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CHARACTERIZATION OF A NADPH-DEPENDENT COENZYME A-SS-GLUTATHIONE REDUCTASE FROM YEAST*

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SUMMARY

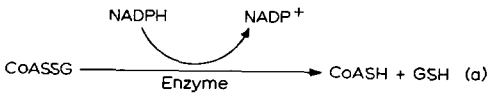
A new NADPH-dependent CoA-SS-glutathione reductase from yeast cells, as from commercially purified yeast GSSG reductase, has been established.

According to the data obtained from polyacrylamide column gel electrophoresis and isoelectric-focusing technique, the yeast GSSG reductase (EC 1.6.4.2) can be separated from the new enzymic activity. This enzyme has the following characteristics: (1) pH optimum = 5.5; (2) $K_m = 20 \cdot 10^{-5}$ M; (3) mol. wt. = $108 \cdot 10^3$; (4) produces GSH and CoASH from CoASSG as substrate in the presence of NADPH and does not require GSH; (5) catalyzes an irreversible reaction; (6) does not reduce CoASSCys, GSSCys, or CysSSCys; (7) is partially inhibited at 25 mM phosphate ion concentration.

INTRODUCTION

A NADPH-dependent CoA-SS-glutathione reductase activity from rat liver and from purified yeast GSSG reductase has been previously demonstrated[1]. This finding opened the question of whether (a) the purified yeast enzyme has some contaminating CoASSG-reducing activity or (b) the yeast GSSG reductase might not be as specific as originally described by PIHL, ELDJARN AND BREMER[2].

In this work we present data in order to characterize this enzyme as different from GSSG reductase. The proposed reaction in the presence of a hydrogen coenzyme donor for this new enzyme is as follows:



Other authors have found a different enzyme from bovine kidney[3] which uses

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CoASSG as substrate but requires free GSH. It is a CoASSG–GSH transhydrogenase and catalyzes a reversible reaction producing free CoASH and GSSG.

MATERIALS AND METHODS

Substrates

GSSG. A solution of GSSG (Boehringer, Mannheim, chromatographically pure), 7.5 mM (pH 6.6) adjusted with 0.1 M NaOH, was utilized for the estimation of GSSG reductase activity.

CoASSG. This substrate was prepared from a reaction mixture of 20 μmoles of CoASH and 80 μmoles of GSSG[1], previously adjusted at pH 7.0 with NaOH, and was incubated in the cold in a final volume of 2 ml of 0.05 M phosphate buffer (pH 7.6) in the presence of oxygen at 35° for 3 h. The mixture was resolved into its components by passage through a Dowex-1 (Cl^-) column (see ref. 4). The eluted peak corresponding to the CoASSG was concentrated by adsorption on charcoal and was further purified by low- and high-voltage electrophoresis. The yield of CoASSG was 35%. ERIKSSON[5] has described a different technique and reports 43% of yield. The identity of the compound was checked by studying the acid hydrolysis products with the automatic amino acid analyzer of MOORE AND STEIN[6]. It was shown also that the purified compound does not contain free GSH, since it did not form *S*-lactylglutathione in the presence of pyruvaldehyde and Glyoxalase I (ref. 7).

The production of $CoASO_3^-$ and GSO_3^- was demonstrated by paper chromatography when the compound is treated with performic acid[8] which splits the disulfide bridge.

Enzymic preparations

GSSG reductase. A commercial crystalline suspension (with 80 units) from Boehringer (Mannheim) was used. This preparation contains an appreciable activity of CoASSG reductase.

CoASSG reductase. The enzymic extract was prepared for preliminary studies from yeast *Saccharomyces cerevisiae* ATCC-1946, cultivated and grown as described by DE KLOET *et al.*[9]. The cultures were harvested during the logarithmic phase, and the extract was made as follows. Approx. 10 g of packed yeast cells were suspended in 20 ml of 0.068 M sodium phosphate buffer (pH 6.8) and were broken by sonication for 30 min with an MSE ultrasonic apparatus. The free cell extract was obtained after centrifugation at 15 000 $\times g$ for 20 min and was fractionated with ammonium sulfate between 40–60% saturation.

