THE NATURE OF THE ENZYMATIC REDUCTION OF THE MIXED DISULFIDE OF COENZYME A AND GLUTATHIONE

Stellan ERIKSSON, Claes GUTHENBERG and Bengt MANNERVIK
Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, Stockholm, Sweden

Received 7 December 1973

1. Introduction

The biological reduction of mixed disulfides and related compounds in various organisms has been investigated, and different mechanisms of the reduction have been considered (cf. [1,2]). The mixed disulfide of coenzyme A and glutathione, CoASSG, is naturally occurring [3–5] and its concentration level in rat liver has been quantitatively estimated [6]. The enzymatic reduction of this compound has been considered to involve either GSH and an enzyme catalyzing thiol–disulfide interchange [7], or NADPH and a specific CoASSG reductase [8,9] or glutathione reductase [10]. Recent research in our laboratory concerns the enzyme systems involved in the reduction of disulfides and thiosulfate esters [1], and it was consequently of interest to study the alternative mechanisms proposed for the reduction of CoASSG, and attempt to evaluate their relative importance. Some of the results have been presented [1].

2. Materials and methods

Glutathione reductase (yeast 5 mg/ml) was obtained from Boehringer; CoA, GSH, GSSG, and NADPH from Sigma; Ampholine carrier ampholytes from LKB; Sephadex G-25 Fine from Pharmacia; CM-cellulose CM32 from Whatman. CoASSG [10] and S-sulfocysteine, $CySSO_3H$, [11] were synthesized according to published procedures.

Livers (20 g) from male Sprague–Dawley rats were homogenized in 4 vol. 0.25 M sucrose and centrifuged at 105 000 *g* for 60 min. The supernatant was passed through a Sephadex G-25 Fine column. The Sephadex gel (500 ml) was equilibrated with 10 mM phosphate buffer (pH 6.1), 1 mM EDTA for subsequent CM-cellulose chromatography. When the supernatant was prepared for isoelectric focusing, the gel was packed in water. CM-cellulose chromatography was performed on 2 × 10 cm glass columns equilibrated with the phosphate buffer described. After application of the sample, the column was washed with buffer until no protein left the column. A linear salt gradient: 250 ml 10 mM phosphate buffer (pH 6.1), 1 mM EDTA + 250 ml 50 mM phosphate buffer (pH 6.1), 1 mM EDTA, 0.2 M NaCl, was used to elute the adsorbed material.

Isoelectric focusing was carried out in a 110 ml column according to the instructions of the manufacturer (LKB). The ampholyte (1%) covered a pH range of 3–10 or 4–7 in a 0–50% sucrose gradient. The sample, containing 50–100 mg of protein, was introduced into the middle of the gradient to avoid denaturation by contact with the strong electrolytes.

Glutathione reductase was assayed spectrophotometrically at 340 nm by recording the NADPH oxidation at 30°C. The reaction system contained 1 mM GSSG, 0.1 mM NADPH, 0.17 M phosphate buffer (pH 7.6), 1 mM EDTA, and enzyme (10–50 μl) in a total volume of 1 ml. The system for measuring the reductase activity with CoASSG as disulfide substrate contained 0.15 mM CoASSG and 50 mM sodium phosphate (pH 5.5), instead of 1 mM GSSG and 0.17 M sodium phosphate (pH 7.6), respectively, and 50–100 μl enzyme.

The thioltransferase activity was measured by

 North-Holland Publishing Company – Amsterdam

coupling to glutathione reductase, using CoASSG or $CySSO_3H$ as acceptor substrates. The reaction system contained 0.5 mM GSH, 0.15 mM CoASSG (or 2.5 mM $CySSO_3H$), 0.1 mM NADPH, 0.4 unit of yeast glutathione reductase, 0.125 M phosphate buffer (pH 7.6), 1 mM EDTA, and enzyme (50–100 μl) in a final volume of 1.0 ml. The reaction was started by addition of the acceptor substrate 3 min after mixing the other component.

3. Results

Ion-exchange chromatography and isoelectric focusing were used to find out if enzymes active with CoASSG could be resolved from glutathione reductase and from the enzyme, thioltransferase, catalyzing thiol–disulfide interchange of low molecular weight substances. (Thioltransferase has recently been proposed as a more adequate group name than transhydrogenase for enzymes catalyzing thiol–disulfide interchange [12]). The thioltransferase, which is active with symmetrical and unsymmetrical disulfides and thiosulfate esters [1], catalyzes the following type of reactions:

$$RSSR' + GSH \rightleftharpoons RSSG + R'SH \qquad \text{eq. (1)}$$

and glutathione reductase acts according to:

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \qquad \text{eq. (2)}$$

Two reactions involving CoASSG have been studied in the present investigation:

$$CoASSG + GSH \rightleftharpoons GSSG + CoASH \qquad \text{eq. (3)}$$

and

$$CoASSG + NADPH + H^+ \rightarrow CoASH + GSH + NADP^+ \qquad \text{eq. (4)}$$

These reactions are expected to be catalyzed by a thioltransferase and a reductase, respectively.

