BBA 45874

THE CELLULAR LOCALISATION OF GLUTATHIONE PEROXIDASE AND ITS RELEASE FROM MITOCHONDRIA DURING SWELLING

R C GREEN AND P J O'BRIEN

Department of Biochemistry, Memorial University of Newfoundland, St John's, Newfoundland (Canada)

(Received July 28th, 1969)

SUMMARY

1. The intracellular and intramitochondrial localisation of rat liver GSH peroxidase (GSH: H_2O_2 oxidoreductase, EC 1.11.1.9) and its release from mitochondria have been investigated.

2. The peroxidase was localised in the mitochondria and the cytosol.

3. The matrix was established as the intramitochondrial site of GSH peroxidase.

4. GSH peroxidase was released from the mitochondria during the swelling initiated by GSH, GSH + GSSG, ascorbate and oleate. The extent of the release was proportional to the degree of swelling. The peroxidase was not released during the swelling induced by phosphate, Ca^{2+} or a mixture of phosphate + GSH.

5. The release of peroxidase during mitochondrial swelling was not specific since the specific activity of the enzyme released was similar to that released by sonication.

6. The significance of GSH peroxidase in the swelling–contraction cycle is discussed.

INTRODUCTION

LEHNINGER AND GOTTERER[1] and LEHNINGER[2] first showed that a heat-labile non-dialyzable factor necessary for contraction (C-factor) was released into the medium when mitochondria were exposed to GSH. The C-factor became sufficiently diluted on release thereby limiting contraction. The C-factor could also be completely released into solution by sonic treatment. As the specific activity of C-factor appearing in the medium from GSH-swollen mitochondria was reported to be some 20 times greater than in sonic extracts[1], the loss of C-factor from mitochondria by contact with GSH was relatively specific. These results suggested that C-factor may be associated with the outer mitochondrial membrane.

HUNTER *et al.*[3] also reported that lipid peroxide formation was associated with GSH + GSSG-induced mitochondrial swelling. NEUBERT *et al.*[4] later showed that the GSH peroxidase accounted for approx. 80–85 % of the activity of C-factor. They suggested that GSH peroxidase may function in contraction by catalysing the reduction, by GSH, of lipid peroxides formed in the lipid bilayer of the mitochondrial

membrane with subsequent changes in the mechanochemical properties of the membrane. Recently LITTLE AND O'BRIEN[5,6] have been able to show that GSH peroxidase (GSH: H_2O_2 oxidoreductase, EC 1.11.1.9) is effective at reducing lipid peroxide and also that the peroxidase is probably the principal mechanism by which lipid peroxides are decomposed in the liver cell.

In the following the release of GSH peroxidase during GSH-induced mitochondrial swelling, and other types of swelling associated with lipid peroxidation, has been compared with the peroxidase release by ultrasonication, detergents and swelling associated with electron transport. The locus of GSH peroxidase within the mitochondria has been identified.

METHODS AND MATERIALS

Preparation of mitochondria

Female rats were used for all experiments since it is reported that their livers contain significantly more GSH peroxidase than those from males[7]. The liver was homogenized in 0.25 M sucrose using four up-and-down strokes of a Teflon-tipped homogenizer to yield a 10% (w/v) homogenate. Nuclei and debris were removed by centrifugation at 600 × *g* for 10 min. The mitochondria were then sedimented at 7500 × *g* for 10 min. Unless otherwise stated the mitochondrial pellet was washed once by gentle re-homogenization in half the original volume of 0.25 M sucrose, followed by centrifugation at 7500 × *g* for 10 min. The final mitochondrial suspension contained about 10 mg protein per ml.

Treatment of mitochondria with swelling agents

An aliquot of mitochondria (corresponding to about 2 mg of protein) was added to a mixture containing 0.25 M sucrose, 20 mM Tris–HCl (pH 7.4) and swelling agent in a final volume of 10 ml. Tubes were incubated for 30–45 min at 23°. The degree of swelling was estimated by measuring the decrease in absorbance at 520 nm and the tubes were then immediately centrifuged at 100 000 × *g* for 30 min in order to sediment the mitochondria together with any membrane fragments. The supernatant was decanted from the well-packed pellet and the pellet then suspended in 3 ml of water by sonicating for 15 sec. The suspension and supernatant were then assayed for enzymic activity.

In some instances the medium used contained 0.125 M KCl in place of 0.25 M sucrose. In this case the mitochondria were washed once in 0.125 M KCl and suspended in 0.125 M KCl prior to use.

