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The Disulphide-Reducing Capacity of Liver Mitochondria

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1. Isolated mammalian mitochondria have been shown to reduce a number of small molecular disulphides in the presence of citric acid cycle intermediates.
2. The physiologically occurring CSSC, GSSG and homocystine are not reduced.
3. The α-keto acids of the citric acid cycle are the most efficient reducing substrates.
4. The reduction is stimulated by AMP, ADP, magnesium ions and by oxygen.
5. On the basis of these results it is suggested that the disulphides are reduced *via* exchange reactions with the thioctic acid.

Glutathione reductase is the only well characterized disulphide-reducing system of mammalian cells. This system requires $NADPH_2$ as coenzyme and is only active with GSSG as substrate. Even mixed disulphides containing glutathione are inactive as substrates[1]. Upon disintegration of rat liver cells, the glutathione reductase is found in the particle-free supernatant fraction[2].

Most mammalian cells demonstrate the ability to reduce a variety of low molecular disulphides[3]. This reduction may be brought about by the glutathione reductase *via* spontaneous exchange reactions between glutathione and the disulphide in question[1].

The reduction of homocystine[4] and of insuline disulphide groups[5] observed in rat liver extracts, are also ultimately caused by glutathione and glutathione reductase.

Romano and Nickerson[6] have described a GSH-independent but $NADH_2$-dependent cystine reductase in extracts of hog liver. A similar cystine-reductase activity has been found in acetone powder preparations of liver[7]. These systems have a relatively low activity, but closer characterization is lacking.

In the present investigation we report on a disulphide reducing system present in mammalian mitochondria. Isolated mitochondria rapidly reduce a number

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of low molecular disulphides, provided substrates for the citric acid cycle are present. This reduction does not require glutathione as a cofactor, and data are presented which indicate that the disulphides are reduced at the thioctic acid level of the α-keto acid oxidation.

MATERIALS AND METHODS

Cystamine and cystamine derivatives were obtained and prepared as previously described[3]. All other chemicals used were commercial products of highest purity.

Mithochondria were prepared from grown rats of mixed breed. Both female and male rats were used. The rats were sacrificed by a blow to the neck and bled from the neck vessels. The isolated organs were cooled on crushed ice, weighed and homogenized in ice cold 10 % sucrose in a teflon-glass Potter-Elvehjem homogenizer. After removal of cell debris and nuclei by centrifugation at $800 \times g$ for five min the mitochondria were isolated by centrifugation at $20\,000 \times g$ for 15 min, washed once in the same volume of homogenizing medium, and resuspended in 10 % sucrose in a concentration corresponding to about 1 g of fresh tissue per ml.

Incubations were performed at 30°C with air as the gas phase. Where not otherwise stated, the following additions were used (expressed as μeqv. per 3 ml): glycylglycine buffer of pH 7.6, 40; potassium phosphate, 40; AMP, 10; magnesium chloride, 20. The appropriate disulphide was added as stated under the individual experiments. All additions were added as 0.1 M solutions. Mitochondria were added in amounts corresponding to approximately one gram of liver tissue (1 ml mitochondria suspension in sucrose) per 3 ml of incubation mixture. The volume of the incubation mixture was adjusted to 3 ml with 0.15 M potassium chloride.

Sulphhydryl groups were determined by titration with 1 mM iodine after precipitation of the proteins with trichloroacetic acid. Soluble starch was used as indicator.

To check the titration method, incubations were performed with ^{35}S-labelled cystamine. After acidification of the reaction mixture cystamine and cysteamine were separated by electrophoresis as previously described[8]. This method also would have revealed a number of possible cystamine metabolites such as hypotaurine, taurine and sulphate. The only metabolite found was cysteamine, and in amounts corresponding closely to the iodine titration values.

Some variations in the amounts of SH formed could be observed from one experiment to the other. The reason for this is not entirely clear, but small variations in pH could in part account for these differences.

Preincubation experiments also showed that a rapid inactivation of the mitochondria took place in the presence of disulphides and in the absence of a reducing substrate. Furthermore, it must be stressed that the reported iodine titration data represent minimum values for the disulphide reduction, since cysteamine and cysteamine derivatives undergo rapid spontaneous oxidation by atmospheric oxygen.