Fig. 1 shows the results of fractionation of a post-microsomal rat liver supernatant on a CM-cellulose column. Thioltransferase activity was measured with $CySSO_3H$ and GSH or with CoASSG and GSH (eqs. 1 and 3), and the activity was in both cases distributed in two peaks, which coincided for the two substrates. Two peaks of thioltransferase activity are normally found under the experimental conditions

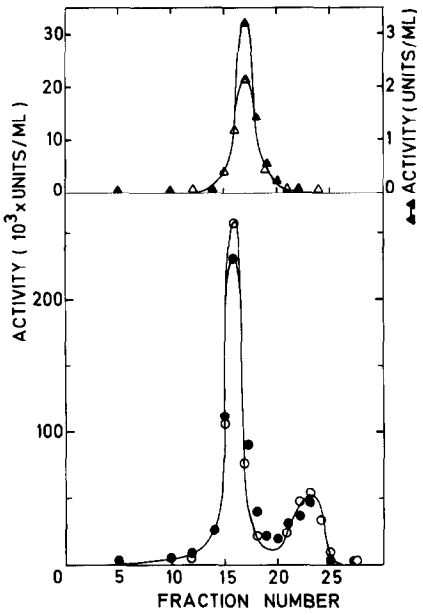

Fig. 1. CM-cellulose chromatography of rat liver supernatant. Symbols of activities: CoASSG–reductase (△-△-△) GSSG–reductase (▲-▲-▲) CoASSG–thioltransferase (○-○-○) $CySSO_3H$–thioltransferase (●-●-●). The formation of 1 μmole of product per min is used as a unit of enzymatic activity.

used, and the two peaks probably correspond to GSH-containing and GSH-deficient forms of the same enzyme [1]. The NADPH-dependent reductions of GSSG and CoASSG (eqs. 2 and 4) were determined, and the activity profiles were similar, having their peaks in identical positions in the chromatogram (fig. 1). The thioltransferase activity was about 10-fold higher than the NADPH-dependent reductase activity at the same (0.15 mM) CoASSG concentration in the assay systems.

To account quantitatively for the CoASSG-reducing activities found in the rat liver supernatant, the recovery of these activities were determined after the different purification steps and compared with the corresponding values obtained with glutathione reductase and with $CySSO_3H$ as a substrate for the thioltransferase. Table 1 shows that the recoveries obtained with CoASSG as a substrate were not different from the figures of the alternative substrates used in the determination of thioltransferase and reductase activities. The yields of thioltransferase and glutathione reductase are in the range normally

Table 1
Recovery of enzymatic activities in rat liver after different purification steps.

Fraction	Thioltransferase activity (%)[a]		Reductase activity (%)[b]	
	CoASSG	$CySSO_3H$	CoASSG	GSSG
Supernatant	100	100	100	100
Sephadex G-25	81	78	100	103
CM-cellulose	42	42	72	70
Not adsorbed on CM-cellulose	2	[c]	3	3

[a] The absolute values of the total activities obtained in a homogenate of 20 g rat liver were 32.1 and 22.9 μmoles/min for CoASSG and $CySSO_3H$, respectively.

[b] The absolute values in the homogenate were 5.4 and 97 μmoles/min for CoASSG and GSSG, respectively.

[c] An exact value was not obtainable due to the low activity. The best estimate was about 2. $CySSO_3H$ is reduced via CySSG and only the second partial reaction can be followed spectrophotometrically. The reaction of CoASSG and GSH is monitored directly and is therefore more easily determined.

obtained with the procedure used. No significant amount of the activities passed unadsorbed through the CM-cellulose column. Furthermore, no components in addition to those shown in fig. 1 have been observed when a rat liver supernatant was fractionated in the first step by DEAE-cellulose chromatography or isoelectric focusing. We therefore conclude the CoASSG reducing activities recorded in fig. 1 and table 1 account for essentially all the activity of a rat liver supernatant, which can be measured under the conditions used.

For further analysis of the activities separated by the CM-cellulose chromatography, fractions no. 15–22 were pooled and subjected to isoelectric focusing (fig. 2). By this technique the thioltransferase and the NADPH-dependent reductase activities were more clearly resolved, but neither in this case could an enzyme specific for CoASSG be identified. It was found that the quotient of the reductase activities obtained with GSSG and CoASSG was the same within the experimental error after CM-cellulose chromatography and isoelectric focusing. Similarly, the ratio of the thioltransferase activities obtained with $CySSO_3H$ and CoASSG was the same after the two purification steps.

