Glutathione *S*-conjugates stimulate ATP hydrolysis in the plasma membrane fraction of rat hepatocytes

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Incubation of a rat hepatocyte plasma membrane fraction with micromolar concentrations of either glutathione disulfide or various glutathione *S*-conjugates resulted in a several-fold increase in the rate of ATP hydrolysis. This stimulation was further enhanced when the plasma membrane fraction had been pretreated with agents that arylate or oxidize sulfhydryl groups, suggesting that this ATPase activity is modulated by the protein thiol status of the plasma membrane. It is proposed that this newly discovered ATPase may function in the cellular extrusion of both glutathione disulfide and glutathione *S*-conjugates.

Hepatocyte *Plasma membrane* *ATPase* *Glutathione* *Thiol*

1. INTRODUCTION

Glutathione (GSH) plays an important role in the catabolism of various hydroperoxides and inactivation of electrophilic drugs and drug metabolites in mammalian cells [1]. Whereas GSH functions as a reductant in the metabolism of hydroperoxides catalyzed by glutathione peroxidase, the inactivation of electrophilic compounds is achieved by the formation of glutathione *S*-conjugates catalyzed by a family of inducible enzymes, the glutathione transferases [2].

The intracellular formation of glutathione *S*-conjugates is followed by their active extrusion from the cell [3–5]. In liver, glutathione *S*-conjugates and glutathione disulfide (GSSG) are secreted preferentially into bile [4,6] by, what appears to be, a common transport system [7]. A similar transport system, involved in the extrusion of intracellular GSSG and glutathione *S*-conjugates, has also been found to be present in heart [8] and in erythrocytes [5,9]. Detailed studies of GSSG transport in erythrocytes have revealed the involvement of an ATP-dependent process exhibiting kinetic parameters suggestive of two distinct components [9,10].

We have recently demonstrated that the plasma membrane fraction from rat hepatocytes contains an ATPase which is activated in the presence of GSSG and mixed disulfides containing a glutathione moiety [11]. Here, we report that several glutathione *S*-conjugates also stimulate ATP hydrolysis in the hepatocyte plasma membrane fraction, and that this activity is modulated by the protein thiol status of the plasma membrane. We propose that the GS-ATPase may function in the active extrusion of intracellular glutathione disulfide and *S*-conjugates.

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Abbreviations: GSH, glutathione (reduced form); GSSG, glutathione disulfide; GS-ATPase, glutathione disulfide- or glutathione *S*-conjugate-stimulated ATPase

2. MATERIALS AND METHODS

Collagenase (grade II) was obtained from Boehringer, Mannheim, and Affi-Gel 731 polyacrylamide beads, coated with polyethylene-

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imine, were from Bio-Rad Laboratories (Richmond, VA). Glutathione, glutathione disulfide, ATP, 1-chloro-2,4-dinitrobenzene, diamide, *N*-ethylmaleimide and *p*-chloromercuribenzoate were purchased from Sigma (St. Louis, MO). Other reagents were commercial products of the highest available grade of purity.

Hepatocytes were isolated from male Sprague-Dawley rats (180–200 g, allowed food and water ad libitum), by collagenase perfusion as described [12]. For isolation of the plasma membrane fraction, hepatocytes were attached to polyacrylamide beads (Affi-Gel 731) and treated as described in [11]. This procedure yields a highly purified preparation of plasma membrane fragments attached to the beads, and contamination with mitochondria and microsomes is negligible [11]. Protein was determined by the method of Lowry et al. [13].

The glutathione *S*-conjugate of 1-chloro-2,4-dinitrobenzene was prepared by incubating 10 mM GSH and 20 mM 1-chloro-2,4-dinitrobenzene in 0.1 M Tris-maleate buffer, pH 6.5, at 37°C for 1 h in the presence of rat liver cytosolic fraction (1.5 mg protein/ml). The formation of *S*-dinitrophenol glutathione was monitored by recording the light absorption at 334 nm in a Shimadzu UV-240 spectrophotometer, as reported in [14]. The *S*-conjugate was then purified [15] and isolated by high-performance liquid chromatography using the method of Reed et al. [16].

