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SOME PROPERTIES OF MITOCHONDRIAL GLUTATHIONE

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SUMMARY

1. The presence of GSH in rat liver mitochondria is confirmed. GSH diffuses from the suspended particles in the presence of phosphate but respiratory inhibitors inhibit the diffusion.

2. GSH is oxidised in situ by oxidants including *t*-butyl hydroperoxide. The products formed include GSSG and GSS-protein mixed disulphides. The oxidation occurs at lower oxidant concentrations if phosphate or oxaloacetate are also present. Respiratory inhibitors abolish their effect.

3. With phosphate, the GSSG produced is found chiefly outside the mitochondria whereas with oxaloacetate, it is found chiefly inside.

4. The GSSG formed by the oxidation is reduced by Krebs-cycle acids with the exception of the ketoacids. Exogenous GSSG is reduced by these substrates only after lysis. Intact particles, however, catalyse the reduction of GSSG by either $NADH_2$ or $NADPH_2$.

INTRODUCTION

Fresh rat liver mitochondria contain a small amount of non-protein thiol. This fraction consists chiefly of a substance assaying as GSH [1, 2] which does not appreciably escape from intact particles during washing and remains stable during short term incubations.

Further study of this thiol is now presented which establishes that GSH can, under suitable conditions escape from mitochondria and also be oxidised and reduced in situ.

MATERIALS AND METHODS

Mannitol (0.25 M) containing 3-(*N*-morpholino) propane sulphonate (10^{-2} M; pH 7.4) and ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (10^{-4} M) is described throughout as 'the medium'. Washed mitochondria were obtained as previously described [1]. The amount present in 2 g rat liver was suspended in 1 ml of the medium.

Incubations. Mitochondrial suspensions with the requisite additions stated in the tables were incubated for 10 min with shaking. The medium and pellet were separated where indicated by centrifuging for 15 min at 6000 × *g*. The volume of supernatant medium was obtained by weighing and the pellet was suspended in 0.5 vol. of water. Proteins were precipitated by adding $HClO_4$ (0.1 vol.; 12 %).

Identification of mitochondrial thiol as GSH. The *N*-[^{14}C]ethylmaleimide derivatives of the mitochondrial non-protein thiol fraction were separated by electrophoresis [1] and the radioactivity coincident with that of the authentic derivative of GSH (at least 70 % of the total non-protein thiol) was eluted with 0.1 M acetic acid, concentrated, then applied as a streak to Whatman No. 1 paper. The paper was subjected to descending chromatography in butanol/acetic acid/water (4 : 1 : 5, v/v) and the area corresponding to the authentic GSH derivative again eluted and concentrated. The residue was hydrolysed for 20 h at 110 °C with 7 M HCl then, after evaporation of the acid, the amino acid content was determined with a Beckman Analyser. The only amino acids found in significant amounts were glutamic acid and glycine present at 0.92 and 0.94 mol, respectively, per mol of ^{14}C. The presence of γ-glutamyl was shown by keeping the unhydrolysed peptide for 24 h in water at 100 °C (0.1 μmol/ml) to release 5-oxoproline [3]. After evaporation, the fraction soluble in ethanol was then hydrolysed with 2 M HCl for 2 h at 100 °C and the presence of glutamic acid shown by dansylation and electrophoresis at pH 4.35 [4].

Specific assay of GSH. Protein-free extracts (0.1 ml) were neutralised with phosphate buffer (pH 7.4; 0.5 M; 75 μl), the mixture stirred 1 min with *N*-[^{14}C]ethylmaleimide (Radiochemical Centre, Amersham; 17.5 μmol/ml; 1.07 Ci/mol; 25 μl) then diluted with water (75 μl) and acidified with 20 % trichloroacetic acid (25 μl). A solution of the unlabeled *N*-ethylmaleimide adduct of GSH (10 μmol/ml) containing excess imide (1 μmol/ml) in 2 % trichloroacetic acid was added as carrier and the mixture frozen until required. Mixtures were spotted onto Whatman No. 3 paper using an automatic applicator which delivered about 90 μl. The paper was subjected to electrophoresis in pyridine/acetic acid buffer at pH 3.6 and 1.7 kV for 4 h. Radioactivity was located in the ninhydrin-positive spots due to the carrier. The spots were cut out, covered with scintillator (POPOP; 1 mg/ml; 4 ml) and counted in a Packard liquid scintillation spectrometer. Standard solutions of GSH treated in this way gave the results shown in Fig. 1.