For the process of purification, a larger amount (400 g) of fresh commercial baker's yeast cells (82 g of dried cells) were used. The selective precipitation procedure as indicated by RACKER[10] was followed, except that the heating step at 55° was avoided and the disruption of the fresh cells was made using a mechanical cell Braun homogenizer for 2 min at 4000 oscillations/min in the cold.

The protein content of the different enzymic extracts was determined using the method of LOWRY *et al.*[11], with bovine serum albumin as standard.

Estimation of CoASSG- and GSSG-reducing activities. The incubation mixture for the estimation of CoASSG reductase is basically the same as that described for rat liver[1]. In a quartz cuvette with 1-cm light path, approx. 100 nmoles of NADPH were

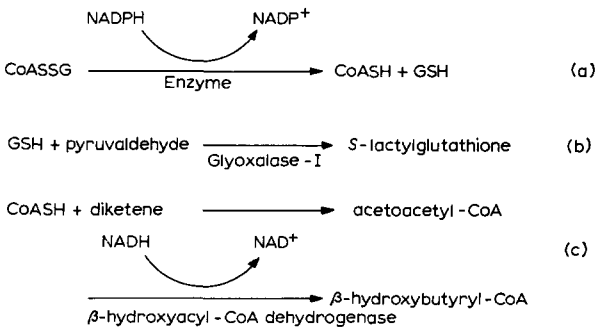
incubated with the yeast enzyme extract in a final volume of 1000 μl of 0.005 M to 0.068 M sodium phosphate buffer (pH 5.5–6.0) with 1 mM EDTA, depending on the conditions of the experiment. The reactions were followed at 340 nm in a DK-2 Beckman recording spectrophotometer at 24°, after the addition of about 60 nmoles of CoASSG. For the GSSG reductase similar conditions were used[12] but at a concentration of 0.068 to 0.225 M phosphate buffer (pH 6.8).

The initial velocities of the reactions were measured and calculated in units according to WEBB[13].

Glyoxalase I for the estimation of GSH. The method described by KLOTZSCH *et al.*[7] was followed by adding 10 μl (dilution 1:50) of distilled pyruvaldehyde (Baker Chem. Co.) to the enzymic incubation mixture and 10 μl (dilution 1:5) of commercially prepared glyoxalase I (EC 4.4.1.5) (Boehringer; 1 mg/ml). The formation of *S*-lactylglutathione was recorded spectrophotometrically at 240 nm; control mixtures were made with known amounts of GSH.

β-Hydroxyacyl-CoA dehydrogenase for the estimation of CoASH. The method by MICHAL AND BERGMEYER[14] was utilized in order to measure free CoASH produced by the yeast reductase; the CoASH was transformed into acetoacetyl-CoA by incubation in the cold for 20 min with 2 μl of distilled diketene at pH 7.4 (Sigma Chem. Co.), then treated with 125 nmoles NADH and 5 μl (dilution 1:80) of β-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) (Boehringer; 2 mg/ml). The reaction was automatically recorded with a DK-2 Beckman spectrophotometer at 340 nm.

Coupling of the enzymic reactions to show the products of CoASSG reductase. It was possible to measure stepwise the three enzymic reactions (CoASSG reductase, glyoxalase I and β-hydroxyacyl-CoA dehydrogenase) in the same mixture by coupling the different enzymes with their substrates as follows:



The reaction was initiated with 65 nmoles of CoASSG as substrate, 100 nmoles of NADPH and 67 μg of purified yeast enzyme in a final volume of 1 ml of 0.068 M sodium phosphate buffer (pH 6.0) without EDTA. The buffer was prepared with redistilled water in order to eliminate inhibiting ions[15]. The change at 340 nm for the completed reactions with CoASSG reductase was estimated, and the amount of substrate degraded was calculated from the molar extinction coefficient for NADPH of 6220.

The use of EDTA in this experiment was avoided because it would interfere with the spectrophotometric measurement of *S*-lactylglutathione at 240 nm.