The glutathione *S*-conjugates (mono and bis) of the *p*-phenetidine metabolite *N*-(4-ethoxyphenyl)-*p*-benzoquinoneimine were synthesized as described in [17], except that the incubation was performed for 2 h at room temperature in 100 mM ammonium carbonate, pH 8. After extraction with ethyl acetate, the aqueous phase was subjected to freeze-drying and the residue was spotted on silica-gel plates (2 mm Merck, Darmstadt), developed in *n*-propanol/water, and the bands corresponding to the mono- and bis-conjugates were isolated. The glutathione *S*-conjugate of hexachlorobutadiene was a generous gift from Dr T. Jones of this Department.

The GS-ATPase assay was performed as described in [11]. Enzyme activity was monitored in a medium containing 0.3 ml of a suspension of polyacrylamide beads coated with plasma membrane fragments (70–90 μg protein), 1 mM ATP, 1 mM ouabain, 20 mM NaN_3, 50 mM Tris-maleate buffer, pH 6.5, in the absence or presence of glutathione *S*-conjugates, GSSG or other substrates. P_i release was measured by the method of Carter and Karl [18].

To investigate the effect of modification of the protein thiol status of the plasma membrane fraction on GS-ATPase activity, a 50% (v/v) bead suspension, coated with plasma membrane fragments was incubated at 37°C for 10 min in Tris-HCl buffer, pH 7.4, in the presence or absence of thiol oxidizing, arylating or reducing agents. The bead suspension was then washed twice with the same buffer and finally suspended at 50% (v/v) in the assay medium, and GS-ATPase activity was measured as described above.

3. RESULTS

As shown in fig.1, incubation of the hepatocyte plasma membrane fraction with 1 mM ATP and micromolar concentrations of either GSSG or *S*-dinitrophenyl glutathione resulted in a several-fold stimulation of the rate of ATP hydrolysis observed in the absence of glutathione derivative. The reaction was linear for up to 1 h, and no stimulation of

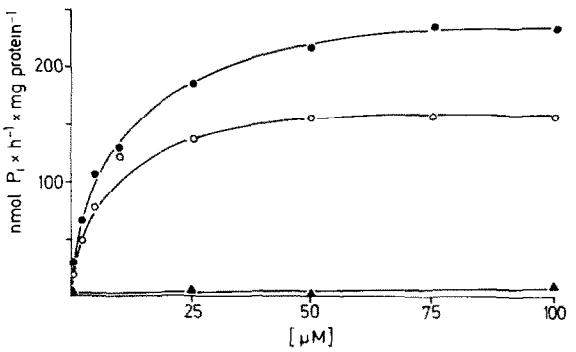

Fig.1. Stimulation of ATP hydrolysis by glutathione disulfide and *S*-dinitrophenyl glutathione in the plasma membrane fraction from rat hepatocytes. Beads coated with plasma membrane fragments were incubated with 1 mM ATP and various concentrations of GSSG (○), *S*-dinitrophenyl glutathione (●) or 1-chloro-2,4-dinitrobenzene (▲) at 37°C for 1 h as described in section 2. When calculating the stimulation of ATP hydrolysis, the rate of P_i release in absence of added substrate (50 nmol·h^{-1}·mg protein^{-1}) was subtracted. One experiment typical of 3.

ATPase activity was seen with the unconjugated drug, 1-chloro-2,4-dinitrobenzene.

