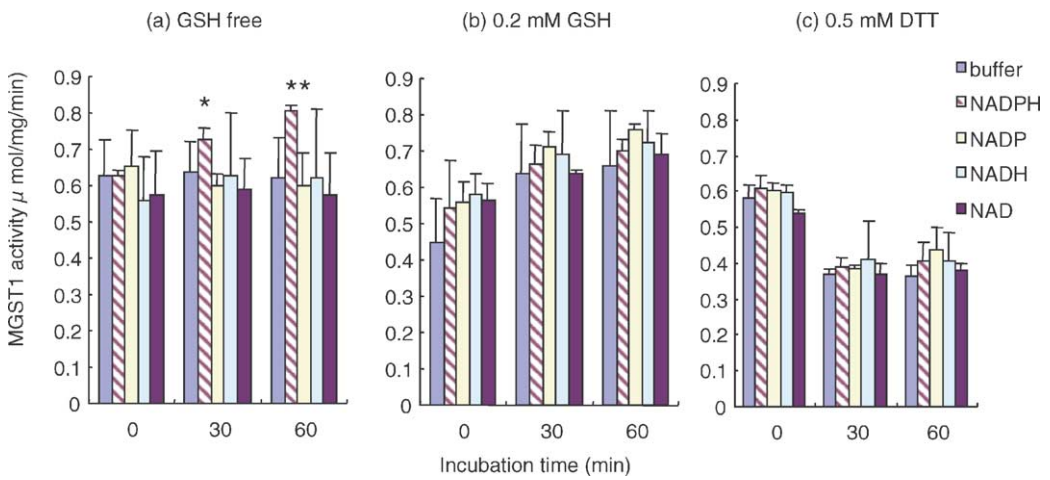

Fig. 5. Activation of purified MGST1 by NADPH and reversal by thiols. (a) Activation of GSH free MGST1 by NADPH, but not NADP, NADH and NAD. Each compound (1 mM) was dissolved in 0.05 M potassium phosphate (pH 7.4) and incubated at room temperature. The GST activity was determined in 0.1 M potassium phosphate, pH 7.0, containing 20% glycerol, 0.1 mM EDTA and 0.1% Triton X-100. The columns show mean and S.D. of triplicate measurements. Two independent experiments showed similar results. Statistically different from 0 min control ($^{*}P < 0.05$ and $^{**}P < 0.01$). (b) Reversal of activation by 0.2 mM GSH. GST activity was assayed in the presence of each chemical (except at zero time). Details are described under (a) and Section 2.2. (c) Reversal of activation by 0.5 mM DTT. Details were as described under (a, b) and Section 2.2.

Table 2
DTT reverses the activation of purified MGST1 by NADPH or ATP

Incubation	MGST1 activity (μmol/mg min)		
	0 min	60 min (% change)	60 min + DTT (% change)
Buffer	0.550 ± 0.012	0.540 ± 0.090 (97)	0.460 ± 0.054 (83)
NADPH	0.580 ± 0.008	0.630 ± 0.046 (109)	0.580 ± 0.036 (99)
ATP	0.550 ± 0.054	0.770 ± 0.004 (140)	0.570 ± 0.052 (104)

GSH free purified MGST1 was incubated with 1 mM NADPH, 3 mM ATP or 0.05 M potassium phosphate buffer (pH 7.4) at room temperature. After preincubation for 60 min at room temperature, incubation with 10 mM DTT for 10 min at room temperature followed. MGST1 activity toward 5 mM GSH and 0.5 mM CDNB was measured. Results are shown as mean ± S.E.M. ($n = 3$).

lower activity than that of controls (Figs. 1c and 5c) indicating that the enzyme becomes slightly activated during purification and storage. Other dinucleotides, NADP, NADH and NAD that were tested did not yield activation (Fig. 5) demonstrating specificity. Even higher activation of purified enzyme was observed when incubating MGST1 with ATP (Table 2). Activation was sensitive to the inclusion of a sulfhydryl (DTT) also in this case. Ligand activation of MGST1 is not without precedent, since bromosulphophtalein was demonstrated as a reversible non-covalent activator of MGST1 [8,28]. In the case of bromosulphophtalein however, activation did occur instantaneously when the compound was included in the assay where 5 mM GSH is present. For NADPH and ATP the activation takes at least 30–60 min to reach maximum. Inhibition by sulfhydryl compounds indicates that a chemical process might be involved. Perhaps these ligands influence the autoxidation capacity of MGST1 (which is known to self-activate very slowly upon exposure to air, unpublished observation). In addition, results in the literature indicate that the different oxidation states of MGST1 might become activated to different degrees and some not at all [11] with the added complexity of interactions within the homo-trimer [29]. Therefore these nucleotides might function as ligands that guide oxidation and stabilise activated oxidation states. Clearly, these speculations will require further experiments. It is interesting to note that the most avid ligand of MGST1 known ($K_d = 5$ nM) leukotriene C_4 [30] also binds very strongly to the nucleotide binding Rossman fold of glyceraldehyde-3-phophate dehydrogenase [31] suggesting similarities, at least, between nucleotide and glutathione conjugate binding domains.

Microsomal glutathione transferase 1 becomes activated by most compounds that can react with sulfhydryls and has therefore been suggested and investigated as a system to detect reactive intermediates during the metabolism of drug candidates [12,27]. Here we define experimental conditions relevant to the background in this type of assay. In earlier experiments where we used MGST1 activation to detect reactive intermediates from phenol we noted, and could diminish, activation by NADPH itself simply by using lower concentrations of the latter [13]. For compounds that cause oxidative stress, careful consideration has to be given to the experimental system (and positive controls included) to be able to discriminate from the formation of covalent reactive intermediates and possible activation by oxidative stress. The work presented here defines these conditions in terms of microsome preparation methodology.

In conclusion, we have defined a new ligand dependent activation pathway for MGST1 that could occur at low thiol concentrations, such as would occur in a stressed cell.

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