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PURIFICATION BY AFFINITY CHROMATOGRAPHY OF YEAST GLUTATHIONE REDUCTASE, THE ENZYME RESPONSIBLE FOR THE NADPH-DEPENDENT REDUCTION OF THE MIXED DISULFIDE OF COENZYME A AND GLUTATHIONE

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Summary

Glutathione reductase (NAD(P)H : oxidised-glutathione oxidoreductase, EC 1.6.4.2) was purified from baker's yeast by a new procedure involving affinity chromatography on 2′,5′-ADP-Sepharose 4B. The yield was 65% of essentially homogeneous enzyme.

The activity was assayed with both glutathione disulfide (GSSG) and the mixed disulfide of coenzyme A and glutathione (CoASSG). The two disulfide substrates gave coinciding activity profiles and a constant ratio of the activities in different chromatographic and electrophoretic systems. No evidence was obtained for the existence of a reductase specific for CoASSG distinct from glutathione reductase. It is concluded that normal baker's yeast contains a single reductase active with both GSSG and CoASSG.

Introduction

It is well established that an NADPH-dependent enzymatic reduction of the mixed disulfide of coenzyme A and glutathione (CoASSG) takes place in both rat liver [1] and yeast [2,3]. It was originally assumed that this enzymatic activity was associated with a reductase distinct from the well-characterized glutathione reductase (NAD(P)H : oxidised-glutathione oxidoreductase, EC 1.6.4.2) [1,3]. However, this assumption has been challenged [4,5] and a consensus is now established on the nature of the reductase in rat liver, i.e., glutathione disulfide (GSSG) and CoASSG are both reduced by the same enzyme, glutathione reductase [5—7]. It should be added that in rat liver the

Abbreviations: CoASSG, the mixed disulfide of coenzyme A and glutathione; GSSG, glutathione disulfide.

thioltransferase-catalyzed reduction of CoASSG by GSH is about 20-fold more efficient than the reaction catalyzed by glutathione reductase [5].

However, in the case of yeast, agreement has not yet been reached. Mannervik and coworkers [5] demonstrated that the CoASSG-reducing activity could not be separated by isoelectric focusing from the GSSG-reducing activity in a commercial glutathione reductase preparation. The kinetics obtained by using competing alternative substrates with the commercial yeast glutathione reductase also seemed to exclude the existence of a separate CoASSG reductase [8]. However, Ondarza and coworkers have carried out a purification from yeast cells and reported separation of the two activities by isoelectric focusing under conditions of high ampholyte concentrations [3,6,7]. It could consequently be argued that the commercial preparation used in our laboratory was lacking a specific CoASSG reductase because it had been lost in the purification.

The successful application of affinity chromatography on 2′,5′-ADP-Sepharose 4B to the purification of glutathione reductase from erythrocytes [9] promised to provide an efficient procedure for purification of the enzyme from yeast cells and thereby to settle the question of the nature of the CoASSG-reducing enzyme.

Experimental Procedure

Materials. NADPH, $NADP^+$, and coenzyme A were obtained from Sigma; GSSG from Boehringer; Sephadex G-25, G-200, and 2′,5′-ADP-Sepharose 4B from Pharmacia; DEAE-cellulose DE 52 from Whatman; hydroxyapatite from Bio-Rad Laboratories. CoASSG was synthesized according to B. Mannervik (formerly B. Eriksson) [2]. The purity of this compound was checked by paper electrophoresis. Commercial baker's yeast (*Saccharomyces cereviseae*) was used as enzyme source.

Assay of enzyme activities. Enzyme activity was measured as the decrease in absorbance at 340 nm and 30°C, due to oxidation of NADPH with GSSG or CoASSG as oxidant. The reaction system contained in 1 ml: 0.1 M sodium phosphate buffer (pH 7.6), 0.5 mM EDTA, 0.1 mM NADPH, 0.5 mM GSSG or 0.17 mM CoASSG, and a suitable amount of enzyme. The low concentration of CoASSG used, as compared to GSSG, was necessitated by limitations in the amounts of CoASSG available, but the concentration was nevertheless higher than that used in ref. 3 (0.06 mM). CoASSG-reducing activity was 1.7 times higher in a system at lower pH: 0.1 M sodium phosphate (pH 5.5). The activity values given in the text refer to pH 7.6.

