MECHANISM OF ACTION OF ENZYMES CATALYZING THIOL–DISULFIDE INTERCHANGE. THIOLTRANSFERASES RATHER THAN TRANSHYDROGENASES

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1. Introduction

Several enzymes catalyzing reduction of a disulfide (RSSR) with a thiol such a glutathione (GSH) have been discovered. In accordance with the nomenclature introduced by Racker [1] these enzymes have been referred to as 'transhydrogenases'. However, in contrast to the hydrogen transfer of pyridine–nucleotide transhydrogenases [2], it is likely that the essential feature of the mechanism is thiol transfer, as in the non-enzymatic thiol–disulfide interchange [3]. Thus, two alternatives exist: a transhydrogenase mechanism, which is a three-substrate reaction,

$$RSSR + 2\,GSH \rightleftharpoons GSSG + 2\,RSH, \quad (1)$$

and a thioltransferase mechanism, which involves reduction of RSSR via consecutive two-substrate reactions

$$RSSR + GSH \rightleftharpoons RSSG + RSH, \quad (2)$$

$$RSSG + GSH \rightleftharpoons GSSG + RSH. \quad (3)$$

In this paper evidence is put forward to support the second of the alternatives. Some of the data have been presented previously [4].

2. Materials

A partially purified rat liver enzyme [5] was used, which is active with a variety of low-molecular-weight disulfides and S-nucleophiles (cf. [6]). [^{35}S]GSH was obtained by Schwarz, and [^{35}S]GSSG was prepared by aerating an aqueous solution (pH 7) of [^{35}S]GSH.

3. Methods

Radioactive assay of thiol–disulfide interchange

The progress curves of labeled reactants formed from [^{35}S]GSH and 5,5′-dithiobis(2-nitrobenzoate) (ArSSAr) were obtained by analysis of aliquots of the reaction system. The system contained 20 μl 1 mM ArSSAr, 10 μl 1.3 mM [^{35}S]GSH (spec. act. 35 mCi/mmole), 50 μl enzyme (or H_2O when the non-enzymatic reaction was studied), and 170 μl 0.2 M sodium phosphate (pH 5.5). The reaction was started by addition of GSH. Aliquots (2 μl) were withdrawn from the system and instantly dried on a 20 × 20 cm thin-layer plate (MN-Polygram Sil G) with a hair-drier. The chromatographic system was acetic acid: *n*-butanol:H_2O, 3:12:5. The GSH-containing compounds were well separated and the radioactive components were localized by means of references, developed with ninhydrin The radioactive spots were cut out, placed in a vials containing 5 ml of scintillation fluid (4 g PPO, 0.1 g dimethyl-POPOP per liter toluene), and counted. The reaction between cystine and [^{35}S]GSH was followed similarly. The reaction system contained 50 μl 10 mM cystine, 50 μl 1.3 mM [^{35}S]GSH, 20 μl enzyme (or H_2O), and 130 μl 0.2 M sodium phosphate (pH 7.7). The reaction was initiated with GSH, and aliquots (1 μl) were dried on a 20 × 20 cm plate of MN-cellulose 300. The application area was earlier impregnated with 5 μl 1 M NEM in ethanol to convert GSH into the NEM adduct, in order to prevent further oxidation of GSH. The chromatographic system was acetic acid: *n*-butanol:H_2O:phenol, 8:15:15:5 (v/v/v/w). The compounds were developed with ninhydrin, scraped into scintillation vials, and decolorized [7] before counting in 5 ml scintillation fluid.