RESULTS

Fig. 1 A shows the time course for the reduction of cystamine and for glutathione by rat liver mitochondria with α-ketoglutarate as the substrate. The maximum reduction of the disulphide was usually obtained after 30 to 40 min. In the experiments presented in Fig. 1, 35 and 50 % of the disulphide was reduced. With higher concentrations of mitochondria (Fig. 2), a complete reduction was generally obtained. It is evident that oxidized glutathione is not reduced to any significant extent by rat liver mitochondria (see also Table 2). No reduction of cystamine was observed when α-ketoglutarate was omitted. Anaerobiosis also impaired the efficiency of the reduction (Fig. 1 B) in spite of the fact that the spontaneous oxidation of cysteamine is slowed down by this treatment.

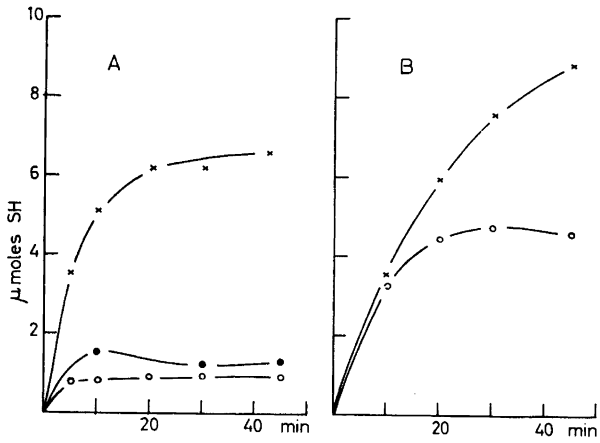

Fig. 1. Time curves for the reduction of cystamine (3 mM) and glutathione (3 mM). Mitochondria from 1 g of rat liver was incubated with the disulphides in the presence of α-ketoglutarate (6.7 mM). A: x, cystamine; o, glutathione; ●, cystamine without α-ketoglutarate. B: x, cystamine in the presence of oxygen; o, cystamine in the absence of oxygen.

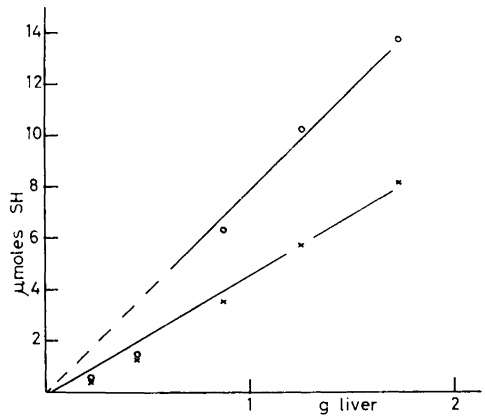

Fig. 2. The effect of mitochondria concentration on the reduction velocity of cystamine (3 mM). Mitochondria from the given amounts of rat liver tissue was incubated for 10 min (x) and 20 min (o) with α-ketoglutarate (6.7 mM) as substrate.

Fig. 2 demonstrates that except for the lowest concentrations the reduction velocity under the described experimental conditions is proportinal to the mitochondrial concentration for at least 20 min. At low mitochondrial concentrations a more rapid inactivation of the reduction was observed.

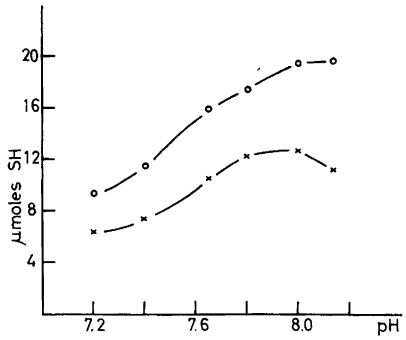

Fig. 3. The effect of pH on the reduction velocity of cystamine (3 mM). Mitochondria from 1 g of rat liver was incubated for 10 min (x) and 30 min (o) with α-ketoglutarate (6.7 mM) as substrate.

