THE REDUCTION OF THE L-CYSTEINE-GLUTATHIONE MIXED DISULFIDE IN RAT LIVER. INVOLVEMENT OF AN ENZYME CATALYZING THIOL-DISULFIDE INTERCHANGE

Stellan A.ERIKSSON and Bengt MANNERVIK*

Department of Biochemistry, University of Stockholm, Stockholm, Sweden

Received 19 January 1970

1. Introduction

The naturally occurring mixed disulfide of cysteine and glutathione (CySSG)** can readily be synthesized in large quantities, free from the corresponding symmetrical disulfides, cysteine (CySSCy) and glutathione disulfide (GSSG) [1]. This has made it possible to study the enzymatic reduction of this compound, which, in addition, may have relevance to the biological reduction of cystine. It has been claimed that similar reductions require only GSH and glutathione reductase and that no catalytic effect on the thiol-disulfide interchange could be demonstrated [2, 3]. However, two highly purified enzymes catalyzing such exchange reactions of low molecular weight compounds have been described [4, 5].

We have reported earlier that we have evidence for an enzymatic reaction between glutathione (GSH) and CySSG [1] and have recently presented data on a partially purified enzyme [6]. The present communication demonstrates the presence of this enzyme in rat liver.

2. Materials and methods

CySSG was prepared as earlier described [1]. Stock solutions (10 mM) in water could be kept for several days in a refrigerator without decomposition. GSH (Sigma Chemical Co.) contained about 1% GSSG, which was usually reduced in the enzymatic assay (see below) before the addition of CySSG. GSSG was obtained from Boehringer & Soehne GmbH; NADPH and glutathione reductase from yeast (NAD(P)H: glutathione disulfide oxidoreductase, EC 1.6.4.2) from Sigma Chemical Co.; CM- and DEAE-cellulose from Whatman.

Glutathione reductase was assayed spectrophotometrically by recording the NADPH oxidation at 340 nm. The reaction system contained 0.4 mM GSSG, 0.1 mM NADPH, 0.1% human serum albumin, 0.15 M sodium phosphate buffer pH 7.0 (containing 0.8 mM EDTA), and enzyme in a total volume of 1 ml. A unit of enzyme is defined as the amount which catalyzes the oxidation of 1 μmole of NADPH per min at 30°C in the assay described above.

The GSH-dependent reduction of CySSG was tested in the system described above for GSSG reduction with the addition of 0.25 mM GSH and 0.20 unit of glutathione reductase from yeast. The reaction was started by the addition of CySSG (final concentration 0.25 mM) 3 min after mixing the other components. This period of time was sufficient for reduction of GSSG present in the commercial GSH preparation. Control experiments, in which the enzyme catalyzing thiol-disulfide interchange was omitted, were always carried out, to allow corrections for the enzyme independent reaction, which accounts for about 40% of the total activity under the conditions of the assay (fig. 1).

* Formerly Bengt Eriksson

** Abbreviations: CySH, cysteine; CySSCy, cystine; CySSG, CySH-GSH mixed disulfide; GSH, glutathione; GSSG, glutathione disulfide.

North-Holland Publishing Company – Amsterdam

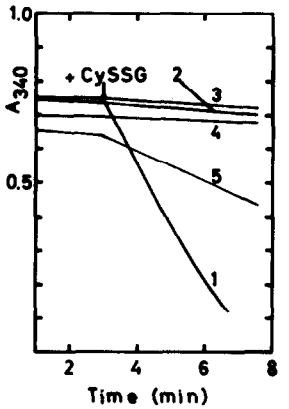

Fig. 1. GSH-dependent reduction of CySSG. Conditions, see text; 1. complete system, 2. CySSG, 3. GSH, 4. yeast glutathione reductase, 5. enzyme catalyzing thiol-disulfide interchange.

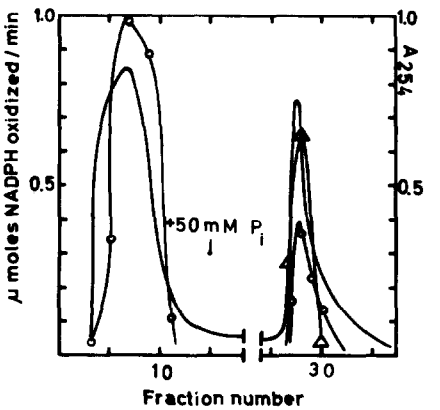

Fig. 2. Separation of enzyme catalyzing thiol-disulfide interchange and glutathione reductase on a 2 X 30 cm DEAE-cellulose column. A freeze-dried sample (18 ml, 960 mg protein) from a CM-cellulose chromatography was applied. The effuent was collected in 3 ml fractions. Activities: thiol-disulfide interchange (- o -), GSSG reduction X 10^{-2} (- Δ -); A_{254}(—).