Assay of enzymes

Mitochondrial preparations were always sonicated for two periods of 30 sec prior to enzyme assay. GSH peroxidase was assayed at 23° using a system containing 0.06 M Tris–HCl (pH 8.5), 1 mM EDTA, 0.25 mM GSH, 0.15 mM cumene hydroperoxide, 0.1 mM NADPH, 0.5 enzyme unit GSSG reductase, in a final volume of 3 ml. The rate of NADPH oxidation was followed spectrophotometrically at 340 nm. The rate of the non-enzymic reaction was subtracted.

Succinate dehydrogenase (succinate–2-(*p*-iodophenyl)-3-*p*-nitrophenyl-5-phenyltetrazolium chloride reductase) was assayed by the method of PENNINGTON[8]. Urate

oxidase was assayed using the method of BAUDHUIN *et al.*[9]. Malate dehydrogenase was assayed by the method of OCHOA[10]. Monoamine oxidase was assayed with benzylamine substrate as described by SCHNAITMAN *et al.*[11]. Adenylate kinase was assayed by the method of SCHNAITMAN AND GREENAWALT[12].

Protein was measured by the methods of HÜBSCHER *et al.*[13] and LOWRY *et al.*[14], using bovine serum albumin as standard.

Cumene hydroperoxide (Technical Grade) was obtained from Matheson, Coleman and Bell, Cincinnati; GSH reductase Type III (100 enzyme units/mg); GSH and GSSG were obtained from Sigma Chemical Co., St. Louis; polyvinyl pyrrolidone was supplied by British Drug Houses, Toronto

RESULTS

Subcellular distribution

Subcellular fractions of rat liver were prepared by the method of SEDGWICK AND HÜBSCHER[15]. The fractions were assayed for protein, GSH peroxidase, succinate dehydrogenase and urate oxidase (see Table I). It is seen that 60% of the peroxidase content of the nuclei-free homogenate was recovered in the particle-free supernatant. The particle-bound peroxidase was associated with the mitochondrial fraction with much smaller amounts being recovered in the lysosomal and microsomal fractions. From a comparison of the distribution of peroxidase with that of the peroxisomal enzyme urate oxidase[16] and the mitochondrial marker succinate dehydrogenase, it is apparent that GSH peroxidase is not associated with the peroxisomes but is truly mitochondrial.

TABLE I

SUBCELLULAR DISTRIBUTION OF GSH PEROXIDASE

Subcellular fractions of rat liver were prepared in 0.3 M sucrose with 2 mM EDTA by the method of SEDGWICK AND HÜBSCHER[15]. All fractions were activated by sonication prior to enzyme assay.

Fraction	*GSH peroxidase*		*Urate oxidase*		*Succinate dehydrogenase*	
	% of total recovered	*units*/mg protein*	*% of total recovered*	*units*/mg protein*	*% of total recovered*	*units**/mg protein*
Mitochondrial	28	17.4	37	0.63	72	80
Lysosomal	5	5.8	51	1.59	5	11
Microsomal	7	5.9	12	0.29	2	4
Soluble	60	31.3	0	0	20	18

* 1 unit is equivalent to an absorbance change of 0.1 per min
** 1 unit is equivalent to an absorbance change of 1.0 per 15 min.

Extractability of GSH peroxidase from mitochondria

NEUBERT *et al.*[17] also found that washing the mitochondria with 0.25 M sucrose extracted "significant" C-factor activity and suggested that the soluble portion of C-factor activity was derived in part from the mitochondria. It was found that four washings with 0.25 M sucrose, however, released only 12% of the total peroxidase activity. It was also found that 0.125 M KCl was able to remove 25% of the per-

oxidase activity from mitochondria in four washes. This may indicate that the peroxidase was not tightly membrane-bound or that some cytosol peroxidase was adsorbed. When washed mitochondria were subjected to the extraction procedure of JACOBS AND SANADI[18], which effects complete removal of cytochrome *c*, only 38 % of the peroxidase was lost.

A method was used for subfractionation of mitochondria in which the mitochondria were treated with different concentrations of Triton X-100 and then centrifuged at 100000 × *g* for 1 h. Only the completely solubilized enzymes should appear in the supernatant while all membrane-bound enzymes remain in the pellet. Mitochondria were treated with the detergent as described in METHODS AND MATERIALS. Fig. 1 shows the pattern of release of four enzymes. Adenylate kinase, a marker enzyme for the intermembrane space[12,19], was released the most easily. Indeed 35 % was released in the absence of detergent during the incubation at room temperature. GSH peroxidase and malate dehydrogenase, a matrix marker[19], were released at the same rate. Monoamine oxidase, a membrane-bound enzyme[12,19], was solubilized much more slowly.