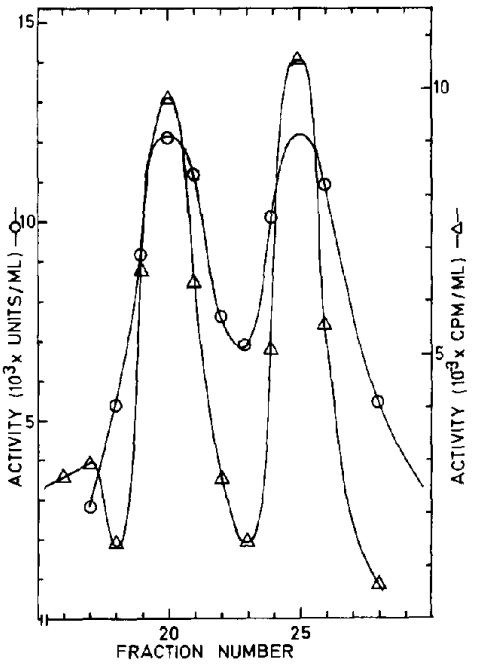

Fig. 1. Demonstration of coincidence of enzymatic thiol–disulfide interchange (o—o—o) and sulfitolysis (△—△—△) after CM-cellulose chromatography of a rat liver preparation. The enzyme was applied on a column (2 × 6 cm) equilibrated with 10 mM sodium phosphate (pH 6) and was eluted with a linear gradient of KCl (0–0.2 M). The two peaks of enzyme activity are regarded as different forms of the same enzyme [9]. The non-enzymatic reaction was subtracted from the values plotted in the diagram. The thiol–disulfide interchange was measured with GSH and the mixed disulfide of cysteine and GSH as earlier described [5]. The sulfitolysis of [^{35}S]-GSSG was assayed by determination of the [^{35}S]S-sulfoglutathione formed. The reaction system contained 50 μl 5 mM [^{35}S]GSSG, 50 μl 10 mM Na_2SO_3, 300 μl 0.2 M sodium phosphate (pH 7.0) – 0.1 mM EDTA, and 100 μl of the chromatographic fraction tested. The reaction was stopped by addition of 5 μl conc. H_2SO_4. S-sulfoglutathione was isolated by chromatography on Dowex 50W (H^+-form) and measured by liquid scintillation counting [10].

4. Results and discussion

In the transhydrogenase mechanism [eq. (1)], both reducing equivalents are transferred from the donor to the acceptor substrate in the course of one catalytic cycle. Consequently, both moieties of the acceptor must appear in reduced form when released as products from the enzyme. The donor substrate, on the other hand, dimerizes by giving off two reducing equivalents. However, a single step of the thioltransferase mechanism [e.g. eq. (2)] releases only one half of the acceptor in a reduced form and the donor is linked to the remaining half of the acceptor substrate. These two alternative mechanisms have both chemical and kinetic consequences.

4.1. Chemical evidence

A chemical consequence of the transhydrogenase mechanism is that sulfitolysis of GSSG, which is catalyzed by the same enzyme that catalyzes thiol–disulfide interchange between GSH and cystine or related compounds [8, 9] (fig. 1), would be expected to give GSH and dithionate

$$GSSG + 2\,HSO_3^- \rightleftharpoons {}^-O_3SSO_3^- + 2\,GSH. \qquad (4)$$

However, the enzymatic sulfitolysis of GSSG yields S-sulfoglutathione and GSH [10], as does the non-enzymatic reaction [11]

$$GSSG + HSO_3^- \rightleftharpoons GSSO_3^- + GSH. \qquad (5)$$

A reaction catalyzed according to the transhydrogenase mechanism, which gives (5) as the net reaction, viz.

$$GSSG + GSH + HSO_3^- \rightleftharpoons GSSO_3^- + 2\,GSH, \qquad (6)$$

would utilize simultaneously two dissimilar donor substrates, GSH and HSO_3^-, but as no GSH is present initially this reaction cannot operate unless GSH is produced non-enzymatically according to (5). In this case a lag or an acceleration would appear in the enzymatic reaction, which, however, has not been observed, Thus, the chemical evidence obtained with GSSG and sulfite strongly favors the thioltransferase mechanism.

4.2. Kinetic evidence

According to the transhydrogenase mechanism the disappearance of the oxidized substrate (RSSR) must be concurrent with the appearance of the oxidized product [GSSG in eq. (1)]. The thioltransferase mechanism also has the over-all stoichiometry of eq. (1), when both partial reactions, (2) and (3), have reached equilibrium. However, transient accumulation of an intermediate (RSSG) may be demonstrable before the

