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DISULFIDE-DISULFIDE INTERCHANGE CATALYZED BY A LIVER SUPERNATANT ENZYME

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Summary

An enzyme widely distributed in rabbit tissues which catalyzes an interchange between *N,N*-di-dinitrophenyl-L-cystine and oxidized glutathione to form the mixed disulfide is described. D-Penicillamine disulfide can be substituted for oxidized glutathione and the mixed disulfide of cysteine and glutathione can serve as the sole substrate giving as one product of interchange, oxidized glutathione. The enzyme is very labile and only limited purification of it has been achieved. The activity increases with increasing pH above 6.6, the K_m for *N,N*-di-dinitrophenyl-L-cystine is 0.2 mM and for oxidized glutathione 0.8 mM. The enzyme is inhibited by SH reagents with protection against iodoacetamide inactivation provided by *N,N*-di-dinitrophenyl-L-cystine. Evidence is presented that disulfide-disulfide interchange enzyme is a different activity from the previously described protein disulfide isomerase and thiol transferase.

Introduction

Glutathione (GSH) is the major low molecular weight thiol found in cells. In its reduced form it can react with either low molecular weight disulfides (RSSR) such as cystine or protein disulfides to form mixed disulfide compounds. In its oxidized form (GSSG) it can react with the corresponding reduced compounds. The reactions proceed by a sulfhydryl-disulfide interchange as depicted in the equations below catalyzed by a thiol transferase enzyme [1].

$$GSH + RSSR \rightleftharpoons GSSR + RSH \quad (1)$$

$$GSSG + RSH \rightleftharpoons GSSR + GSH \quad (2)$$

In this paper we describe a disulfide-disulfide interchange as depicted in the equation below catalyzed by a liver supernatant enzyme at neutral or alkaline pH values.

$$GSSG + RSSR \rightleftharpoons 2GSSR \quad (3)$$

Such an enzyme has not been previously described. Disulfide-disulfide interchange reactions have been observed to occur non-enzymatically in strong acid solution [2].

Methods and Materials

Glutathione reductase, Type III from yeast, D-penicillamine and *N,N*-didinitrophenyl-L-cystine (bis-DNP-cystine) were purchased from the Sigma Chemical Co., St. Louis, MO. The mixed disulfide of cysteine and GSH was synthesized from cystine thiosulfonate and GSH as described by Eriksson and Eriksson [3]. D-Penicillamine disulfide was prepared by oxidizing D-penicillamine with $FeCl_3$ at pH 8.0. The D-penicillamine disulfide was recovered from solution by precipitating it with cold acetone.

Enzyme assays. The assay mixture for disulfide-disulfide interchange activity, adapted from Ryle and Sanger [4] was 1 mM bis-DNP-cystine and 3 mM GSSG in 1 ml 0.1 M Tris-HCl buffer (pH 7.5). The reaction was started by adding enzyme in 0.1 ml or less. After 10 min at 37°C the reaction was stopped by adding 2 ml 2 M HCl, the supernatant fluid recovered by centrifugation and extracted twice with 3 ml portions of diethyl ether. To remove the last traces of ether the aqueous layer was incubated, with shaking, at 37°C for 15 min. The absorbances were then measured at 350 nm to estimate their dinitrophenyl content which is a measure of the amount of mixed disulfide of DNP-cysteine and GSH formed. Specific activity of the interchange enzyme is expressed as nmol mixed disulfide formed/mg protein. Protein concentration of preparations was measured by the method of Lowry et al. [5] and a molar extinction coefficient for the mixed disulfide at 350 nm of $1.75 \cdot 10^6\ M^{-1} \cdot cm^{-1}$ was used. Linearity of product formation with time of incubation or amount of enzyme in mixtures was maintained up to 70 nmol product.

Protein disulfide isomerase was assayed as previously described [6]. Thiol transferase was assayed in a manner similar to that described for the disulfide-disulfide interchange enzyme except that GSSG in mixtures was replaced by GSH at 0.2 mM final concentration. An appreciable rate of mixed disulfide synthesis was obtained without enzyme.

Preparation of disulfide-disulfide interchange enzyme. Rabbit liver was homogenized in 0.88 M sucrose (30%, w/v). The homogenate was centrifuged at 15 000 × *g* for 20 min and the supernatant retained. This supernatant was in some cases recentrifuged at 105 000 × *g* for 1 h to obtain the 105 000 × *g* supernatant. For further purification the first supernatant was heated with stirring for 30 min at 55°C. The precipitate which formed was removed by centrifugation. To the supernatant, 2 vols. saturated $(NH_4)_2SO_4$ (at 24°C) was added and after 30 min at 0°C the resulting precipitate was removed by centrifugation. This preparation is referred to as liver supernatant.