An excess of pyruvaldehyde was added to the same quartz cuvette containing

the above reaction mixture for the evaluation of GSH. After 5 min the enzymic reaction was started with glyoxalase I, and an increase at 240 nm was observed. A blank containing an equivalent amount of NADPH was prepared in order to compensate for the absorbance of the enzymic mixture; the molar extinction coefficient for *S*-lactylglutathione is 3370 at 240 nm.

In order to estimate free CoASH liberated from CoASSG, 2 μl of diketene were added to the mixture previously adjusted at pH 7.4 and kept on an ice bath for 20 min; after this period approx. 125 nmoles of NADH and 5 μl (dilution 1:80) of β-hydroxyacyl-CoA dehydrogenase were mixed, and the change at 340 nm was recorded. The amount of CoASH could be calculated by using the molar extinction coefficient of 6220 for NADH. In this experiment a blank of sodium phosphate buffer was utilized.

Ultracentrifugation in sucrose gradient. The sucrose gradient method (7–25%) designed by MARTIN AND AMES[16] has been followed for the establishment of the molecular weight of both reductases of yeast (CoASSG and GSSG reductases) and for their possible separation in the ultracentrifuge Model L with the SW-39L rotor at 36 000 rev./min (105 000 $\times g$) for 16 h at 4°. Bovine serum albumin was used as standard (mol. wt. 65 000).

Polyacrylamide gel electrophoresis. In order to separate both activities, the polyacrylamide gel electrophoresis technique according to the method of RAYMOND AND NAKAMICHI[17] was utilized. Samples of enzymic yeast extracts or purified GSSG reductase containing from 2 to 6 mg of protein were processed for 14 h at 40 mA on a 13 cm $\times$ 1.5 cm column of 7.5–10% polyacrylamide, in 0.1 M Tris–maleate buffer-8 mM EDTA (pH 6.5–7.6). The column was cut into 3.5-mm disc pieces which were homogenized for 1 min in the cold with 1 ml of 0.10 M sodium phosphate buffer (pH 7.0) with 1 mM EDTA. After standing 4 h in the cold, the homogenates were centrifuged, and the supernatants were assayed for activity.

Isoelectric-focusing technique. The method developed by VESTERBERG AND SVENSON[18] was followed for a better separation of both enzymes and to determine their p*I*. An electrophoresis column (LKB 8100, of 110 ml of capacity) with a density gradient of carrier ampholytes from 6 to 2% within a pH range of 3–6 was used. The cathode at the bottom contained 2% of ethanolamine in a solution with 16 g of sucrose *plus* 12 ml of water. The anode solution contained 10 ml of 1% phosphoric acid. The initial charge of the column was 440 V, 3 mA (1.32 W); after 63 h of running, the charge decreased to 0.5 W. The experiment was carried out at 5°.

1.5-ml samples were collected for enzymic activity and estimation of pH (Fig. 2).

In order to evaluate the approximate isoelectric point of these enzymes, a preliminary run was performed within a pH range of 3–10.

RESULTS AND DISCUSSION

After the partial purification of the yeast extract using the selective precipitation procedure described by RACKER[10] but eliminating the heating step at 55°, both reductases from *S. cerevisiae* remained in the ammonium phosphate precipitate with an increase of 27.2-fold for GSSG reductase and 143-fold for CoASSG reductase. The results appear in Table I which shows an activities ratio of GSSG reductase/CoASSG reductase = 1.17 units/0.384 unit = 3, quite different from the ratio present in commercially purified GSSG reductase which is 80 units/2 units = 40, due probably

TABLE I

SUMMARY OF PARTIAL PURIFICATION OF YEAST GSSG REDUCTASE AND CoASSG REDUCTASE

The GSSG reductase and CoASSG reductase were measured at the conditions specified under MATERIALS AND METHODS: GSSG reductase in 0.225 M phosphate buffer (pH 6.8) with 1 mM EDTA; CoASSG reductase in 0.05 M phosphate buffer (pH 5.5) with 1 mM EDTA. The activities were calculated in munits according to WEBB[13].