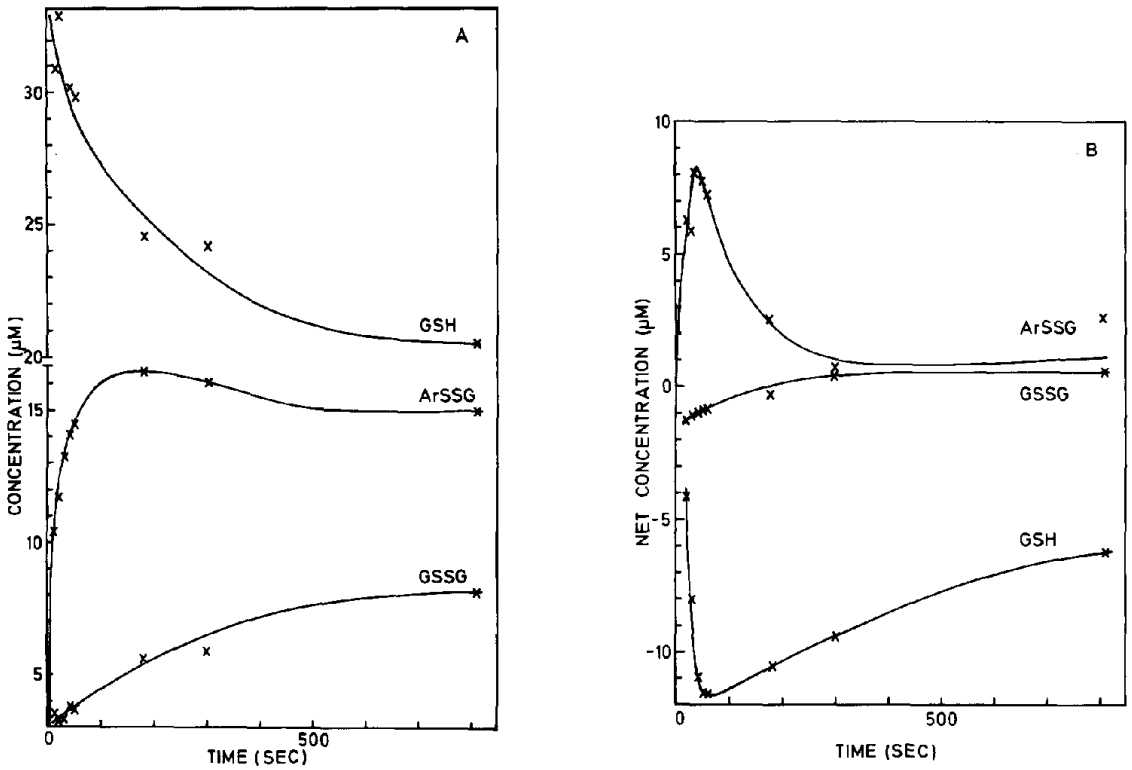

Fig. 2. (A) Progress curves of the enzyme-catalyzed reaction of 5,5-dithiobis(2-nitrobenzoate) (ArSSAr) and [^{35}S]GSH. Experimental details are given in Methods; (B) net enzymatic reaction of ArSSAr and GSH obtained by subtraction of the non-enzymatic progress curves from the curves given in (A).

final concentration is reached. A consequent lag in the formation of the oxidized product will appear. Such results will be obtained if the second reaction catalyzed by the enzyme [eq. (3)] is sufficiently slow. The sulfitolysis of GSSG evidently filfils this requirement, because the reaction corresponding to eq. (3) ($GSSO_3^- + HSO_3^- \rightleftharpoons {}^-O_3SSO_3^- + GSH$) is infinitely slow.

The enzyme used also catalyzes thiolysis of 5,5′-dithiobis(2-nitrobenzoate) (ArSSAr) with GSH [6]

$$ArSSAr + GSH \rightleftharpoons ArSSG + ArSH, \quad (7)$$

$$ArSSG + GSH \rightleftharpoons GSSG + ArSH. \quad (8)$$

These reactions have been studied separately by determination of initial rates in the absence and presence of enzyme. In both cases reaction (7) was more rapid than (8); non-enzymatically 4-fold and enzymatically about 9-fold at the enzyme concentration used [6]. Consequently, a biphasic progress curve is obtained when the enzyme-catalyzed liberation of ArSH is followed spectrophotometrically. The limb of the progress curve pertaining to the slow reaction (8) extrapolated to zero time, gives an absorbance equivalent to the initial concentration of ArSSAr.

The accumulation of ArSSG in the enzymatic reaction was demonstrated directly when [^{35}S]GSH was used. Fig. 2A shows progress curves of the reactants in the presence of enzyme; fig. 2B gives the net enzymatic reaction curves after subtraction of the non-enzymatic progress curves. Fig. 2B clearly shows an enzymatic accumulation of ArSSG, which cannot be explained by the transhydrogenase mechanism. However, the thioltransferase mechanism readily accounts for the results.