Release of GSH peroxidase from mitochondria during swelling

It was observed that the GSH-induced swelling was not reproducible when determined by the change in absorbance over a 30-min period. This was mainly due to the different lengths of the lag phase between different samples of mitochondria. A change in the length of the lag period has also been found during "ageing" of mitochondria[20]. However, in all cases the degree of swelling observed was related to the amount of peroxidase released. The results with combinations of GSH and GSSG were much more reproducible.

Expt. 1 in Table II shows the effect of GSH and GSSG. 5 mM GSH brought about a small degree of swelling, accompanied by a loss of peroxidase from the mitochondria. GSSG alone produced neither effect but in combination, GSH and GSSG were very effective in promoting swelling. An almost complete loss of peroxidase from the mitochondria occurred at the same time. In the presence of EDTA, GSH failed to swell mitochondria and no peroxidase was lost.

Ascorbate is known to bring about mitochondrial swelling by a process associated with peroxidation of mitochondrial lipids[21] and similar to that initiated by GSH. Ascorbate was therefore tested for its effectiveness in releasing peroxidase Expt 2 in Table II shows that ascorbate brought about extensive swelling accompanied by a large loss of peroxidase from the mitochondria. GSH appeared to offer some protection against ascorbate-induced swelling

The swelling caused by phosphate or Ca^{2+} is linked to the electron transport chain of the mitochondria[22,23]. It was found that little peroxidase was released by these agents in comparison to that released by GSH (Expt. 3, Table II). Also, with GSH and phosphate together, the amount of peroxidase release was much less than with GSH alone. Swelling initiated by sodium oleate, however, resulted in extensive release of peroxidase from the mitochondria presumably as a result of its surface active properties.

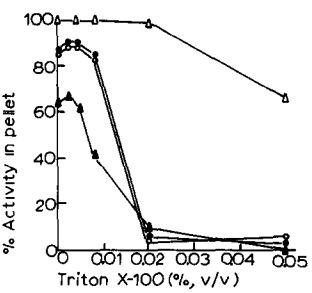
The question arises as to whether the peroxidase is released only upon disruption of the mitochondrial membranes However, Fig. 2 shows that the release of peroxidase during GSH + GSSG swelling closely followed the absorbance change at

TABLE II

RELEASE OF GSH PEROXIDASE FROM MITOCHONDRIA DURING SWELLING

The mitochondrial suspension was incubated with additive for 35 min The absorbance at 520 nm was measured, the suspension centrifuged at 100000 × *g* for 30 min and the peroxidase activity of the pellet measured

Additive	*Relative absorbance at 520 nm*	*Peroxidase released* (%)
Expt. 1		
None	100 ($A_{520\ nm} = 0.89$)	0
5 mM GSH	76	52
5 mM GSSG	105	0
5 mM GSH + 5 mM GSSG	16	97
5 mM GSH + 10 μM EDTA	102	0
Expt 2		
None	100 ($A_{520\ nm} = 0\ 83$)	0
0 2 mM ascorbate	46	85
10 mM GSH	86	19
0 2 mM ascorbate + 10 mM GSH	77	50
10 μM sodium oleate	33	78
Expt 3		
None	100 ($A_{520\ nm} = 1\ 00$)	0
10 mM GSH	31	90
5 mM potassium phosphate (pH 7 4)	60	5
10 mM GSH + 5 mM potassium phosphate (pH 7 4)	58	13
0 5 mM $CaCl_2$	60	13



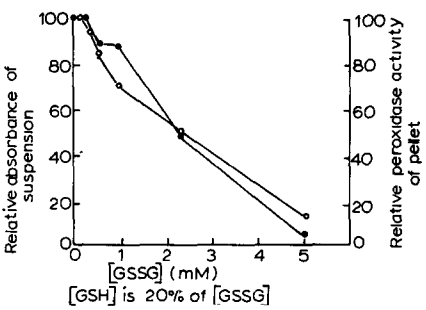


Fig 1 Release of enzymes from mitochondria by Triton X-100 An aliquot of mitochondria (about 2 mg protein) was added to a mixture containing 0 25 M sucrose, 20 mM Tris–HCl (pH 7 4) and Triton X-100 in a final volume of 10 ml After 30 min at room temperature the mixtures were centrifuged at 100000 × *g* for 30 min. The pellet was suspended in 3 ml of water by sonication and the suspension and supernatant were assayed for enzyme activities △—△, monoamine oxidase; ●—●, GSH peroxidase, ○—○, malate dehydrogenase, ▲—▲, adenylate kinase