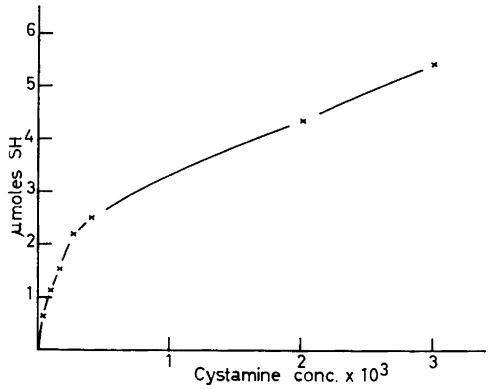

Fig. 4. The effect of cystamine concentration on the reduction velocity. Mitochondria from 1 g of rat liver was incubated for 10 min in the presence of α-ketoglutarate (6.7 mM) with the given cystamine concentrations in equilibrium with a glutathione containing "SS buffer system" of total SS concentration 10 mM.

Fig. 3 shows that an increase in pH up to about 7.8 causes a rapid rise in the reduction velocity. This effect must be even greater than here observed, since the increase in pH also is known to increase the spontaneous oxidation of the thiol formed. Increase of the pH above 8 did not give further rise in the reduction rate.

Fig. 4 shows the effect of cystamine concentration on the reduction velocity. In these experiments "disulphide buffer systems"[9] were used in order to obtain significant amounts of SH formation even with the 10^{-5} M concentrations of cystamine. These SS buffers consisted of equilibrium mixtures of varying amounts

Table 1. The effect of cofactors on the reduction of cystamine (3 mM) by rat liver mitochondria with α-ketoglutarate as substrate. The complete system contained (μeqv. per 3 ml.): glycylglycine buffer of pH 7.6, 40; potassium phosphate, 40; AMP, 10; magnesium chloride, 20.

Cofactor omitted	μmoles SH produced by mitochondria from 1 g of tissue. Incubation time		
	10 min	20 min	45 min
None	5.4	6.6	6.8
Phosphate	4.5	6.8	7.5
Mg++	4.6	4.8	4.7
AMP	3.8	3.8	4.1
Cystamine	0.7	0.7	0.8

of cystamine with oxidized glutathione and with a total SS concentration of 10 mM. It could be deduced from the relationship between concentration and reduction velocity that of the three disulphides present in these mixtures (GSSG, cystamine and their mixed disulphide) only cystamine was reduced by mitochondria, whereas the other two served as disulphide stores which replaced cystamine as it became reduced. The initial equilibrium concentration of cystamine as calculated from the known equilibrium constants[8] is plotted on the abscissa of Fig 4. In the course of the experiment these values became lowered with less than 10 % of the initial values owing to the buffering capacity of the system. It appears that with rising cystamine concentrations there is a rapid increase in the reduction velocity until in the mM range the reduction rate levels off.

Table 1 shows that no cofactor was found which had any significant effect on the initial reduction rate, but both magnesium and AMP (as well as ADP) were repeatedly observed to maintain the reduction for a longer period of time. The reduction was also found to proceed for a longer period of time under aerobic conditions (Fig. 1 B). The omission of phosphate had no significant effect. In other experiments, AMP was found on a molar basis to have approximately twice the effect of ADP. ATP had far less effect than AMP and ADP. The addition of NAD, NADP or nicotinamide had no effect on the reducing velocity. Of particular significance is the finding that NADP plus citrate had no effect on the reduction of GSSG, thus excluding contamination of the mitochondria with the extramitochondrial isocitric dehydrogenase and the soluble glutathione reductase of rat liver[2].

Table 2 demonstrates that cystamine, its N-alkyl derivatives and cystine diethylester are all efficiently reduced. NN′-diacetylcystamine and bis(hydroxyethyl) disulphide are reduced to some extent, whereas with GSSG, homocystine, tetrathionate and NNN′N′-cystamine tetraacetic acid no significant reduction was observed. Table 3 shows the substrate requirements for the reduction of cystamine by rat liver mitochondria. Choline effected no reduction, although

Table 2. The reduction of different disulphides by rat liver mitochondria with α-ketoglutarate as substrate (6.7 mM).