The enzyme-catalyzed thiol–disulfide interchange of [^{35}S]GSH and a natural disulfide substrate, cystine, was studied by the same method. In this case, however,

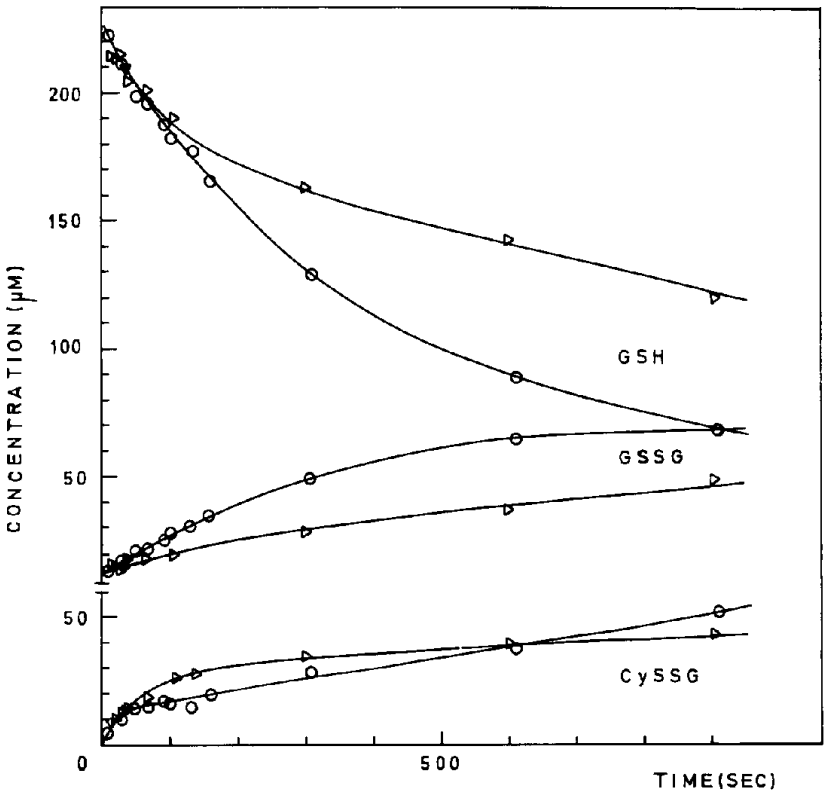

Fig. 3. Progress curves of the enzyme-catalyzed (○—○—○) and non-enzymatic (△—△—△) reactions of cystine and [^{35}S]GSH. Experimental details are given in Methods. Note that for 600 sec the concentration of the mixed disulfide (CySSG) is higher in the absence than in the presence of enzyme.

no transient accumulation of an intermediate (RSSG) could be demonstrated (fig. 3). This results is required by the transhydrogenase mechanism, but may be anticipated also according to the thioltransferase mechanism, because reaction (3) (where RSH = cysteine) was at least 4 times more rapid than (2) at equal reactant concentrations ([5] and unpublished experiments). Although no positive evidence derives from this experiment, it is suggested that the enzyme acts according to the thioltransferase mechanism also with cystine. This statement has support from the finding that maximal velocity of GSSG formation from cystine and GSH is obtained only after a time period during which the rate increases, as measured by coupling to glutathione reductase. Our interpretation of this observation is that a steady-state concentration of the intermediate (RSSG) must be reached before maximal GSSG production ensues. No such effect is obtained when the mixed disulfide of cysteine and glutathione (RSSG) is used as a substrate. These results have no simple explanation in terms of the transhydrogenase mechanism.

5. Conclusion

The collected data on the enzyme catalyzing thiol–disulfide interchange of cystine and glutathione or other low-molecular-weight compounds strongly indicate that the catalytic process is a two-substrate mechanism, the essence of which is thiol transfer. Earlier results (cf. [7]) strengthen this conclusion. We therefore suggest the trivial name of *thioltransferase* for this enzyme. The available evidence supports the proposition that all enzymes catalyzing thiol–disulfide interchange should properly be regarded as thioltrans-

ferases rather than transhydrogenases. The corresponding non-enzymatic reactions are known to follow the same principal mechanism [3].

Acknowledgements

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