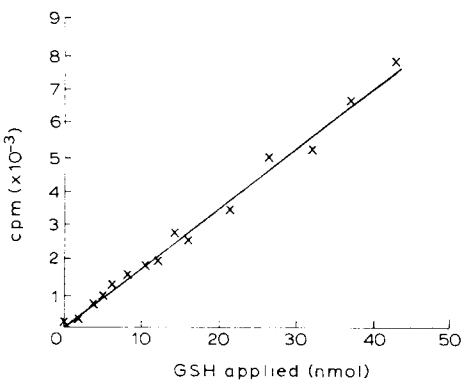

Fig. 1. Assay of GSH with *N*-[^{14}C]ethylmaleimide after electrophoresis. See Materials and Methods.

Other assays. Thiol was estimated in protein-free extracts with 5,5'-dithiobis-(2-nitrobenzoic acid) [5, 6]. Disulphide was assayed from the increase in thiol resulting after incubation with glutathione reductase and $NADPH_2$ [7].

Protein was assayed by a modification of the Biuret method [8].

RESULTS

The non-protein thiol of rat liver mitochondria consists chiefly of GSH. This was previously suggested [1] and has now been confirmed by amino acid analysis (see Materials and Methods). Assays of this GSH have been made directly by counting radioactivity of the *N*-[^{14}C]ethylmaleimide derivative and indirectly by determining the total non-protein thiol. The latter method gives greater precision since GSH is the chief non-protein thiol [1] but it requires stronger mitochondrial suspensions because of the low concentration present.

Export of mitochondrial GSH

These methods show that when mitochondria are incubated in suspension in the medium they lose some of their endogenous GSH by outward diffusion. The loss at 30 °C is not significantly affected by various added Krebs-cycle acids but it is considerably increased by the presence of phosphate. The amounts of phosphate required vary with the strength of the suspension presumably due to non-specific binding by the anion. Using 7 mg protein/ml (Table I) $3.3 \cdot 10^{-3}$ M phosphate is sufficient whereas at 40 mg/ml (Table II) $2.5 \cdot 10^{-2}$ M is necessary. Only a minimal amount of swelling occurs either under the first conditions as shown by turbidity measurements (Table I) or under the second as shown by measurements of water entry [9]. Some Krebs-cycle acids (e.g. pyruvate and malate) have no significant influence on this

TABLE I

EFFECT OF VARIOUS ANIONS ON SWELLING AND GSH CONTENT OF MITOCHONDRIAL PELLETS AFTER INCUBATION

Mitochondrial suspensions (approx. 7 mg protein/ml) were incubated for 10 min in the medium containing the potassium salts of the anions shown ($3.3 \cdot 10^{-3}$ M). GSH assays were performed as described in Fig. 1 and Materials and Methods. Absorbances were determined before and after incubation with samples of the suspensions of volume (approx. 0.1 ml) sufficient to give initial absorbances of about 1.0 when diluted to 3 ml with ice-cold medium. GSH values are given as a percentage of the values found prior to incubation. The superscript shows number of estimations; the value in brackets shows ±S.D. The absorbances are measured at 720 nm.

Anion	Temperature of incubation (°C)	GSH (%)	Fall in absorbance (%)
Nil	0	80^{11} (9)	—
Nil	37	60^{3} (11)	—
Nil	30	65^{11}(11)	Nil^{5} (8)
Citrate	30	57^{3} (19)	12^{5} (16)
Ketoglutarate	30	62^{4} (9)	5^{5} (10)
Succinate	30	68^{3} (12)	8^{5} (13)
Phosphate	30	24^{3} (11)	16^{4} (9)

TABLE II

EFFECT OF SOME ANIONS AND ELECTRON TRANSPORT INHIBITORS ON THE PHOSPHATE-INDUCED ESCAPE OF NON-PROTEIN THIOL FROM PELLETS

The anions (sodium salts) or inhibitors were added in 0.05 ml to 1 ml of mitochondrial suspension (40–50 mg protein/ml) containing phosphate ($2.5 \cdot 10^{-2}$ M) to give the final concentrations shown. After incubation at 30 °C, non-protein thiol level was assayed on the protein-free pellet extract. The results are expressed as a percentage of the value before incubation.