Fraction	*Vol. (ml)*	*Protein (mg/ml)*	*GSSG reductase*		*CoASSG reductase*	
			Specific activity (munits)	*Yield (%)*	*Specific activity (munits)*	*Yield (%)*
Crude extract	475	12.0	43	100.00	2.68	100.00
Acetone ppt.	190	14.0	92	91.30	5.76	100.50
1st alcohol ppt.	39	22.4	126	40.50	—	—
2nd alcohol ppt.	12	11.2	210	10.08	4.32	38.10
Gel eluates	12	5.0	258	5.76	32.20	12.60
Ammonium phosphate ppt.	1.3	8.0	1170	4.15	384.00	26.10

to the absence of the heating step in our extract and also to the different properties of each enzyme.

We had reported[1] a ratio of 303 for the commercial GSSG reductase, but this value was due to the measurement of CoASSG reductase under suboptimal conditions (without EDTA).

The determination of the pH optimum was made with two types of preparation: (a) the purified yeast extracts from the final step of the selective precipitation procedure and (b) with the above yeast extract but previously passed through the polyacrylamide

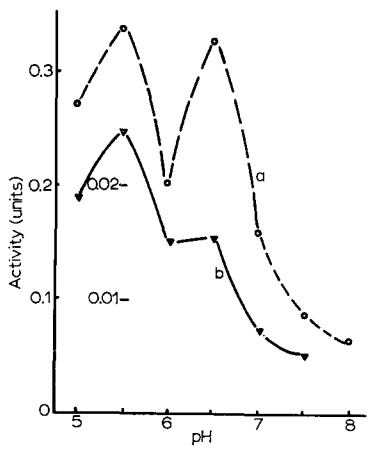


Fig. 1. Effect of pH on the CoASSG reductase from yeast. (a) 990 μl of 0.068 M sodium phosphate buffer prepared with redistilled water (at the indicated pH), 5 μl of yeast enzyme (33.5 μg of protein), and 10 μl of NADPH (100 nmoles); after 5 min, 20 μl of CoASSG (64.5 nmoles). (b) 950 μl of 0.068 M sodium phosphate buffer prepared with redistilled water (at the indicated pH), 50 μl of yeast enzyme passed through polyacrylamide gel electrophoresis column (the amount of protein was roughly estimated since it was very low after elution of the gel disc cut from the column), and 10 μl of NADPH (100 nmoles); after 5 min, 20 μl of CoASSG (64.5 nmoles). All the results were obtained by measuring the initial velocities of the reactions at 22–24°.

gel electrophoresis column. The pH curves for both experiments are shown in Fig. 1. The CoASSG reductase (Prep. a) shows two peaks of activity: one at pH 5.5 and the other at 6.5. However, this second peak decreases if the enzyme extract has been purified on the polyacrylamide gel electrophoresis column. We think that the pH optimum at 6.5 could correspond (when yeast extract has not passed through gel electrophoresis column) to the combined activities of CoASSG–GSH transhydrogenase[3], CoASSG reductase and GSSG reductase. Under such conditions (pH 6.5) the enzymic reaction product (GSH) of CoASSG reductase will enhance the CoASSG–GSH transhydrogenase which forms GSSG, a substrate that is broken down by GSSG reductase. These two enzymes last mentioned display optimum activities at pH 8.2 and 7.0, respectively.

The pH optimum of GSSG reductase at 7.0 obtained in our laboratory agrees well with data reported by others[19–21] and does not change when the enzyme is further purified by gel electrophoresis.

The apparent Michaelis constant for yeast CoASSG reductase activity at fixed value of NADPH (100 nmoles) at pH 5.5 was obtained by plotting the reciprocal of initial velocities against substrate concentration according to the LINEWEAVER–BURK[22] method. The enzymic activity was measured in each experiment with varying amounts of CoASSG which ranged from 11.3 to 191.5 nmoles. The constant value calculated by this procedure for the yeast enzyme purified in this laboratory is $20.0 \cdot 10^{-5}$ M and $19.8 \cdot 10^{-5}$ M for the CoASSG reductase activity present in GSSG reductase from Boehringer.