Stimulation of ATP hydrolysis by GSSG and *S*-dinitrophenyl glutathione was concentration dependent (fig.1), and analysis of the data demonstrated that they conformed to Michaelis-Menten kinetics with an apparent K_m value of ~6 μM for both GSSG and *S*-dinitrophenyl glutathione (table 1). As also shown in the table, other glutathione *S*-conjugates exhibited similar affinity for the ATPase, although the apparent V_{max} differed between the various *S*-conjugates. It is of interest to note that the glutathione bis-conjugate of *N*-(4-ethoxyphenyl)-*p*-benzoquinoneimine caused a significantly higher rate of ATP hydrolysis than the corresponding mono-conjugate (table 1).

We have previously reported that, in addition to GSSG, mixed disulfides containing a glutathione moiety stimulated ATP hydrolysis in a hepatocyte plasma membrane fraction, whereas cystine was without effect [11]. Here, we have tested cystamine and dithiodipyridine and found them to be also inactive, suggesting that the glutathione moiety is required for the stimulation of ATP hydrolysis.

As shown in fig.2, the GS-ATPase activity appears to be modulated by the thiol status of the plasma membrane. Thus, the stimulation of ATP hydrolysis by the glutathione mono-conjugate of *N*-(4-ethoxyphenyl)-*p*-benzoquinoneimine was further enhanced by pretreatment of the plasma membrane fraction with thiol oxidizing (diamide) or arylating (*p*-chloromercuribenzoate and *N*-ethylmaleimide) agents. Under our experimental conditions, pretreatment of the plasma membrane fraction with either *p*-chloromercuribenzoate, *N*-

Table 1

Kinetic parameters of glutathione disulfide- and *S*-conjugate-stimulated ATP hydrolysis in rat hepatocyte plasma membrane fraction[a]

	K_m (μM)	V_{max} (nmol $P_i \cdot h^{-1} \cdot mg\ protein^{-1}$)
GSSG	6.24 ± 0.69 (4)	162 ± 20.7
Glutathione *S*-conjugate of:		
Hexachlorobutadiene	6.49 ± 0.81 (3)	208 ± 22.2
1-Chloro-2,4-dinitrobenzene	6.17 ± 1.01 (3)	209 ± 49.1
N-(4-Ethoxyphenyl)-*p*-benzoquinoneimine		
mono-conjugate	6.25 ± 0.42 (4)	204 ± 14.4
bis-conjugate	6.34 ± 0.53 (4)	324 ± 21

[a] Data are expressed as mean ± SD. The number of experiments is given within parentheses

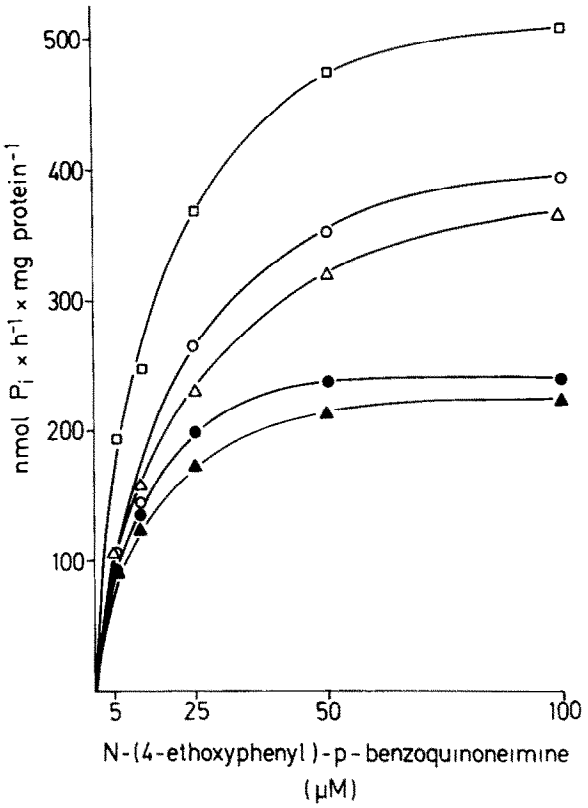

Fig.2. Activation of glutathione *S*-conjugate-stimulated ATP hydrolysis by pretreatment of the rat hepatocyte plasma membrane fraction with thiol-depleting agents. Aliquots of beads coated with plasma membrane fragments were preincubated in the absence (●) or presence of 1 mM diamide (△), 250 μM *N*-ethylmaleimide (○), 50 μM *p*-chloromercuribenzoate (□), or 5 mM ascorbate (▲) at 37°C for 10 min. The suspension was then washed with buffer, and the rate of ATP hydrolysis was assayed in presence of various concentrations of the glutathione mono-conjugate of *N*-(4-ethoxyphenyl)-*p*-benzoquinoneimine as described in section 2. One experiment typical of 4.