	Disulphide	μmoles SH produced by mitachondria from 1 g of tissue Incubation time 10 min	20 min	45 min
Exp. I	None	0.8		0.9
	Cystamine	7.8		12.9
	NN'-Tetraethylcystamine	6.9		10.0
	NN'-Tetramethylcystamine	6.4		11.4
	Bis(hydroxyethyl)disulphide	1.8		4.2
	GSSG	1.0		1.3
	NNN'N'-Cystaminetetraacetic acid	0.9		1.1
Exp. II	None	0.7	0.9	
	Cystamine	8.9	10.5	
	Cystine diethyl ester	6.4	5.4	
	NN'-Diacetylcystamine	2.4	3.3	
	Sodium tetrathionate	1.4	1.5	
	L-Homocystine	1.3	1.5	
	CSSC	1.2	1.3	

oxygen uptake showed that the compound was rapidly metabolized by the mitochondria. All the intermediates of the citric acid cycle could serve as reducing substrates. Although the differences were not great, it was constantly observed that the α-keto acids were superior to the other substrates. Citrate showed an intermediary efficiency, whereas malic and succinic acid were found to be the poorest substrates.

DISCUSSION

The mitochondrial system for disulphide reduction reported on in the present paper, most likely is different from the previously known disulphide reducing systems of mammalian tissues. Thus, the finding that GSSG is not reduced, even in the presence of citrate and NADP, strongly indicates that the glutathione reductase is not involved in the mitochondrial system. This conclusion is further supported by the finding that the latter enzyme is lacking in rat liver mitochondria[2].

Succinate belongs to the relatively poor substrates, and choline could not bring about any reduction at all (Table 3). These substrates are oxidized directly by flavoproteins and the electrons are fed directly into the electron transport chain[10]. The electrons required for the reduction of disulphides by mitochondria, therefore, most likely are not derived from this respiratory chain.

The participation of $NADH_2$ in the mitochondrial reduction cannot be exclu-

Table 3. Reducing substrate requirement for the reduction of cystamine by rat liver mitochondria. Substrate concentration 6.7 mM, cystamine concentration 3 mM.

Substrate	μmoles SH produced by mitochondria from 1 g of tissue Incubation time	
	10 min	20 min
None	2.1	2.1
Choline	2.4	2.2
Fumaric acid	7.2	10.8
Malic acid	7.6	11.2
Succinic acid	7.8	11.3
Citric acid	8.7	12.6
α-Ketoglutaric acid	9.7	15.5
Glutamic acid	10.8	15.2
Oxaloacetic acid	9.6	14.4
Pyruvic acid	10.2	15.6
Oxaloacetic + pyruvic acids	11.3	16.5

ded with certainty, since this coenzyme is known to be retained by mitochondria even after washing. However, the finding that malate, which is oxidized in the mitochondria by a $NADH_2$ dependent dehydrogenase, is a relatively poor substrate for the reduction, indicates that $NADH_2$ is not involved. It should be recalled that the slow acting "cystine reductase" of Romano and Nickerson[6] is $NADH_2$-dependent.

The α-keto acids of the citric acid cycle were repeatedly found to be the best reducing substrates. This fact points to the thioctic acid step of the α-keto-acid oxidation to be the immediate donor of electrons for the reduction of disulphides. Thioctic acid is known to be reduced at the aldehyde oxidation step, and it is claimed to be reoxidized by transfer of electrons to a protein disulphide group[11]. The added small molecular disulphides may become reduced by spontaneous interaction with these groups in the thiol form. If such a view is correct, higher concentrations of the foreign disulphide would be expected to bring about a "disulphide poisoning" of the α-keto acid oxidation of mitochondria. That this is indeed the case, will be shown in a forthcoming communication. The observed effect of pH on the reduction velocity is also in accordance with the proposed reduction mechanism. It is known that the ionized thiol is the reactive species in the exchange reactions between SH and SS compounds[8]. Since the pK_{SH} of the thiols in question most likely are above 8, an increase in pH will be expected to speed up the reaction rate.

The effects of AMP, of magnesium ions and of oxygen in maintaining the mitochondrial reduction for a longer period of time is difficult to explain. Most

probably these effects are connected with the preservation of the integrity of the mitochondrial structure and with the reformation of α-keto acids of the citric acid cycle.

The results shown in Table 2 demonstrate that rat liver mitochondria do not reduce the physiologically occurring GSSG, CSSC and homocystine to any significant extent. This can not be explained by differences in the oxidation-reduction potentials of these disulphides[8]. Most probably these compounds do not reach the reducing site in the mitochondria (the thioctic acid SH groups), and impermeability of the mitochondrial membrane appears to be the most likely explanation.

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