A K_m of $6.41 \cdot 10^{-5}$ M for yeast GSSG reductase from Boehringer and of $4 \cdot 10^{-5}$ M for yeast GSSG reductase purified in this laboratory and previously separated by gel electrophoresis were obtained.

In an attempt to separated GSSG reductase from CoASSG reductase, a sucrose gradient (7–25%) was utilized[16]; however, both maximum enzymic activities appeared in the same peak. The calculated molecular weight of the two enzymes corresponds to 107 790. Similar results were obtained for a commercially purified GSSG reductase and appear on Table II. The CoASSG-reducing activity present in this preparation did not separate from GSSG reductase after ultracentrifugation. The experiments were performed each time against bovine serum albumin as standard (mol. wt. 65 000).

Since the two enzymes were not separated by ultracentrifugation in sucrose gradient, the commercial GSSG reductase (Boehringer) as well as the partially purified

TABLE II

DATA FOR THE MOLECULAR WEIGHT OF CoASSG REDUCTASE AND GSSG REDUCTASE OBTAINED BY ULTRACENTRIFUGATION WITH THE SUCROSE GRADIENT METHOD OF MARTIN AND AMES[16]

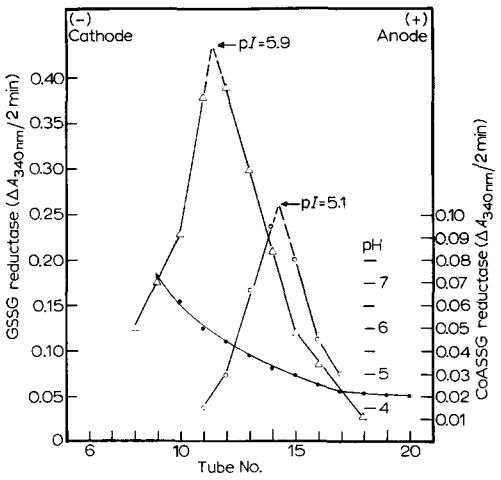
Source of enzyme	*(I) Distance of serum albumin to meniscus (cm)*	*(II) Distance of maxima enzymic activities to meniscus (cm)*	*Ratio I/II*	*Mol. wt. calc. for CoASSG reductase and GSSG reductase*
Baker's yeast cells prepared in this laboratory	1.382	1.937	1.401	107 790
Yeast GSSG reductase (Boehringer)	1.690	2.402	1.421	110 090

TABLE III

SEPARATION OF PARTIALLY PURIFIED YEAST EXTRACT BY 10% POLYACRYLAMIDE GEL ELECTROPHORESIS[17]

A sample of partially purified yeast enzyme (4 mg) was separated at pH 7.6 on the conditions specified under MATERIALS AND METHODS. The protein migrated from top of the column (cathode) towards the anode (increasing tube No.). For GSSG reductase 50 μl from the gel homogenate supernatant were used (at pH 7.0) and 300 μl for CoASSG reductase (at pH 5.5). The activities are expressed from the initial velocity as $\Delta A_{340\ nm}$ per 2 min.

Tube No.	$\Delta A_{340\ nm}$/2 min		*Ratio a/b*
	(a) GSSG reductase	*(b) CoASSG reductase*	
6	0.040	0.030	1.33
7	0.040	0.065	0.61
8	0.085	0.103	0.82
9	0.130	0.085	1.53
10	0.015	0.010	1.5



