Molecular and Cellular Biochemistry **101**: 175–187, 1991.
© 1991 *Kluwer Academic Publishers. Printed in the Netherlands.*

Metals are directly involved in the redox interconversion of *Saccharomyces cerevisiae* glutathione reductase

José Peinado, Javier Florindo, C. García-Alfonso, E. Martínez-Galisteo, Antonio Llobell, and J. López-Barea
Departamento de Bioquímica y Biología Molecular, (Unidad de Veterinaria), Universidad de Córdoba, Avda. de Medina Azahara s/n, 14071 Córdoba, Spain

Received 12 April 1990; accepted 30 July 1990

Key words: glutathione reductase, *Saccharomyces cerevisiae*, redox interconversion, metals, cell-free extracts

Summary

Redox inactivation of glutathione reductase involves metal cations, since chelators protected against NADPH-inactivation, 3 μM EDTA or 10 μM DETAPAC yielding full protection. Ag^{+}, Zn^{2+} and Cd^{2+} potentiated the redox inactivation promoted by NADPH alone, while Cr^{3+}, Fe^{2+}, Fe^{3+}, Cu^{+}, and Cu^{2+} protected the enzyme. The Zn^{2+} and Cd^{2+} effect was time-dependent, unlike conventional inhibition. Glutathione reductase interconversion did not require dioxygen, excluding participation of active oxygen species produced by NADPH and metal cations. One Zn^{2+} ion was required per enzyme subunit to yield full NADPH-inactivation, the enzyme being reactivated by EDTA. Redox inactivation of glutathione reductase could arise from the blocking of the dithiol formed at the active site of the reduced enzyme by metal cations, like Zn^{2+} or Cd^{2+}.

The glutathione reductase activity of yeast cell-free extracts was rapidly inactivated by low NADPH or moderate NADH concentrations; $NADP^{+}$ also promoted rapid inactivation in fresh extracts, probably after reduction to NADPH. Full inactivation was obtained in cell-free extracts incubated with glucose-6-phosphate or 6-phosphogluconate; the inactivating efficiency of several oxidizable substrates was directly proportional to the specific activities of the corresponding dehydrogenases, confirming that redox inactivation derives from NADPH formed *in vitro*.

Abbreviations: DETAPAC – diethylenetriaminepentaacetic acid, 2′,5′-ADP-Sepharose-N^6-(6-aminohexyl) adenosine-2′,5′-bisphosphateSepharose

Introduction

Glutathione reductase is a dimeric flavoenzyme [1], with one FAD molecule per monomer, catalyzing the reaction: $GSSG + NADPH + H^{+} \rightleftharpoons 2GSH + NADP^{+}$. The human erythrocyte enzyme has been sequenced [2], the 3-dimensional structure studied by X-ray crystallography at 1.54 Å resolution [3, 4], the NAD(P)H and glutathione binding sites studied in detail [5–7], and the catalytic mechanism settled by X-ray analysis of reaction intermediates [7, 8]. After an N-terminal flexible arm, each subunit has 4 domains: FAD-binding, NADPH-binding, central and interface [3–7]. The electrons are transferred from the nicotinamide ring, located at the *Re* face of the isoalloxa-

cine ring, to flavin and then to a strained redox-active disulfide placed on its *Si* face in the GSSG-binding site; the transient active-site dithiol reduces the GSSG, aided by a His residue from the other subunit [3–8].

Glutathione reductase is inactivated under reducing conditions. Worthington and Rosemeyer attributed the inactivation of the human enzyme by NADPH to aggregation through the redox-active dithiols [9]. We have studied the NAD(P)H-inactivation of glutathione reductase from several organisms, its protection and reactivation, and attributed the process to the formation and disappearance of an additional intramolecular disulfide between the proximal thiolate of the reduced enzyme and other close Cys residue [10–16]. The physiological significance of glutathione reductase interconversion has been established with the *E. coli* enzyme, in cell-free extracts [13], permeabilized, and intact cells [14]. Redox inactivation of *E. coli* glutathione reductase has been confirmed, especially at very low NAD(P)H concentrations [21, 22]: the inverse relationship between NAD(P)H-inactivation and enzyme concentration prompted the proposal that inactivation is due to dissociation of the dimeric enzyme after reduction.

Based on the detailed knowledge of human glutathione reductase [3–8], other possible regulatory mechanisms have been proposed, such as the opening of a disulfide linking both monomers at the twofold molecular axis [3], or the formation of an additional disulfide between the N-terminal arm and the active site [3, 4, 8]. The *E. coli* gene coding for glutathione reductase has been recently cloned and sequenced [17]: the bacterial enzyme lacks the intersubunit disulfide bridge and the N-terminal flexible arm which is present in its human homologue. The cloned gene is being used to test the role of aminoacid residues on catalysis [18, 19] and the regulatory mechanism [20] by site-directed mutagenesis. An intersubunit disulfide bridge has been engineered at the 2-fold axis by changing Thr-75 into Cys: this mutant enzyme is inactivated by NADPH at the same rate as the wild type, although it maintains its dimeric form after inactivation [20].