Addition	Amount ($\times 10^{-2}$ M)	Non-protein thiol in pellet (%)
Nil		44^{6} (12)
Pyruvate	2.5	50^{4} (12)
Citrate	2.5	65^{4} (12)
Isocitrate	2.5	69^{4} (4)
Ketoglutarate	2.5	63^{4} (2)
Succinate	2.5	69^{4} (3)
Fumarate	2.5	59^{4} (10)
Malate	2.5	55^{4} (6)
Antimycin A	25 μg/ml	75^{3} (6)
Dicoumarol	50 μg/ml	59^{2} (2)
EDTA	$2.5 \cdot 10^{-4}$ M	72^{4} (6)
Rotenone	25 μg/ml	72^{3} (8)

effect of phosphate but succinate and isocitrate reduce or prevent it in common with EDTA and respiratory inhibitors (Table II).

There appears to be little entry of exogenous GSH into mitochondria with or without phosphate as shown by light-scattering in isosmolar solutions of the ammonium salt of GSH or by comparison with [^{14}C]sucrose penetration into the pellet [10].

Oxidation of mitochondrial GSH

During short term incubations there is no significant net oxidation of the GSH of mitochondrial suspensions unless it is provoked by the addition of a thiol oxidant such as ferricyanide, azoformate [11] or *t*-butyl hydroperoxide [12]. At high concentrations of one of these oxidants the oxidation then occurs without the need for further additions. At low concentrations, however, (5 nmol/mg protein) the oxidant is only effective after the further addition of phosphate or oxaloacetate to the suspension of intact mitochondria or after lysis with deoxycholate (Fig. 2). If phosphate is added, most of the GSSG produced by the oxidation is found in the suspension medium whereas in the case of oxaloacetate, it remains within the mitochondrial pellet (Table III).

The oxidation of GSH by *t*-butyl hydroperoxide in the presence of either phosphate or oxaloacetate is inhibited by EDTA and rotenone but not by cyanide and azide (Table IV). The amount of GSSG assayed after treatment with these oxidants is appreciably less than the amount of GSH lost. Part of the deficit consists of mixed disulphides with protein SH groups as shown by reduction of the washed protein precipitate with dithiothreitol and specific assay of the released GSH (Table V). Small amounts of mixed disulphide are present before the oxidation but afterwards there is a 3–4-fold increase. This increase is prevented by succinate which also prevents the

formation of GSSG (see later). Mixed disulphides assayed do not account fully for the GSH lost and not recovered as GSSG but it is possible that some of them are resistant to reduction by dithiothreitol under the experimental conditions chosen.

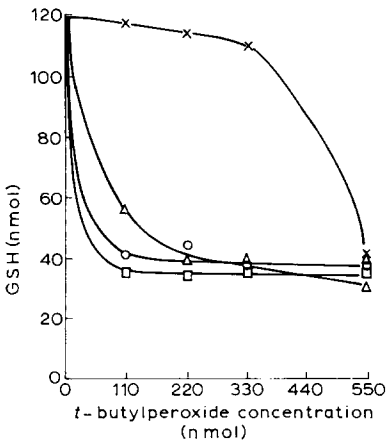

Fig. 2. The oxidation of mitochondrial non-protein thiol by *t*-butyl hydroperoxide in the presence of phosphate, oxaloacetate or deoxycholate. To the suspension (1 ml) at 0 °C was added nicotinamide (0.05 M) and as indicated 0.05 ml of solutions to give the final concentrations shown: water (×), phosphate ($2.5 \cdot 10^{-2}$ M) (△), oxaloacetate ($2.5 \cdot 10^{-2}$ M) (□), deoxycholate (0.66 %) (○). After shaking, butyl hydroperoxide was added in ethanol (25 μl). After incubation at 30 °C non-protein thiol was assayed on the protein-free extracts.

TABLE III

THE DISTRIBUTION OF NON-PROTEIN THIOL (NPSH) AND NON-PROTEIN DISULPHIDE (NPSS) BETWEEN PELLET AND MEDIUM AFTER TREATMENT WITH OXIDANTS IN THE PRESENCE OF PHOSPHATE OR OXALOACETATE

Mitochondrial suspensions (1 ml) were mixed at 0 °C with phosphate or oxaloacetate ($2.5 \cdot 10^{-2}$ M) and the oxidant (250 nmol) added. Methyl phenyl azoformate and *t*-butyl hydroperoxide were added in ethanol (10 μl) and ferricyanide in water. After incubation at 30 °C, the protein-free extracts from pellet and medium were assayed for non-protein thiol and non-protein disulphide. Values given are as percentage of the total (NPSH + 2 NPSS) found in the untreated and unincubated suspension.