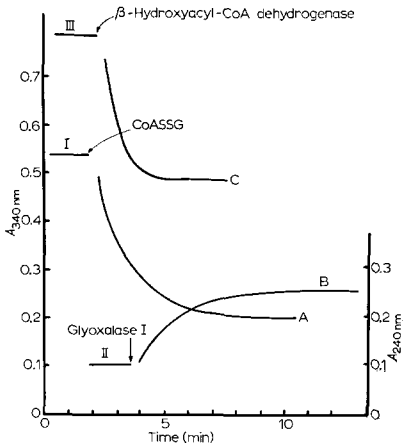


Fig. 2. Isoelectric-focusing separation of CoASSG reductase from yeast GSSG reductase. A sample of 200 μl (1 mg) of commercially purified GSSG reductase, (dialyzed against 4 l of water, during 3 h) in a concentration gradient from 6 to 2% of carrier ampholytes, within a pH range from 3 to 6, was applied to an electrophoresis column (see MATERIALS AND METHODS). For evaluation of CoASSG reductase (measured in 0.05 M phosphate buffer with 1 mM EDTA, at pH 5.5) 100 μl were used, and 20 μl for GSSG reductase (0.1 M phosphate buffer with 1 mM EDTA, at pH 7.0) (see METHODS). Each point represents the initial velocity expressed as a change in absorbance at 340 nm per 2 min.

Fig. 3. Coupling of enzymic reactions to establish the products of CoASSG reductase. Control I: 1000 μl of 0.068 M phosphate buffer (pH 6.0), without EDTA, 10 μl of NADPH (100 nmoles), and 10 μl of yeast enzyme (67 μg of protein). (A) Incubation mixture of I *plus* 65.4 nmoles of CoASSG. Control II: The same incubation mixture of A, *plus* 10 μl pyruvaldehyde (approx. 820 nmoles). (B) Incubation mixture of control II, *plus* 10 μl of glyoxalase I (1 mg/ml), diluted 1:5. A blank containing 10 μl of NADPH was used. Control III: The same incubation mixture of B, previously adjusted to pH 7.4, 2 μl of diketene incubated for 20 min in the cold, *plus* 10 μl of NADH (125 nmoles). (C) Incubation mixture of control III, *plus* 5 μl of β-hydroxyacyl-CoA dehydrogenase (2 mg/ml), diluted 1:80.

preparation from baker's yeast cells were also studied in polyacrylamide gel electrophoresis. The results in Table III show that the two reducing activities could be separated. There was a correspondence between enzymic activity and the stained area obtained after treatment with Amido black, but the staining did not discriminate the two enzymes, probably because of their close vicinity.

In order to differentiate both enzymes further, the isoelectric-focusing technique with a carrier ampholytes gradient from 6 to 2% was followed within a pH range of 3–6. As can be noticed in Fig. 2, the separation is quite clear; a distance of at least 7.2 mm between the two activity peaks was achieved (1.5 ml in the column corresponds to 3.6 mm). It is to be observed that, while GSSG reductase activity diminishes, the other enzyme (CoASSG reductase) increases.

A pI of 5.9 for GSSG reductase and a pI of 5.1 for CoASSG reductase were calculated by extrapolation of the experimental data.

To estimate the reaction products of Eqn. a of the introduction coupled enzymic reactions were used. A stoichiometric relationship was found between the amount of substrate degraded (55.4 nmoles of CoASSG), when measured as NADPH consumption, and the amount of products formed (48.4 nmoles of CoASH and 47.5 nmoles of GSH) (Fig. 3).

It was also demonstrated that the enzymic reaction shown in Eqn. a is irreversible under the following conditions. A mixture of CoASH and GSH in the presence of $NADP^+$, a purified yeast extract, which contains an appreciable activity of CoASSG reductase at 24° and 0.068 M phosphate buffer (at three different pH's: 6.0, 6.8 and 8.0) does not show an increase in the absorbance at 340 nm. The GSH solution used in these experiments was freshly made but contained a small amount of GSSG.

It can be assumed that the CoASSG reductase reaction is GSH independent, since purified CoASSG is free of GSH as has been described under MATERIALS AND METHODS. Moreover when GSH is added in competing amounts to the reaction mixture,

