ON THE ACTIVE SITE OF THE NADPH-DEPENDENT CoA-SS-GLUTATHIONE REDUCTASE FROM YEAST AND RAT LIVER

Raúl N. ONDARZA and Rebeca ABNEY
Departamento de Bioquímica, Facultad de Medicina, C.U.
Universidad Nacional Autónoma de México (México)

Received 27 February 1970

1. Introduction

We have described a new NADPH-dependent CoASSG*-reductase from rat liver and yeast [1, 2]. Since this enzyme catalyzes a reaction similar to those of lipoamide dehydrogenase (EC 1.6.4.3), GSSG*-reductase (EC 1.6.4.2) and thioredoxin reductase, which are well-known pyridine nucleotide-dependent flavoproteins [3–5], we decided to see if the new reductase contains an active site with the particular characteristics of this group of enzymes.

Our studies with arsenite and 5-nitrofuran derivatives, utilised as inhibitors, indicate that the CoASSG-reductase contains a disulfide bridge in its active site and suggest the presence of FAD as prosthetic group.

2. Materials and methods

The preparation and purification of CoASSG, and the conditions for the estimation of the enzymic activity have been previously described [1, 2, 6].

A commercially purified crystalline suspension of yeast GSSG-reductase (5 mg of protein/ml, 80 units) from Boehringer was used as source of yeast CoASSG-reductase (this preparation contains an appreciable activity of the new enzyme as contaminant, 2–5 units).

CoASSG-reductase was also prepared from 100 g of rat liver homogenized with 0.1 M sodium phosphate buffer (pH 7.4) and centrifuged at 105,000 *g* for 30 min. The supernatant was precipitated with ammonium sulfate between 40 and 60% saturation, for one hr in the cold. After centrifugation at 15,000 *g* for 15 min, the precipitate was suspended in 0.1 M phosphate buffer (pH 7.4) and dialysed. The dialysate was passed twice with the same buffer through a Sephadex G-75 column (5 × 75 cm). The fractions containing both reductase activities were precipitated with ammonium sulfate at 60% saturation as before. The precipitate was dissolved in minimum volume of 0.1 M sodium phosphate buffer (pH 7.0) and dialysed overnight. This preparation was used for enzyme assay and contained 22 mg of protein per ml (177 mU of CoASSG-reductase). The protein content of the extracts was determined by the method of Lowry et al. [7].

* *Abbreviations:*
Co-SS-Glutathione, CoASSG
Glutathione-SS-glutathione, GSSG.

3. Results

Since arsenite is known to react with a NADPH-dependent reducible SS bridge, forming covalent bonds, such as in GSSG-reductase and lipoamide dehydrogenase, with resulting inhibition of enzyme activity [3, 4], the effect of this compound was assayed on CoASSG-reductase activity from the yeast and rat liver. Arsenite was found to inhibit the CoASSG-reductase activity at 10^{-1}, 10^{-2} and 10^{-3} M only when it was preincubated with the NADPH-dependent reduced enzyme for a minimum period of 3 min. The results of incubation for 5 min in the presence of arsenite at 10^{-2} M, with yeast and rat-liver preparations, with or without NADPH, appear in figs. 1 and 2.

It is interesting to note that the CoASSG-reductase from rat liver is more sensitive to arsenite than is the

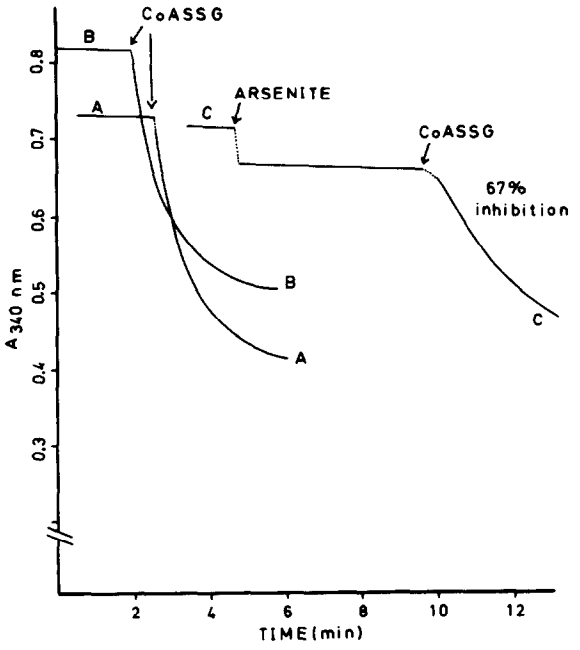

Fig. 1. Inhibition of reduced CoASSG-reductase from yeast (commercial preparation of yeast GSSG-reductase) by 10^{-2} M arsenite. The incubation mixtures were prepared as follows:

(A) 970 μl of 0.05 M sodium phosphate buffer (pH 5.5) containing 1 mM EDTA, 10 μl of NADPH (125 nmoles), 20 μl of GSSG-reductase (Boehringer, 5 mg/ml, dil. 1 : 100); after 2 min of incubation, 10 μl of CoASSG (78 nmoles) were added (curve A).