3. Results

A rat liver homogenate, prepared in 10 mM sodium phosphate buffer pH 6.0 (containing 1 mM EDTA) and centrifuged at 15000 *g* for 30 min, could be applied directly onto a CM-cellulose CM 32 column equilibrated with the same buffer. The effluent was shown to stimulate the CySSG reduction slightly when assayed in the presence of glutathione reductase. This effect was due to GSH present in the effluent and was hardly detectable if additional GSH was present in the reaction mixture or if the effluent was too dilute. When the ion-exchange column was eluted with 0.12 M KCl in the starting buffer, two overlapping protein fractions were obtained, which both catalyzed the CySSG reduction in the presence of GSH, NADPH and glutathione reductase from yeast. These fractions could be clearly separated by chromatography of the effluent from the CM-cellulose column on a DEAE-cellulose DE 32 column equilibrated with 10 mM sodium phosphate buffer pH 7.4 (containing 1 mM EDTA and 1 mM mercaptoethanol) (fig. 2). One component was not absorbed under these conditions and catalyzed the thiol-disulfide exchange:

$$CySSG + GSH \rightleftharpoons CySH + GSSG$$

It was completely free from glutathione reductase, but could be assayed by coupling to the yeast glutathione reductase system (cf. Methods and fig. 1). The specific activity was 0.12 μmole.min^{-1}.mg^{-1}. A similar thiol-disulfide oxidoreductase activity was first described by Racker under the name of glutathione-homocystine transhydrogenase [7].

The second component was released by increasing the phosphate concentration to 50 mM. It contained the glutathione reductase of the liver, which in the assay system containing GSH catalyzed the CySSG reduction in the absence of added yeast glutathione reductase (specific activity: 0.15 μmole.min^{-1}.mg^{-1}; total activity: 7.4 μmoles.min^{-1}). The total activity of this fraction, when tested in the presence of yeast glutathione reductase, was 10.1 μmoles.min^{-1}, which is only half that of the enzyme catalyzing thiol-disulfide interchange (20.0 μmoles.min^{-1}). It also reduced CySSG without the addition of GSH to the reaction system, but this reaction was considerably slower (specific activity: 2.2.10^{-3} μmole.min^{-1}.mg^{-1}; total activity 0.11 μmole.min^{-1}). This activity is due to the NADPH-dependent reduction of CySSG by glutathione reductase, the rate of which is about 1% of the reduction of GSSG. Essentially the same rate was obtained with crystalline enzyme from yeast (cf. also [4]) or highly purified glutathione reductase from porcine erythro-

cytes (B.Mannervik, unpublished experiments). However, it is possible that glutathione reductase can also catalyze the thiol-disulfide interchange directly, but this cannot be demonstrated by the present assay method, as we monitor the reaction with the aid of this enzyme.

No other fractions catalyzing the reduction of CySSG have been eluted from the CM-cellulose even by increasing the pH to 8 at 0.15 M NaCl. Neither have any other components been detected by using the anion-exchanger DEAE-cellulose.

A more than 100-fold purification of the enzyme catalyzing the thio-disulfide interchange has been carried out. However, this preparation was not free from glutathione reductase. This is a serious objection, since it was found that the thiol-disulfide interchange activity measured in the system was not independent of the concentration of glutathione reductase (a finding reported also by Nagai and Black [5]). We are presently trying to develop an improved purification procedure including a DEAE-cellulose chromatography step to remove all glutathione reductase.

4. Discussion

The data in the present paper demonstrate that an enzyme catalyzing the reaction between GSH and CySSG is present in rat liver. The enzyme is localized in the cytosol. This is confirmed by using supernatants from high speed centrifugation of homogenates made in 0.25 M sucrose (free from microsomes and heavier subcellular particles). These supernatants have the same activity and chromatographic properties as the preparations in hypotonic phosphate buffer described in the present paper. We have also tested intact mitochondria, submitochondrial particles, and microsomes and found them to be without CySSG-reducing activity. This is consistent with the finding that GSSG and cystine are not reduced by mitochondria [8]. The enzyme catalyzing thiol-disulfide interchange can also catalyze the reaction between GSH and cystine, but this probably takes place in two steps:

$$CySSCy + GSH \rightarrow CySH + CySSG$$
$$CySSG + GSH \rightarrow CySH + GSSG$$

The first of these cannot be observed by the assay method used, which makes the kinetics of the reaction more complicated, as it is monitored by the NADPH-dependent reduction of GSSG. The observed activity is only 25% of that obtained with CySSG. It is tempting to suggest that the enzyme catalyzing thiol-disulfide interchange and glutathione reductase are responsible for the biological reduction of cystine. Similar enzymatic systems for the cystine reduction have previously been described in human liver [9], rat tissues [10], bovine kidney [4] and yeast [5]. The bovine thiol-transferase [4] has also been shown to have activity with CySSG.

In spite of the rapid nonenzymatic thiol-disulfide interchange, it is evident that the thiol-transferase exerts a catalytic effect on the reduction of CySSG, which in other respects is fully consistent with the glutathione reductase dependent mechanism advanced by Pihl et al. [2] for symmetrical disulfides. The direct reduction of CySSG by glutathione reductase is too slow to be of biological significance.

References

[1] B.Eriksson and S.A.Eriksson, Acta Chem. Scand. 21 (1967) 1304.
[2] A.Pihl, L.Eldjarn and J.Bremer, J. Biol. Chem. 227 (1957) 339.
[3] T.S.Woodin and I.H.Segel, Biochim. Biophys. Acta 167 (1968) 78.
[4] S.H.Chang and D.R.Wilken, J. Biol. Chem. 241 (1966) 4251.
[5] S.Nagai and S.Black, J. Biol. Chem. 243 (1968) 1942.
[6] S.A.Eriksson and B.Eriksson, Abstracts 6th Meeting, FEBS, Madrid 1969, p. 77.
[7] E.Racker, J. Biol. Chem. 217 (1955) 867.
[8] L.Eldjarn and J.Bremer, Acta Chem. Scand. 17 Suppl. (1963) S 59.
[9] A.D.Patrick, Biochem. J. 83 (1962) 248.
[10] P.L.Wendell, Biochim. Biophys. Acta 159 (1968) 179.