Determination of protein concentration. Protein concentration was calculated from the absorbances at 260 and 280 nm [10]. For fractions containing $NADP^+$ the method of Lowry et al. [11] was used after precipitation of the protein by 20% trichloroacetic acid.

Isoelectric focusing. Isoelectric focusing was carried out in a 110-ml column at 4°C according to the instructions of the manufacturer (LKB). The conditions described by Ondarza et al. [3] were accurately reproduced. The carrier ampholyte concentration was 6—2% and covered a pH-range of 4—6. The enzyme sample was dialyzed against 5 mM Tris · HCl buffer (pH 7.4) con-

taining 1 mM EDTA before each run and applied into the middle of the gradient. The amount of protein applied was 0.5 mg. After each run fractions of 1.5 ml were collected for enzyme activity and pH determinations. Measurements of pH were made at 4° C.

Gel electrophoresis. Polyacrylamide gel electrophoresis was performed essentially according to Ornstein [12] and Davis [13]. Gels were stained for protein with Coomassie Brilliant Blue G-250 and for enzyme activity according to Kaplan [14].

Purification of glutathione reductase from yeast

Preparation of yeast supernatant. Commercial baker's yeast (50 g) was suspended in 150 ml 20 mM Tris · HCl buffer (pH 8.2), containing 1 mM EDTA. The cells were broken in a Ribi Cell Fractionator at 25 000 lb/inch^2 and centrifuged at 20 000 × *g*.

DEAE-cellulose chromatography. The supernatant was passed through a Sephadex G-25 column (4 × 40 cm) before application on a DEAE-cellulose column (4 × 7 cm) equilibrated with the same buffer (20 mM Tris · HCl, pH 8.2, 1 mM EDTA). The enzyme was eluted by a linear gradient of 0—0.4 M NaCl.

2',5'-ADP-Sepharose chromatography. The buffer of the pooled glutathione-reductase-containing fractions from the previous purification step was exchanged for 50 mM phosphate buffer, pH 7.6, 1 mM EDTA, by repeated concentration and dilution on a Diaflo PM 10 membrane filter. The enzyme (190 ml, 2.3 mg/ml) was then applied to a column (2 × 3.6 cm) of 2',5'-ADP-Sepharose (50 mM phosphate, pH 7.6, 1 mM EDTA) and elution was effected by a linear $NADP^+$-gradient (0—5 mM).

Hydroxyapatite chromatography. The pooled fractions from the 2',5'-ADP-Sepharose step were adsorbed on a hydroxyapatite column (2 × 2 cm) equilibrated with 10 mM potassium phosphate, pH 6.7, 1 mM EDTA, and the enzyme was eluted with a linear gradient of potassium phosphate (10—350 mM, pH 6.7). Active fractions were pooled and concentrated on a Diaflo PM 10 membrane.

Sephadex G-200 chromatography. The concentrate (2 ml, 1.4 mg/ml) from the hydroxyapatite column was chromatographed on a Sephadex G-200 column (1.5 × 85 cm) equilibrated with 20 mM Tris · HCl (pH 7.0), 1 mM EDTA, 0.1 M NaCl.

Results

Purification of glutathione reductase

Table I summarizes the results of the purification. The enzyme was purified in five steps to a state close to homogeneity in a yield of 65%. Additional steps involving chromatography on DEAE-cellulose, hydroxyapatite, or 2',5'-ADP-Sepharose 4B did not increase the specific activity above 140 units/mg (protein determined according to Kalckar [10]). Analysis by disc electrophoresis of the purest fraction showed only one minor extra band in addition to the major component. Both bands stained for glutathione reductase activity. Homogeneous glutathione reductase has previously been shown to form