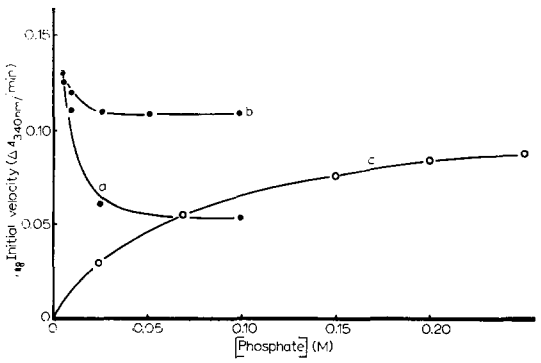


Fig. 4. Effect of phosphate concentration upon yeast purified CoASSG reductase and GSSG reductase. CoASSG reductase: (a) 980 μl of sodium phosphate buffer (pH 5.5) with 1 mM EDTA, 10 μl of yeast enzyme (84 μg of protein) 10 μl of NADPH (100 nmoles); after 5 min: 20 μl (64.5 nmoles) of CoASSG. (b) 990 μl of sodium phosphate buffer (pH 5.5) with 1 mM EDTA, 10 μl of enzyme (84 μg of protein): 10 μl of NADPH (100 nmoles); after 5 min 65 μl (209 nmoles) of CoASSG. GSSG reductase: (c) 990 μl of sodium phosphate buffer (pH 6.8) with 1 mM EDTA, 10 μl of diluted (1:2) yeast enzyme (42 μg of protein), 10 μl of NADPH (100 nmoles); after 5 min 100 μl of GSSG (750 nmoles).

the initial velocity is not affected. On the contrary, if GSSG is added, the reaction is inhibited, probably by competing for the active site of the new enzyme. These experiments were done by measuring the CoASH liberated[14].

There was no reducing activity, under optimal conditions for CoASSG reductase, of the purified yeast extract on CoASSCys, GSSCys or CysSSCys. The two mixed disulfides were produced by using the same principle for the preparation of CoASSG, that is CoASH with CysSSCys forms CoASSCys, GSH with CysSSCys forms GSSCys.

In Fig. 4 is shown a striking inhibition effect on CoASSG reductase by phosphate ion, which is less accentuated under substrate saturation conditions. On the contrary, GSSG reductase can be stimulated by this ion or by ionic strength as has been recently demonstrated also by WOODIN AND SEGEL[21] with GSSG reductase from *Penicillium chrysogenum*. Nevertheless, there is a noteworthy difference between these two enzymes regarding to the influence of phosphate on the substrate enzyme interaction (CoASSG has a phosphate group in 3′ position of the ribose).

The most important properties of GSSG reductase, CoASSG reductase and CoASSG–GSH transhydrogenase are summarized in Table IV in order to show the differences and similarities among the three enzymes.

TABLE IV

COMPARISON OF DATA OBTAINED FOR GSSG REDUCTASE, CoASSG REDUCTASE AND CoASSG–GSH TRANSHYDROGENASE

Properties	*GSSG reductase activity*			*CoASSG reductase activity*		*CoASSG–GSH trans-hydrogenase*[3]
	Partially purified yeast	*Purified yeast (Boehringer)*	*Ref. 20*	*Partially purified yeast*	*Purified yeast (Boehringer)*	
Mol. wt. × 10^{-3}	108	110	56.5*	108	110	12
Isoelectric point	—	5.9	—	—	5.1	—
pH optimum	7.0	7.0	7.0	5.5	5.5	8.2
K_m × 10^5 (M)	4	6.41	8.5	20	19.8	4.5
Cofactors	NADPH	NADPH	NADPH	NADPH	NADPH	GSH
Reversibility	Irreversible	Irreversible	Irreversible	Irreversible	Irreversible	Reversible
Phosphate ion effect	Activation	Activation	Activation	Inhibition	Inhibition	Activation

* Based on one flavin per mole of enzyme.

The presence of CoASSG in yeast[23], rat liver[24,25] and bovine liver[26], as well as the NADPH-dependent enzymic activity which splits the mixed disulfide (CoASSG), in animal and plant cells, justifies their study as possible regulators during aerobic–anaerobic metabolism. It has to be mentioned that mixed disulfides could be involved in the regulation of sulfhydryl enzymes; for example, yeast and rat-liver GSSG reductases are inhibited *in vitro* by disulfide exchange with CoASSG, if these enzymes have been previously reduced by NADPH, provided that no high CoASSG-reducing activity is present[15]. Another example of regulation of a sulfhydryl enzyme is the activation of rat-liver fructose diphosphatase by disulfide exchange with cystamine, as reported by PONTREMOLI *et al.*[27].

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