Fig 2 Release of GSH peroxidase during mitochondrial swelling initiated by a mixture of GSSG and GSH Mitochondria were exposed to a mixture of GSSG and GSH in the molar ratio of 5 1. The medium also contained 0 25 M sucrose and 20 mM Tris–HCl (pH 7.4) in a final volume of 10 ml After 30 min the degree of swelling was estimated by measuring the absorbance at 520 nm The suspensions were then centrifuged at 100000 × *g* for 30 min and the pellet and supernatant assayed for GSH peroxidase activity. ●—●, GSH peroxidase; ○—○, absorbance at 520 nm

520 nm. It was also found that in the case of ascorbate-induced release of peroxidase, the extent of enzyme release was slightly greater than the decrease in absorbance. GEBICKI AND HUNTER[24] found that 95 % of the mitochondria remained intact when the absorbance had fallen to 20 % of the original value during GSH + GSSG swelling. Thus the release of peroxidase occurred before disruption of the mitochondrial membranes.

Since the peroxidase appeared to be localised within the mitochondrial matrix, it was difficult to envisage how the enzyme could be specifically released. It was therefore decided to investigate the specificity of the release by measuring, at the same time, the release of malate dehydrogenase (another matrix enzyme) and of total protein

Table III compares the effects of ascorbate, GSH + GSSG, and oleate on the swelling of mitochondria and on the release of GSH peroxidase, malate dehydrogenase and protein. In all these cases the release of peroxidase was accompanied by a similar release of malate dehydrogenase and release of some protein. It is thus apparent that during swelling the peroxidase is released in the same manner as other matrix components. Sonication resulted in a loss of almost all peroxidase and malate dehydrogenase but of only 57 % of protein. The specific activity of the peroxidase released by sonication was similar to that released during oleate or GSH + GSSG-induced swelling. Results obtained with a KCl medium as used by LEHNINGER AND GOTTERER[1] were similar to that observed in a sucrose medium. The main difference observed with the KCl medium was that much greater amounts of enzyme and protein were lost in the absence of any swelling agent.

TABLE III

RELEASE OF GSH PEROXIDASE, MALATE DEHYDROGENASE AND PROTEIN FROM MITOCHONDRIA DURING SWELLING

Mitochondria were incubated with swelling agents and the degree of swelling was estimated by measuring the absorbance at 520 nm The mixtures were then centrifuged at 100000 × *g* for 30 min and the pellets and supernatants assayed for GSH peroxidase, malate dehydrogenase and protein. Initial absorbance at 520 nm = 0 75.

Additive	*Relative absorbance at 520 nm*	*Peroxidase released* (%)	*Malate dehydrogenase released* (%)	*Protein released* (%)
None	100	9	12	4
11 μM ascorbate	23	69	87	77
3 mM GSSG + 0 6 mM GSH	45	85	85	49
0 1 mM oleate	73	67	53	40
Sonication	5	95	92	57

The release of peroxidase was identical to that of malate dehydrogenase when mitochondria were treated with Triton X-100 or GSH + GSSG. However, the peroxidase was released slightly more readily than malate dehydrogenase upon treatment with oleate. The converse was true during ascorbate-induced swelling. This may indicate small differences in the effects of these agents on the mitochondrial structure.

DISCUSSION

NEUBERT *et al.*[17] reported that C-factor activity was distributed between the mitochondrial and soluble fractions of the cell and that GSH peroxidase was responsible for the majority of the C-factor activity. LITTLE AND O'BRIEN[5] showed a similar distribution for GSH peroxidase activity. However, in neither of these studies were the fractions characterised by measuring marker enzymes. The peroxisomes are known to be the site of much of the hydrogen peroxide metabolism within the liver cell[25] and in particular contain the catalase which was at one time believed to be located in the mitochondria[16]. With the usual methods for separating subcellular fractions the peroxisomes were sedimented in both the mitochondrial and lysosomal fractions[16]. Hence the possibility existed that the particle-bound GSH peroxidase was located in the peroxisomes and not entirely within the mitochondria. However, the results show that specific activity of the lysosomal fraction is 2.2 times that of the mitochondrial fraction with respect to urate oxidase (a peroxisomal marker) while the specific activity of the peroxidase in the mitochondrial fraction is 3 times that of the lysosomal fraction. This would therefore rule out a peroxisomal localisation for GSH peroxidase.