(B) This mixture is similar to A, except that 100 μl of 0.1 M arsenite (10 μmoles) were added to the mixture which did not contain NADPH; after 5 min of incubation the mixture was dialysed for 1 h against water and finally equilibrated with 0.05 M sodium phosphate buffer (pH 5.5) containing 1 mM EDTA; then 10 μl of NADPH were added (125 nmoles) and after 2 min 78 nmoles of CoASSG were mixed (curve B).

(C) Contained the complete incubation mixture as in A plus 100 μl of 0.1 M arsenite (10 μmoles). After 5 min of incubation 78 nmoles of CoASSG were mixed (curve C). The same degree of inhibition of this preparation was obtained after dialysis.

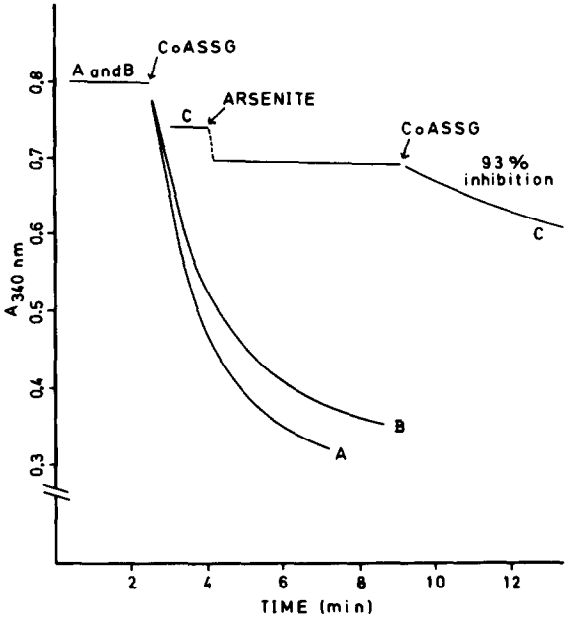

Fig. 2. Inhibition of reduced CoASSG-reductase from rat liver by 10^{-2} M arsenite. The incubation mixtures were prepared as follows:

(A) 950 μl of 0.05 M sodium phosphate buffer (pH 5.5) containing 1 mM EDTA, 10 μl of NADPH (125 nmoles) and 40 μl of partially purified rat-liver extract (22 mg of protein/ml); after 2 min 10 μl of CoASSG (78 nmoles) were added (curve A).

(B) This mixture is similar to A, except that 100 μl of 0.1 M arsenite (10 μmoles) were added to the mixture which did not contain NADPH; after 5 min of incubation the mixture was dialysed for 1 h against water and finally equilibrated with 0.05 M sodium phosphate buffer (pH 5.5) containing 1 mM EDTA; then 10 μl of NADPH were added (125 nmoles) and after 2 min 78 nmoles of CoASSG were mixed (curve B).

(C) Contained the complete incubation mixture as in A, plus 100 μl of 0.1 M arsenite (10 μmoles); after 5 min of incubation 10 μl of CoASSG (78 nmoles) were mixed (curve C). The same degree of inhibition of this preparation was obtained after dialysis.

yeast enzyme (see curves C, figs. 1 and 2). If the enzyme from both preparations is not previously reduced with NADPH (see curves B, figs. 1 and 2) no inhibiting effect was observed.

The inhibitory effect of arsenite is not reversed by dialysis. Longer times of incubation (1 hr) produce total inactivation of yeast and rat-liver preparations.

The 5-nitrofuran derivatives [8] utilised in these studies as inhibitors of CoASSG-reductase and GSSG-reductase were: furadantin (*N*-5-nitro-2-furfurylidene-1-aminohydantoin), furoxone (*N*-5-nitro-2-furfurylidene-3-amino-2-oxazolidone), micofur (5-nitro-2-furfuraldehyde oxime), furaspor (5-nitrofurfuryl methyl ether) and furacin (5-nitro-2-furfuraldehyde semicarbazone) at final concentrations of 10^{-4} M in the reaction mixtures. During the assay of enzymic activity, blanks of buffer containing an equivalent

Table 1
Inhibition of CoASSG-reductase and GSSG-reductase from yeast and rat-liver by 5-nitrofuran derivatives (10^{-4} M).