ethylmaleimide or diamide resulted in a 70–80% decrease in free sulfhydryl groups, and was associated with a 1.5–2.3-fold increase in the *S*-conjugate-stimulated rate of ATP hydrolysis; the basal rate of ATP hydrolysis, observed in the absence of added *S*-conjugate, was not affected by this pretreatment. Fig.2 shows the results obtained with the mono-conjugate of *N*-(4-ethoxyphenyl)-*p*-benzoquinoneimine, and similar results were obtained with the other *S*-conjugates (not shown). In contrast, there was no apparent effect on *S*-conjugate-stimulated ATPase activity by pretreatment of the plasma membrane fraction with a reducing agent, ascorbate (fig.2).

4. DISCUSSION

We have reported that the plasma membrane fraction of rat hepatocytes contains an ATPase whose activity is stimulated by GSSG and low-molecular-mass mixed disulfides containing a glutathione moiety [11]. Similarities between the kinetic properties of this activity and those reported earlier for GSSG transport in erythrocytes [10] led us to propose that this ATPase may be involved in the active extrusion of intracellular GSSG in liver and erythrocytes [11].

Here we have found that several glutathione *S*-conjugates also stimulate ATP hydrolysis in the hepatocyte plasma membrane fraction, and that GSSG and the various *S*-conjugates exhibit similar affinities for stimulation of the ATPase (K_m ~6 μM when measured at pH 6.5); the apparent K_m for GSSG is lower than that reported in [11], due to an increased recovery of the high-affinity component of the ATPase, during plasma membrane purification. In view of the known competition between GSSG and *S*-conjugates for a common transport system in liver [7,19], it appears likely that the ATPase may be a component of this system. It is noteworthy that all the *S*-conjugates investigated exhibited similar affinities for the ATPase, suggesting that the xenobiotic moiety of the conjugate is of minor importance for its interaction with the enzyme.

The concentration of GSSG has been estimated to be as low as 10^{-8} M when the glutathione reductase operates close to its thermodynamic equilibrium [20]. The level of GSSG is also quite low in the intact hepatocyte, but it rises rapidly during oxidative stress [5,21]. It is therefore conceivable that the GS-ATPase operates at a low rate under normal, physiological conditions, but is rapidly activated when either GSSG or *S*-conjugates accumulate within the cell.

Like many other enzymes, several ATPases coupled with ion transport are known to depend on free sulfhydryl groups for activity [22–24]. However, in contrast to the Na^+,K^+-ATPase and several Ca^{2+}-ATPases, which are inhibited by agents that interfere with free sulfhydryl groups, the GS-ATPase appears to be activated by compounds that oxidize or arylate thiol groups. Although the molecular mechanism underlying this effect has not been investigated, activation of this enzyme by GS-disulfides would be expected to have an important protective effect under both physiological and pathophysiological conditions, i.e., a normal GSH/GSSG redox level and secretion of *S*-conjugates could still be maintained within the hepatocyte even under conditions where cellular thiols have been oxidized or arylated [25].

Our data suggest that the newly discovered GS-ATPase may be involved in the active excretion of both GSSG and *S*-conjugates from hepatocytes. It has also been reported that several other cell types actively excrete GSSG and *S*-conjugates [5,6,9,26] and the possible existence of a similar GS-ATPase in the plasma membrane fraction from cells other than hepatocytes is currently under investigation in our laboratory.

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