Results and Discussion

Preparation and distribution of disulfide-disulfide interchange enzyme

Liver homogenates first used in our studies were prepared in 0.25 M sucrose. While interchange activity was found in the 105 000 × *g* supernatant, much activity remained associated with cell particles. When homogenization was carried out in 0.88 M sucrose all the activity measured in the homogenate was recovered in the 105 000 × *g* supernatant. Using a combination of heating and $(NH_4)_2SO_4$ precipitation a preparation of specific activity of 330 nmol/mg protein was obtained. This represents a 10-fold purification over the homogenate with a recovery of about 60% of the activity. Further purification of the enzyme has been hampered by its extreme lability. Use of reducing agents to protect the enzyme has not been examined successfully, as such materials interfere with the subsequent assay of the enzyme.

The disulfide-disulfide interchange enzyme was found in many rabbit tissues. Brain and liver showed the highest activity followed by spleen, kidney, lung, and muscle. No activity was detected in polymorphonuclear leukocytes.

Properties of the enzyme

A prominent feature of the interchange activity is its lability. Storage of the 105 000 × *g* supernatant at 4°C for 24 h resulted in 50% loss of activity. The $(NH_4)_2SO_4$ precipitated preparation is stable at 4°C for at least 3 weeks. The pH of 105 000 × *g* supernatants decreased below 7 by $(NH_4)_2SO_4$ precipitation and this probably accounts for the stability of this preparation and not the removal of protein. Results of other experiments indicate that storing the preparation at a pH below 7.0 is one important factor in maintaining its activity. Interestingly, interchange activity is retained on heating the 105 000 × *g* supernatant at 55°C for 30 min, but on storage the preparation is more labile than the non-heated supernatant. Dialysis or gel filtration of a $(NH_4)_2SO_4$ precipitated preparation resulted in almost complete loss of activity. Adding dialysate back to dialyzed preparations did not restore activity.

The rate of formation of mixed disulfide from bis-DNP-cystine and GSSG catalyzed by liver supernatant was examined from pH 6.0 to 10.5. From pH 6.8 to 9.4 an approximate linear increase in rate with increasing pH was found. Above pH 9.7 no further increase in rate was obtained and below pH 6.6 no product was formed. This pH activity curve differs from the bell-shaped curve found for thiol transferase reactions and also from the curve found for non-enzymic thiol transfer. The pH dependence of the disulfide interchange reaction is not explained by the ionization of a single SH group. No mixed disulfide was formed in the absence of supernatant or with just one substrate plus supernatant. The affinity of the two substrates for the enzyme was next determined by measuring the rate of mixed disulfide formation with varying substrate concentration. A K_m of 0.8 mM was found for GSSG and 0.2 mM for bis-DNP-cystine. The SH blocking agents, *p*-mercuribenzoate, iodoacetamide, and *N*-ethylmaleimide all inhibit the interchange reaction greater than 90% when added to reaction mixtures at 1 mM concentration. A very effective inhibitor of the reaction in Cu^{2+} which inhibited 50% at 15 μM. These data support the essential nature of SH group for disulfide-disulfide interchange. The finding

TABLE I

PROTECTION BY SUBSTRATE OF DISULFIDE-DISULFIDE INTERCHANGE ENZYME INACTIVATION BY SH REAGENTS

Materials were added to mixtures containing buffer and liver supernatant. Preincubation at 0°C was carried out for 1 min with substrates and for 3 min with SH reagents before starting the reaction by adding the remaining substrate. 0.5 mM iodoacetamide and 1 mM *N*-ethylmaleimide were used.

Material added first	Iodoacetamide (% inhibition)	*N*-Ethylmaleimide (% inhibition)
SH reagent	65	95
bis-DNP-cystine	20	80
GSSG	65	95

that substrate protects against one of the reagents indicates that the SH is located at or near the enzyme active site (Table I). As can be seen, only good protection was obtained with bis-DNP-cystine against iodoacetamide inactivation. Results similar to that obtained with *N*-ethylmaleimide were obtained with either *p*-mercuribenzoate or Cu^{2+} as the inhibitor.