Addition	Pellet		Medium	
	NPSH	NPSS	NPSH	NPSS
Butyl hydroperoxide	69^{4} (12)	3^{4} (3)	13^{4} (2)	3^{4} (3)
+phosphate	11^{4} (4)	8^{4} (6)	8^{4} (8)	22^{4} (6)
+oxaloacetate	13^{4} (8)	34^{4} (5)	5^{4} (3)	5^{4} (2)
Methyl phenyl azoformate	60	13	2	14
+phosphate	15	20	2	34
Ferricyanide	58^{2} (2)	5^{2} (4)	5^{2} (3)	4^{2} (1)
+phosphate	14^{2} (13)	10^{2} (8)	20^{2} (8)	12^{2} (10)

TABLE IV

NON-PROTEIN THIOL (NPSH) AND NON-PROTEIN DISULPHIDE (NPSS) CONTENT OF MITOCHONDRIAL SUSPENSIONS INCUBATED WITH *t*-BUTYL HYDROPEROXIDE AND VARIOUS ELECTRON TRANSPORT INHIBITORS

The suspension (1 ml) was mixed at 0 °C with 0.1 ml of solution containing phosphate or oxaloacetate (final concentrations: $2.5 \cdot 10^{-2}$ M) and the inhibitor added. The mixture was then incubated at 30 °C with *t*-butyl hydroperoxide (250 nmol in 10 μl ethanol) and assays were performed on the protein-free extracts. Values are given as the percentage of the total (NPSH+2 NPSS) found in untreated and unincubated suspensions.

Inhibitor	Amount	Phosphate		Oxaloacetate	
		NPSH	NPSS	NPSH	NPSS
Nil		19^{4} (8)	30^{4} (6)	25^{4} (9)	31^{4} (12)
Antimycin	25 μg/ml	38^{4} (15)	28^{4} (12)	21^{3} (4)	37^{3} (7)
Azide	$5 \cdot 10^{-2}$ M	19	33	14	25
Cyanide	$5 \cdot 10^{-5}$ M	15^{2} (1)	28^{2} (3)	18	24
Dicoumarol	25 μg/ml	36^{2} (3)	33^{2} (4)	36^{2} (3)	33^{2} (4)
EDTA	$2.5 \cdot 10^{-4}$ M	63^{2} (2)	16^{2} (5)	—	—
Rotenone	50 μg/ml	66^{4} (9)	9^{4} (3)	51^{2} (14)	18

TABLE V

MIXED DISULPHIDE FORMATION IN MITOCHONDRIAL SUSPENSIONS TREATED WITH OXIDANTS

Mitochondrial suspensions (1 ml) were incubated at 30 °C with or without oxidant (1 μmol). The separated pellet was suspended in water and proteins precipitated with trichloroacetic acid. The precipitate, washed by resuspending first in an *N*-ethylmaleimide solution (1 mM; 10 ml) then in water (10 ml), was dissolved in phosphate buffer (pH 7.4; 0.5 M; 0.1 ml) by stirring with solid urea (about 0.25 g). After incubation for 1 h at 30 °C with dithiothreitol (40 μmol/ml; 0.1 ml), sodium arsenite (160 μmol/ml; 0.05 ml) was added, the volume made up to 1.4 ml and the proteins precipitated with trichloroacetic acid (10 %; 0.1 ml). GSH in the extracts was assayed as described in Fig. 1.

Addition to medium*	GSH assayed (nmol)
Nil	7^{4} (4)
t-Butyl hydroperoxide	26^{3} (6)
t-Butyl hydroperoxide + succinate (0.025 M)	11^{3} (5)
Methylazoformate	25^{3} (7)

* The medium contains phosphate ($2.5 \cdot 10^{-2}$ M).