It was also attempted to identify a specific NADPH-dependent reductase in a commercial glutathione reductase preparation from yeast (cf. [9]). Fig. 3 illustrates the results of an isoelectric focusing experiment undertaken to investigate whether activities with GSSG and CoASSG can be resolved. In this enzyme source a considerable activity was demonstrated with CoASSG, but the activity profiles obtained with GSSG and CoASSG as substrates coincided completely after the isoelectric focusing.

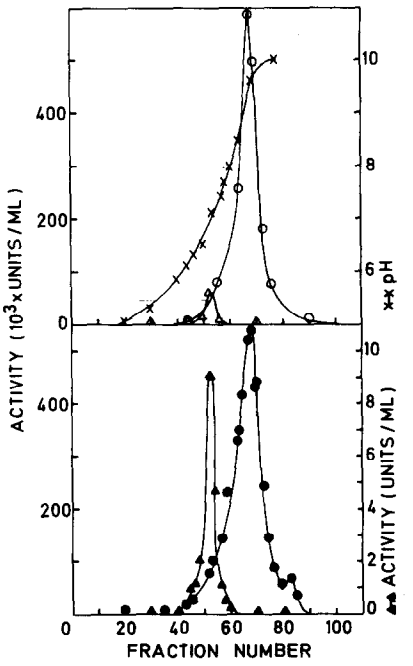

Fig. 2. Isoelectric focusing of rat liver supernatant after chromatography on CM-cellulose. Fractions 15–22 in fig. 1 were pooled, dialyzed overnight against 100 vol. 5 mM sodium phosphate (pH 7) and subjected to isoelectric focusing (pH 3–10) for 35 hr. Symbols: see fig. 1.

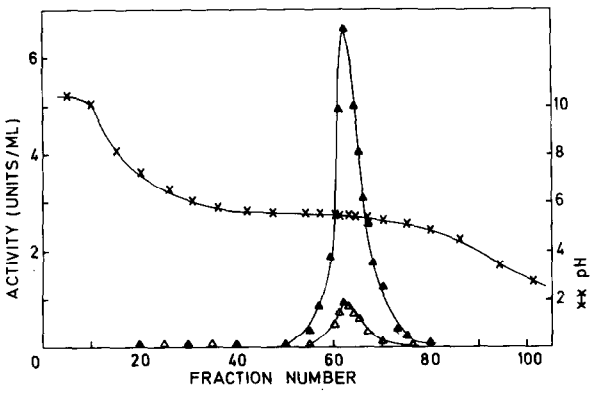

Fig. 3. Isoelectric focusing of commercial yeast glutathione reductase. A sample of 2.5 mg (spec. act. 120 U/mg) was dialyzed against 100 vol. 5 mM sodium phosphate (pH 7) overnight and focused for 40 hr (pH 4–7). Symbols: see fig. 1.

4. Discussion

Two mechanisms for the enzymatic reduction of CoASSG have been demonstrated. One involves a reductase according to eq. (4) and utilizes NADPH as the donor of reducing equivalents. The second alternative, which is catalyzed by a thioltransferase according to eq. (3), has GSH as the immediate reductant but is linked indirectly to NADPH via eq. (2). The reductase activity has been found in rat liver [6,8], yeast [9,10], and erythrocytes [10]; the thioltransferase in rat tissues [6,7] and bovine kidney [7]. Although both enzyme activities have been established beyond doubt, which is also shown by the present investigation, the relative importance of the two reactions in vivo has not been evaluated. Neither has the reductase activity been sufficiently well defined in relation to glutathione reductase.

The nature of the reductase activity has been studied by Ondarza et al. [8,9,13], who conclude that an enzyme distinct from glutathione reductase exists. However, some of their data, such as molecular weight, sensitivity to arsenite and 5-nitrofuran derivatives, are most easily explained by assuming that glutathione reductase effects the CoASSG reduction. The data in favor of the existence of two separate reductases are mainly (a) varying quotients between the GSSG and CoASSG reducing activities during purification or under varying test conditions and (b) different isoelectric points of the activities in yeast [9]. However, it is known that glutathione reductase itself responds in different ways to ionic strength or pH when the activity is measured with NADH instead of NADPH [14], and similar differences are feasible for the disulfide substrates GSSG and CoASSG. The separation of the two reductase activities from yeast by isoelectric focusing [9] is not consistent with the assumption of a single reductase, but Ondarza's results can be explained if a contaminating thioltransferase activity, eq. (3) were partially overlapping the glutathione reductase peak. Our own data on the yeast enzyme have not shown any separation of the two reductase activties by isoelectric focusing (fig. 3).