TABLE I

PURIFICATION OF GLUTATHIONE REDUCTASE FROM BAKER'S YEAST

	Volume (ml)	Total protein (mg)	Total activity (GSSG) (μmol/min)	Total activity (CoASSG) (μmol/min)	Activity ratio $\frac{(GSSG)}{(CoASSG)}$	Specific activity (GSSG) ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	Yield (GSSG) (%)
Supernatant	137	12 880	322	46.6	6.9	0.025	100
DEAE cellulose	236	555	319	33	9.6	0.57	99
2',5'-ADP-Sepharose	52	3.28	251	25.5	9.8	76.5	78
Hydroxyapatite	37	2.78	268	28	9.6	96.4	83
Sephadex G-200	31	1.49 *	209	23	9.1	140 *	65

* Protein determination according to Kalckar [10]. Use of the extinction coefficient at 280 nm reported by Massey and Williams [16] gives a specific activity of 260 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ of our preparation; the corresponding specific activity reported by Massey and Williams is 270.

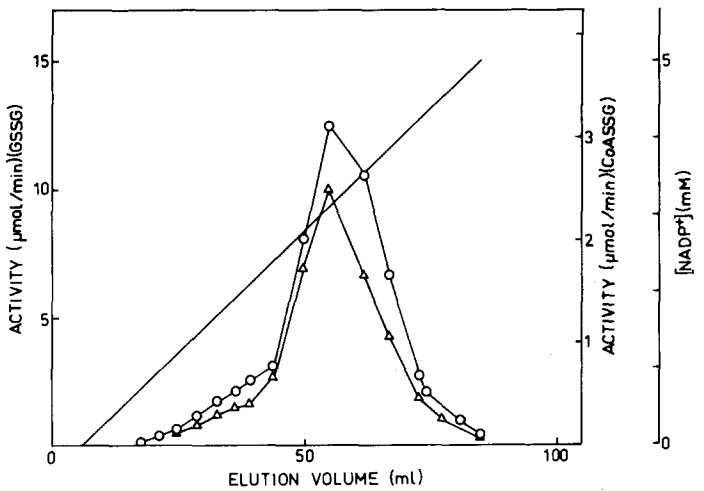

Fig. 1. Chromatography of glutathione reductase from yeast on 2',5'-ADP-Sepharose 4B. The enzyme was applied in 50 mM sodium phosphate (pH 7.6) containing 1 mM EDTA and the column (2 × 3.6 cm) was then washed with the same buffer. Elution was effected by a concentration gradient of $NADP^+$ (0—5 mM). Reductase activity (per ml) assayed with GSSG pH 7.6 (○) and CoASSG pH 5.5 (△); ———, $NADP^+$ concentration.

aggregates under aerobic conditions [15], and it is probable that the yeast enzyme behaves similarly.

GSSG- and CoASSG-reducing activities during purification

Fig. 1 shows the elution profile of chromatography on 2',5'-ADP-Sepharose 4B. The peaks of the GSSG- and CoASSG-reducing activities coincide completely. The same pattern was obtained in every step of the purification.

With the exception of the original supernatant fraction, the two activities showed a constant ratio of 9.1—9.8 throughout the entire purification; in individual fractions of chromatographic separations as well as in pooled fractions (see Table I).

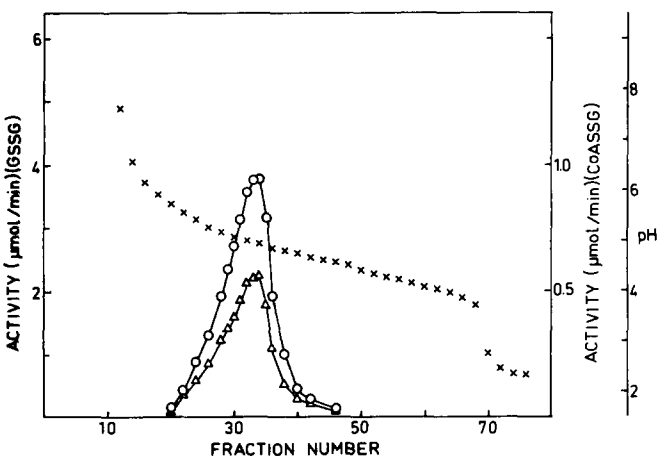

Fig. 2. Isoelectric focusing of purified glutathione reductase from yeast. The experimental conditions are described in the text. Reductase activity (per ml) assayed at pH 7.6 with GSSG (○) and CoASSG (△); ×, pH.