Reduction of mitochondrial GSSG

Depletion of GSH by oxidation is prevented when certain Krebs-cycle acids are added to mitochondrial suspensions containing *t*-butyl hydroperoxide and phosphate or oxaloacetate. Pyruvate, oxaloacetate and ketoglutarate do not possess this property but the other Krebs-cycle acids (citrate, isocitrate, succinate and fumarate) are effective (Table VI). These acids are clearly functioning as disulphide reductants. This has

been shown (e.g. with isocitrate) by recovery of GSH when Krebs-cycle acid is added after preliminary depletion with oxidant. Moreover, the depletion-preventing Krebs-cycle acids can promote the reduction of exogenous GSSG by mitochondria after lysis with or without pretreatment with butyl hydroperoxide. Since $NADH_2$ is also a good

TABLE VI

THE OXIDATION OF MITOCHONDRIAL NON-PROTEIN THIOL BY *t*-BUTYL HYDROPEROXIDE IN THE PRESENCE OF KREBS-CYCLE ACIDS

The suspension (1 ml) was mixed at 0 °C with the sodium salt of the acid plus phosphate or oxaloacetate (all at $2.5 \cdot 10^{-3}$ M) or deoxycholate (0.67 %). *t*-Butyl hydroperoxide (250 nmol in 10 µl ethanol) was then added and the mixture incubated at 30 °C. Assays were performed on the protein-free extracts. Figures are the percentage of non-protein thiol found in the unincubated suspension.

Krebs-cycle acid	Non-protein thiol found in the presence of:		
	Phosphate	Oxaloacetate	Deoxycholate
Nil	19^4 (8)	8	18
Pyruvate	17^3 (7)	8	18
Citrate	73^3 (7)	71	91
Isocitrate	77^3 (13)	63	72
Ketoglutarate	23^3 (4)	8	18
Succinate	78^3 (7)	100	9
Fumarate	63^3 (8)	80	9
Malate	46^3 (10)	80	9
Oxaloacetate	20^3 (2)	—	—

TABLE VII

THE REDUCTION OF EXOGENOUS GSSG BY KREBS-CYCLE ACIDS IN LYSED MITOCHONDRIA

Mitochondrial suspensions (1 ml) were added to GSSG (625 nmol), Krebs-cycle acid (1250 nmol as the sodium salt), nicotinamide (50 µmol), deoxcycholate (3.3 mg) and *t*-butyl hydroperoxide (550 nmol; added last) in a final volume of 1.4 ml. After incubation at 30 °C non-protein thiol was assayed on protein-free extracts. Values are expressed as the non-protein thiol formed in excess of the amount without added Krebs-cycle acid. Amounts formed without added Krebs-cycle acid: oxidant present, 53^3 (50); oxidant absent, 180^3 (90).

Krebs-cycle acid	Non-protein thiol formed (nmol)	
	Oxidant absent	Oxidant present
Pyruvate	57^2 (50)	8^2 (8)
Citrate	—	280^2 (60)
Isocitrate	165^3 (20)	270^2 (40)
Ketoglutarate	29^2 (1)	-16^3 (18)
Succinate	180^3 (50)	177^3 (50)
Fumarate	106^2 (30)	134^2 (50)
Malate	133^2 (65)	120^2 (60)
Oxaloacetate	-14^2 (14)	-6^2 (6)
$NADH_2$*	382^2 (40)	496^2(110)

* Variation of mitochondrial volume and of incubation time downwards showed a linear variation of non-protein thiol formed.

TABLE VIII

THE REDUCTION OF EXOGENOUS GSSG BY SUBSTRATES ADDED TO MITOCHONDRIAL SUSPENSIONS

Mitochondrial suspension (1 ml) was added to GSSG (600 nmol), nicotinamide (50 μmol) and the additions indicated. After incubation at 30 °C, non-protein thiol was assayed on the protein-free extracts. Means are given for two assays.

Addition	Non protein thiol formed (nmol)
Nil	5 (12)
NADP (25 nmol)	9 (5)
Isocitrate* (3 μmol)	2 (8)
Isocitrate* + NADP	76 (6)
$NADH_2$ (3 μmol)	86 (6)
Phosphate (8 μmol)	29 (1)
Phosphate less GSSG	6 (2)

* No significant rate of reduction is obtained when this is replaced by other Krebs-cycle acids.

lysate reductant, these Krebs-cycle acids may act by virtue of their capacity to reduce NAD (Table VII). Substantial reduction of exogenous GSSG by lysed mitochondria also occurs even without added Krebs-cycle acids presumably due to the presence of endogenous substrates. Intact mitochondrial suspensions do not have this capacity [13] but they acquire it if phosphate is added to the medium (Table VIII) presumably by allowing the entry of otherwise impermeable disulphide.