	Yeast				Rat-liver			
	CoASSG-reductase		GSSG-reductase		CoASSG-reductase		GSSG-reductase	
	Δ340 nm/min	Inhibition (%)	Δ340 nm/min	Inhibition (%)	Δ340 nm/min	Inhibition (%)	Δ340 nm/min	Inhibition (%)
Normal activity	0.085	–	0.130	–	0.092	–	0.138	–
Furadantin	0.050	41	0.051	67	0.061	34	0.042	85
Furoxone	0.036	58	0.067	48	0.075	19	0.070	75
Micofur	0.012	86	0.036	72	0.021	77	0.042	85
Furaspor	0.022	74	0.071	45	0.055	40	0.008	97
Furacin	0.031	64	0.045	65	0.041	55	0.025	82

The GSSG-reductase and CoASSG-reductase activated from yeast (commercial GSSG-reductase from Boehringer) and from partially purified rat-liver extracts were measured as follows: for the evaluation of yeast CoASSG-reductase, 20 μl of yeast GSSG-reductase (Boehringer, 5 mg/ml, dil. 1 : 100), 10 μl of NADPH (125 nmoles), plus the corresponding substrate (see below) in a final volume of 1 ml, made up with 0.05 M sodium phosphate buffer (pH 5.5), containing 1 mM EDTA. For the estimation of yeast GSSG-reductase, 5 μl of yeast GSSG-reductase (5 mg/ml, dil. 1 : 500), 10 μl of NADPH, (125 nmoles), plus substrate in a final volume of 1 ml, made up with 0.1 M sodium phosphate buffer (pH 7), containing 1 mM EDTA. For the estimation of liver CoASSG-reductase, 40 μl of partially purified rat-liver extract (22 mg of protein/ml), 10 μl of NADPH (125 nmoles) plus the substrate, in a final volume of 1 ml, made up with 0.05 M sodium phosphate buffer (pH 5.5), containing 1 mM EDTA. For liver GSSG-reductase 5 μl of partially purified rat-liver extract (28 mg of protein/ml), 10 μl of NADPH (125 nmoles), plus substrate in a final volume of 1 ml, made up with 0.1 M sodium phosphate buffer (pH 7), containing 1 mM EDTA. The 5-nitrofuran derivatives and NADPH were added to the enzyme preparations 20 min before the beginning of each experiment; after this period of time, the corresponding substrate was added (750 nmoles of GSSG or 78 nmoles of CoASSG) and the NADPH consumption recorded in an automatic Beckman DK-2 spectrophotometer at 340 nm. The initial velocities of the reactions were measured and appear in the table expressed as change in 340 nm/min.

amount of the respective nitrofuran derivatives were used.

In order to evaluate the reversibility of inhibition, in some experiments the incubation mixtures which contained the enzyme, NADPH and 5-nitrofuran derivatives, were dialysed overnight against cold water, followed by a two-hour dialysis against phosphate buffer. The inhibition was reversed by dialysis and was independent of the presence of NADPH, whether added before or after the incubation of the enzyme with nitrofuran derivatives. The inhibiting effects on both enzymes are shown in table 1.

It may be noted, apart from the main purpose of this work, that the yeast CoASSG-reductase is more sensitive to inhibition by 5-nitrofuran derivatives, than is the rat-liver enzyme, especially with furoxone and furaspor. On the other hand, the GSSG-reductase from yeast is less affected by these inhibitors than the respective enzyme from rat-liver.

4. Conclusions

On the basis of the results of inhibition of CoASSG-reductase obtained under the conditions here established, we conclude that:

(a) There is present a disulfide bridge, as evidenced by the fact that the enzyme is inhibited by arsenite, provided that it has been previously reduced with NADPH. The inhibition by arsenite is not removed after dialysis.

(b) The CoASSG-reductase from rat liver and yeast must be a flavoprotein. This proposition can be made because 5-nitrofuran derivatives are known to compete with flavoproteins, as shown by Buzard and Kopko [8] for the GSSG-reductase from rat-liver. In our studies this effect is also independent of the addition of NADPH and can be reversed by dialysis.

The above findings indicate the existence in CoASSG-reductase of an active site probably similar in its basic

catalytic mechanism to lipoamide dehydrogenase and GSSG-reductase [3, 4]. Direct studies in support of this conclusion can be performed only when highly purified preparations are available.

Acknowledgements

We wish to thank the Norwich Pharmacal Co de México for the gift of 5-nitrofuran derivatives, and Dr. A.Gómez-Puyou for reading the manuscript.

References

[1] R.N.Ondarza and J.Martínez, Biochim. Biophys. Acta 113 (1966) 409.

[2] R.N.Ondarza, R.Abney and A.M.López-Colomé, Biochim. Biophys. Acta 191 (1969) 239.

[3] R.L.Searls, J.M.Peters and D.R.Sanadi, J. Biol. Chem. 236 (1961) 2317.

[4] V.Massey, G.Palmer, C.H.Williams, Jr., B.E.P.Swoboda and R.H.Sands, in: Flavins and Flavoproteins (B.B.A. Library Vol. 8) ed. E.C.Slater (Elsevier, Amsterdam, 1966) p. 132.

[5] E.Colleen Moore, P.Reichard and L.Thelander, J. Biol. Chem. 239 (1964) 3445.

[6] R.N.Ondarza, in S.P.Colowick and N.O.Kaplan, Methods in Enzymology, Vol. 18 (Academic Press, New York) in press.

[7] O.H.Lowry, N.J.Rosebrough, A.L.Farr and R.J.Randall, J. Biol. Chem. 193 (1951) 265.

[8] J.A.Buzard and F.Kopko, J. Biol. Chem. 238 (1963) 464.