Isoelectric focusing

Isoelectric focusing of the purest fraction obtained after chromatography on Sephadex G-200 could not separate the two activities. They gave identical elution profiles; both having an isoelectric point at pH 4.9 (Fig. 2). In two experiments two peaks were obtained; one smaller more basic (isoelectric point at pH 5.9) in addition to the major one, but even in these cases the GSSG- and CoASSG-reducing activities followed each other completely.

Discussion

The purification by the new procedure results in an essentially homogeneous glutathione reductase. The specific activity given in Table I (140 μmol · min^{-1} · mg^{-1}) corresponds closely to that given previously by Massey and Williams [16] provided that correction for use of different methods for protein determinations is applied (see Table I). Throughout the purification the GSSG- and CoASSG-reducing activities closely followed each other. The quotient between the two activities was constant (about 9) except in the supernatant fraction. The explanation of a somewhat higher CoASSG-reducing activity in this fraction is most probably to be found in the presence of thioltransferase [17], which in the presence of endogenous GSH of the yeast cells increases the rate of CoASSG reduction by addition of a parallel reaction pathway:

$$\text{CoASSG} + \text{GSH} \rightarrow \text{CoASH} + \text{GSSG}$$
$$\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+$$

Thioltransferase (transhydrogenase) has been demonstrated in yeast by Nagai and Black [18]. Thus, the apparent loss of CoASSG-reducing activity after chromatography on DEAE-cellulose can be explained by elimination of this additional pathway owing to loss of GSH and/or thioltransferase in the first purification step. The molecular weight of the thioltransferase is only about 15 000 [18] whereas that of glutathione reductase is about 120 000 [19], and these enzymes are consequently readily separated by gel filtration (see Fig. 1 in ref. 20). Therefore, no thioltransferase can remain after chromatography on Sephadex G-200 in the purification. The possibility that thioltransferase plus glutathione reductase were responsible for the CoASSG-reducing activity in purer enzyme fractions was thus excluded. No reductase activity towards CoASSG in addition to that accounted for by glutathione reductase (99% of that found in the supernatant) could be demonstrated in the chromatography on DEAE-cellulose. We therefore conclude that a reductase specific for CoASSG cannot have been lost in the purification.

The remaining question is whether the two activities can be resolved by isoelectric focusing under the conditions reported by Ondarza et al. (see ref. 6, footnote on p. 170). As shown in Fig. 2, no such resolution was obtained. The experiment has been repeated several times with the same result. In two cases two peaks of reductase were obtained but both peaks had the same quotient of the two activities. The values reported here for CoASSG reduction were obtained in an assay having a pH of 7.6. All measurements of this activity have also been repeated with an assay at pH 5.5 (see ref. 3). The results and conclusions from these measurements were the same as reported for pH 7.6 except

that the CoASSG activity was 1.7-fold higher at pH 5.5.

Thus, in spite of the fact that we are unable to explain the results of Ondarza et al., which indicate the existence in yeast of a reductase distinct from glutathione reductase [3], the present results show clearly that only one enzyme is present in our preparation. It should be noted that the yield of our purification procedure is considerably higher and the specific activity of the enzyme more than 100 times higher than the corresponding values reported by Ondarza et al. [3]. In view of the efficiency of the thioltransferase-catalyzed reaction [5], it might be suggested that the presence of thioltransferase in the yeast preparation used by Ondarza et al. could have given rise to a peak of CoASSG-reducing activity in the position of thioltransferase.

On the basis of the results reported in the present paper of a complete purification of glutathione reductase from yeast cells, the previous data obtained with the commercial enzyme [2,5], the results of alternative substrates kinetics [8], and analogy with the results obtained with rat liver [4—6], we conclude that ordinary yeast cells (*S. cerevisiae*) do not contain an NADPH-dependent CoASSG-reducing enzyme distinct from glutathione reductase. The reductase activity demonstrated with CoASSG is the expression of lack of absolute substrate specificity of the glutathione reductase.

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