Mitochondria possess glutathione reductase activity [14] but at least some of this is accessible to extra-mitochondrial substrates. Thus, in the absence of phosphate, exogenous GSSG is reduced by nicotinamide-protected $NADH_2$. High rates of reduction are also attained with NADP plus isocitrate, a substrate for which there is a known extra-mitochondrial (i.e. peroxysomal) NADP-specific dehydrogenase [15] (Table VIII).

DISCUSSION

The increase by phosphate of the rate of spontaneous diffusion of GSH from incubated mitochondria is prevented by respiratory inhibitors. Phosphate is a respiration-dependent swelling agent and as such it promotes the escape of various substances [16] including coenzyme A [17] from mitochondria. Although the escape of GSH does not correlate with swelling, it nevertheless is probably due to an increase in mitochondrial permeability. Thus succinate, and to a lesser extent other substrates which inhibit GSH escape, inhibits phosphate swelling in dense suspensions [9]. An alternative is that mitochondria are permeable to free GSH but actually contain an unstable derivative of it which is phosphorylysed in situ and hydrolysed during the acid extraction procedure. This alternative is not, however, in accordance with the findings that (i) GSH cannot freely enter mitochondria, (ii) extraction of acetone powders from whole mitochondria with water gives only free GSH, (iii) only negligible amounts of thioesters, the most likely form of such a derivative, are present in mitochondria [18].

The effect of phosphate on the oxidation of mitochondrial GSH by small amounts of thiol oxidant may also be due to an effect on GSH permeability. The oxidation would occur between the oxidant and the GSH which had escaped, an explanation consistent with the largely extra-mitochondrial location of the GSSG formed, the effect of respiratory inhibitors and the fact that mitochondrial lysis obviates the need for phosphate. Prevention of oxidation by Krebs-cycle acids would then be attributable to the capacity (established with exogenous disulphide) for GSSG to re-enter the phosphate-treated mitochondria. When the oxidation of GSH by butyl peroxide is promoted by oxaloacetate instead of phosphate, the GSSG produced is largely confined within the mitochondria. This is in contrast to the situation in the cytosol of rat liver [12] and other tissues [19, 20] from which GSSG can escape by diffusion across the intact cell membrane. This intra-mitochondrial oxidation of GSH is probably mediated by glutathione peroxidase since butyl peroxide is known to be a substrate for this enzyme [27]. Opposing the oxidation there will be the concurrent reconversion of GSSG to GSH by endogenous reducing agents known to be present because spontaneous reduction of exogenous GSSG by lysates occurs. It seems possible that oxaloacetate, a known inhibitor of succinate dehydrogenase functions by blocking this reverse reaction [21]. The Krebs-cycle acids which counteract the effect of thiol oxidants in intact mitochondria presumably function either by reducing NAD to $NADH_2$ or, in the case of succinate, by sparing the latter by preferential interaction with the electron transport chain [22].

Glutathione reductase activity has previously been found in mitochondria where it has been reported to be located chiefly within the matrix [14]. However, part of it must also occur outside since intact mitochondria can catalyse the reduction of exogenous GSSG with $NADPH_2$. The mitochondrial enzyme is specific for $NADPH_2$ and it is therefore not clear if it can catalyse the endogenous reduction in mitochondria pretreated with butyl peroxide and oxaloacetate. Eldjarn and Bremer [13] attributed their observed reduction of certain disulphides by Krebs-cycle acids and mitochondria to thiol-disulphide exchange with lipoate reduced during α-keto acid oxidation. This explanation has been questioned [23] but subsequently restated with additional evidence as the mechanism for reduction of cystamine [28]. It is certainly not applicable to the reduction of GSSG, however, because it is precisely α-ketoglutarate, pyruvate and of course oxaloacetate which are ineffective as reductants. Their incapacity, also apparent in lysates where it is not remedied by adding coenzyme A, may be due to an inhibitory reaction of GSSG with apoenzyme SH groups to form a mixed disulphide as established in other systems [24]. Such inhibition may be a factor in mitochondrial metabolism because mixed disulphides are already present to some extent [25] and they form from endogenous GSH during in vitro oxidation.

Since mitochondria lack catalase but contain glutathione peroxidase [26] and also do not readily eliminate their GSSG, they may be especially dependent on oxidation of GSH to remove endogenous peroxides and thus on an intact reducing capaccity to prevent the inhibitory effects of the GSSG formed.

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