The release of C-factor from mitochondria by GSH was reported to be some 20 times more specific than sonication[1]. This, combined with the apparent requirement for GSH peroxidase in the contraction process, suggested that the enzyme may be associated with the mitochondrial outer membrane rather than the matrix where 80 % of the mitochondrial protein is located. However, when the release of GSH peroxidase by Triton X-100 is compared to that of other mitochondrial enzymes it is seen that its release closely follows that of malate dehydrogenase, believed to be a matrix marker. Adenylate kinase, localised in the inter-membrane space, was released much more rapidly. On the other hand the membrane enzyme monoamine oxidase was solubilized much more slowly. It is concluded that the method is effective in separating the enzymes of the matrix and inter-membrane space from each other and from the membrane-bound enzymes. From the application of this method, it is apparent that GSH peroxidase is located within the mitochondrial matrix. If the peroxidase can protect membranes against lipid peroxidation[26], the matrix peroxidase may protect the inner membrane while the cytosol peroxidase may protect the outer membrane.

The release of C-factor has previously been measured only in the case of GSH-induced swelling and has necessitated the use of high concentrations of mitochondrial protein[2]. By assaying the peroxidase activity rather than contraction factor activity, it has been possible to follow the release of this enzyme during all types of swelling while using a protein concentration sufficiently low as to enable the $A_{520\ nm}$ changes to be followed. It is apparent that the mere presence of GSH is not enough to cause release of peroxidase. The peroxidase is released only during the swelling process with the amount of peroxidase released being directly proportional to the extent of swelling. Since the presence of GSSG during GSH-induced swelling obviated the need for C-factor during contraction, NEUBERT AND LEHNINGER[27] suggested that GSSG may prevent loss of contraction factor from mitochondria. However, from Fig. 2 it is clear that GSSG does not prevent loss of peroxidase from mitochondria.

Peroxidase was also readily released during mitochondrial swelling induced by

ascorbate or oleate but not by phosphate or Ca^{2+}. This suggests that the release is associated with membrane damage by peroxidation[25,26] or surface active agents. The presence of phosphate during GSH-induced swelling (Table II) prevented the release, presumably due to the inhibition of peroxidation[28], and confirms that the release is a result of the swelling and not to the action of GSH.

Since the peroxidase was located in the mitochondrial matrix and since its release appears to be related to changes in membrane integrity it is not surprising to find the release is not specific. Other enzymes of the matrix (*e.g.* malate dehydrogenase) were released and the total loss of protein from the mitochondria was large. The use of KCl rather than a sucrose medium had no effect on the overall pattern of the release.

It is known that peroxidase can modify mitochondrial swelling since C-factor preparations can inhibit GSH-induced swelling of mitochondria[17]. The C-factor may act in this case by preventing the accumulation of lipid peroxides that could be responsible for the swelling. Whereas peroxidation may be involved in swelling it is unlikely that peroxidation interferes with contraction since other peroxidation-linked types of swelling do not require C-factor for contraction[21,29,30]; the addition of pure lipid peroxide does not interfere with contraction[31] and it is known that lipoxidase can act as a contraction factor[32]. As peroxidase was readily released during types of swelling not requiring C-factor for contraction, the C-factor requirement for contraction of GSH-swollen mitochondria is not simply because of C-factor loss. The recent findings of LEHNINGER AND BECK[30] may suggest an alternative explanation of the role of C-factor. They confirmed that mitochondrial swelling initiated by commercial samples of GSH is dependent on metal contamination. However, as no C-factor was required for reversal of metal-induced swelling in the absence of GSH, the presence of GSH during the swelling phase may reduce some disulphide links which become exposed during the swelling and which are necessary for contraction. Possibly the presence of GSH during the swelling of mitochondria by other agents may also result in a C-factor requirement for contraction. Treatment with GSSG can obviate the need for C-factor[32]. The action of GSH peroxidase as a contraction factor for GSH-swollen mitochondria may therefore partly result from its effectiveness in catalysing the restoration of essential disulphide groups by lipid peroxides

ACKNOWLEDGMENT

This investigation was supported by the National Research Council of Canada.

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