The present investigation confirms the existence in rat liver supernatants of a reductase and a thioltransferase activity using CoASSG as a substrate. To investigate the possibility that a CoASSG reductase activity distinct from glutathione reductase was present in rat liver, CM-cellulose chromatography was run (fig. 1) under conditions which resolve a number of GSH-linked enzymes [1]. However, neither this procedure nor isoelectric focusing of CM-cellulose fractions (fig. 2) or rat liver supernatant gave any evidence for the presence of more than one reductase. It is conceivable that the thioredoxin system in rat liver (cf. [15]) could also contribute to the reduction of CoASSG. However, most of the reductase activity of a rat liver supernatant is recovered in the glutathione reductase fraction after CM-cellulose chromatography, and the thioredoxin system is therefore probably of minor importance. This conclusion has support by the results of Tietze [16], which indicate that only a small part of the reducing activity towards low molecular weight disulfides is arsenite-sensitive and can be ascribed to the thioredoxin system. We therefore conclude that the main responsibility for the biological reduction of CoASSG resides with a thioltransferase and glutathione reductase.

The remaining question is to what extent the reduction of CoASSG takes place directly according to eq. (4) in comparison with the proportion reduced via thiol–disulfide interchange (eqs. 3 and 2). Under standard assay conditions the thioltransferase reaction has 6-fold higher activity than the reductase activity with CoASSG (cf. table 1, footnotes a and b).

However, the thioltransferase activity with 50 μM CoASSG and 5 mM GSH, which may approximate the situation in vivo (cf. [6]), was 1.70 μmoles/min per g rat liver, and the value of the corresponding reductase activity was only 0.065 μmole/min per g, when measured with 50 μM CoASSG and 0.1 mM NADPH in 0.125 M sodium phosphate (pH 7.6). (The reductase activity in the standard assay system (pH 5.5) is about 10-fold higher.) Furthermore, it is clear that the nonenzymatic thiol–disulfide interchange is negligible (0.008 μmole/min per ml) in comparison with the enzymatic reactions, and that the endogenous concentration of glutathione reductase is sufficient (approx. 5 units/g liver) not to be rate-limiting for the thioltransferase mechanism (eqs. 3 and 2). Consequently, we estimate, by consideration of probable in vivo concentrations of the reactants, that the thioltransferase-mediated CoASSG reduction is at least 20-fold more efficient than the direct reduction (eq. 4) in rat liver cytosol.

Acknowledgements

CoASSG was synthesized by Miss Kerstin Jacobsson. This work was supported by the Swedish Cancer Society.

References

[1] Mannervik, B. and Eriksson, S.A. (1974) in: Glutathione (Flohé, L., Benöhr, H.C., Sies, H., Waller, H.D. and Wendel, A., eds) pp. 121–132. Georg Thieme Verlag, Stuttgart.
[2] Jocelyn, P.C. (1972) Biochemistry of the SH Group, pp. 196–198. Academic Press, London and New York.
[3] Stadtman, E.R. and Kornberg, A. (1953) J. Biol. Chem. 203, 47–54.
[4] Chang, S.H. and Wilken, D.R. (1965) J. Biol. Chem. 240, 3136–3139.
[5] Ondarza, R.N. (1965) Biochim. Biophys. Acta 107, 112–119.
[6] Dyar, R.E. and Wilken, D.R. (1972) Arch. Biochem. Biophys. 153, 619–626.
[7] Chang, S.H. and Wilken, D.R. (1966) J. Biol. Chem. 241, 4251–4260.
[8] Ondarza, R.N. and Martínez, J. (1966) Biochim. Biophys. Acta 113, 409–411.
[9] Ondarza, R.N., Abney, R. and López-Colombé, A.M. (1969) Biochim. Biophys. Acta 191, 239–248.
[10] Eriksson, B. (1966) Acta Chem. Scand. 20, 1178–1179.
[11] Segel, I.H. and Johnson, M.J. (1963) Anal. Biochem. 5, 330–337.
[12] Askelöf, P., Axelsson, K., Eriksson, S. and Mannervik, B. (1974) FEBS Letters 38, 263–267.
[13] Ondarza, R.N. and Abney, R. (1970) FEBS Letters 7, 227–230.
[14] Scott, E.M., Duncan, I.W. and Ekstrand, V. (1963) J. Biol. Chem. 238, 3928–3933.
[15] Larsson, A. (1973) Eur. J. Biochem. 35, 346–349.
[16] Tietze, F. (1970) Biochim. Biophys. Acta 220, 449–462.