The activation energy of the interchange reaction was investigated at saturating levels of substrate by measuring the rate of mixed disulfide formation at different temperatures. A value of 4.6 kcal/mol was found which is comparable to values obtained with other enzyme-catalyzed reactions.

Relationship of disulfide-disulfide interchange enzyme to other disulfide-metabolizing enzymes

For the following reasons the enzyme described here acting on low molecular weight disulfide substrates is not protein disulfide isomerase previously described to act on protein disulfide bonds [6,7]. The protein disulfide isomerase is present in rabbit leukocytic extracts [8], while the disulfide-disulfide interchange enzyme is not. One of the substrates of the disulfide-disulfide interchange enzyme, bis-DNP-cystine, is a potent inhibitor of the protein disulfide isomerase. In rabbit liver microsomes 35 μM bis-DNP-cystine inhibited the enzyme 50%. That the disulfide-disulfide interchange enzyme is the previously described enzyme which catalyzes sulfhydryl-disulfide interchange seems very unlikely. The latter enzyme is present in our preparations and on dialysis the activity is retained, while as noted earlier, the disulfide-disulfide interchange activity is lost. The sulfhydryl-disulfide interchange uses a low molecular weight thiol as a substrate while the disulfide-disulfide interchange enzyme generates a low molecular weight thiol which it uses a reactant with the second substrate. It follows that reduced substrate in the latter reaction is always combined with enzyme and this view is supported by the unavailability of the SH for iodoacetamide when substrate is added first. That a free low molecular weight reduced thiol is not involved in the disulfide-disulfide interchange is also indicated by the finding that no reaction is seen until both disulfide substrates are added and that prior incubation of bis-DNP-cystine with GSH (25 μM) did not increase the rate of subsequent enzyme-catalyzed mixed disulfide formation with GSSG.

A possible arrangement of functional groups at the enzyme active site to

account for interchange is a protein-SH in juxtaposition with a protein disulfide bond. Interchange would proceed via an intermediate mixed disulfide compound with the enzyme participating as the reduced compound.

Interchange activity with substrates other than GSSG

Several disulfide compounds, oxidized dithiothreitol and 5,5-dithiobis-(2-nitrobenzoic acid), did not serve as substrates in the reaction in the place of GSSG. D-Penicillamine disulfide did serve as a substrate. When added to the standard assay mixture at a concentration of 3 mM, it gave about 30% the rate of mixed disulfide formation obtained with GSSG. This result is not surprising as the sulfur of D-penicillamine is sterically hindered by the two methyl groups attached to its β-carbon. The mixed disulfide of GSH and cysteine (CySSG) was also a substrate for the interchange reaction (Table II). With this material only one substrate is needed and the product of the interchange reaction, GSSG, is measured spectrophotometrically by oxidation of NADPH catalyzed by glutathione reductase. Also shown in Table II is the effect of adding D-penicillamine disulfide to reaction mixtures which inhibited the formation of GSSG. Oxidized dithiothreitol which is not a substrate for the interchange enzyme added to reaction mixtures did not inhibit the formation of GSSG. Substrate protection against iodoacetamide inactivation using CySSG showed that it provided, like the results obtained with GSSG, little protection.

Some possible physiological roles for enzyme-catalyzed disulfide-disulfide interchange reactions are: (a) It provides a direct pathway for solubilization of cystine by GSSG through formation of the soluble mixed disulfide. (b) It provides a mechanism for the formation of D-penicillamine disulfide from its mixed disulfide with GSH. The mixed disulfide is formed by reaction of D-penicillamine and GSSG catalyzed by a thiol transferase enzyme. D-Penicillamine is used in the treatment of rheumatoid arthritis [8] and it has been proposed that the disulfide compound in combination with Cu^{2+} is the active material [9].

TABLE II

CySSG AS A SUBSTRATE FOR DISULFIDE-DISULFIDE INTERCHANGE AND THE EFFECT OF D-PENICILLAMINE DISULFIDE ON THE REACTION

Complete reaction mixture contained 0.43 mM CySSG, 0.1 mM NADPH, 3 units glutathione reductase and liver supernatant in 3 ml 0.08 M Tris-HCl (pH 7.5) and were incubated for 10 min at 37°C. The amount of NADPH oxidized was determined by measuring the decrease in absorbance at 340 nm.

Composition of mixture	NADPH oxidized (μmol)
Complete	0.160
—Supernatant	0.023
—Glutathione reductase	0.012
—CySSG	0.014
+0.3 μmol D-penicillamine disulfide	0.080

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