Several metal cations, such as Hg^{2+}, Zn^{2+}, and Cu^{2+}, inhibit glutathione reductase, especially when previously reduced [23–29]. This fact, plus the different mechanisms proposed for redox inactivation, and the detailed knowledge of the human enzyme, led us to investigate the possible role of metal cations. The present paper shows the specific involvement of certain metal cations on the redox inactivation of pure glutathione reductase from *S. cerevisiae*, and also reaffirms the physiological significance of its redox interconversion based on studies performed with yeast cell-free extracts incubated with pyridine nucleotides and different oxidizable substrates.

Materials and methods

Reagents

Peptone, yeast extract, and agar were from Difco (USA). Glucose; the nitrates of lithium, calcium, lanthanum, manganese, cobalt, nickel, copper (II), silver, cadmium, thallium, and bismuth; the chlorides of sodium, potassium, rubidium, magnesium, chromium, and copper (I); and the sulfates of ammonium-iron (II) and (III) were from Merck (Fed. Rep. Germany). The nitrates of strontium, barium, zinc, aluminium, and lead as well as stannous chloride were from Riedel (Fed. Rep. Germany). All the salts employed were of analytical grade. L-Cysteine, DETAPAC, DTT, EDTA, FAD, GSSG, NADPH, $NADP^+$, sodium gluconate, trisodium isocitrate, Trizma base; citric, L-lactic, and malic acids; yeast glutathione reductase (type III), glucose oxidase, catalase, and bovine serum albumin were from Sigma (USA). NADH, gluconate-6-phosphate and glucose-6-phosphate were from Boehringer (Fed. Rep. Germany); the NAD^+ was from Serva (Fed. Rep. Germany); the 2′,5′-ADP-Sepharose and Sephacryl S-300 were from Pharmacia (Sweden).

Organism studied, growth, and cell-free extract preparation

Strain S288c of *S. cerevisiae* was grown at 30° C by orbital shaking at 100 rpm with 1% yeast extract, 1% peptone and 2% glucose. Cells were collected by centrifugation, washed and suspended in 5 ml buffer (100 mM Tris-HCl, pH 7.5, 0.5 mM L-Cys)

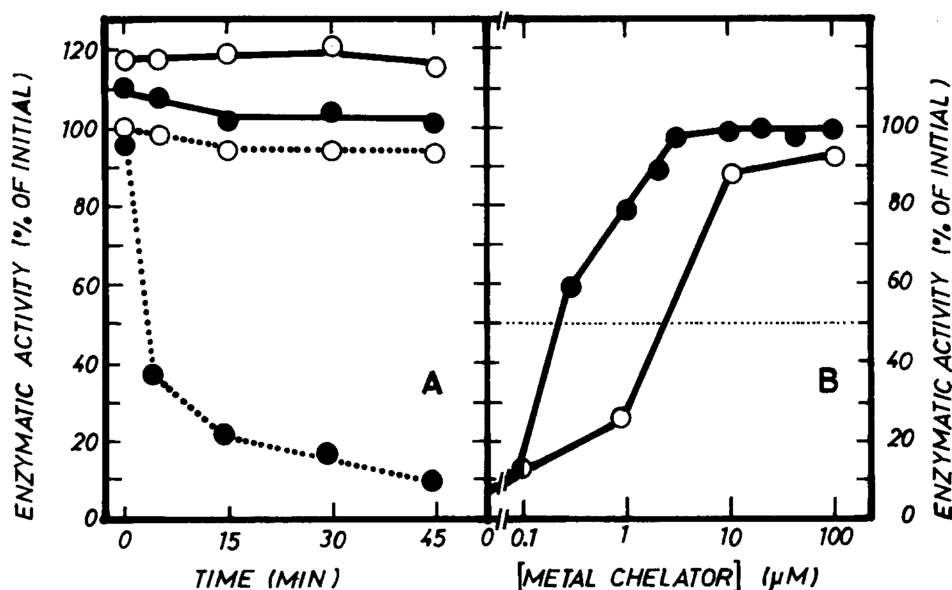

Fig. 1. Protection by metal-chelators against redox inactivation of glutathione reductase by NADPH. A. – Time-course of protection by EDTA: the pure enzyme (2.1 μg) was incubated in buffer A with (●) or without (○) NADPH, either in the presence (solid lines) or the absence (broken lines) of 5 mM EDTA, in 0.15 ml. The time-course of enzymatic activity was followed, and expressed as percentage of the initial control without additions, 162 U/mg. B. – Protection by different EDTA or DETAPAC concentrations: the enzyme (1.5 μg) was incubated in buffer A with NADPH and the indicated concentrations of EDTA (●) or DETAPAC (○) in 0.35 ml. After 30 min the activities were determined and expressed as the percentage of the control without NADPH, 174 U/mg.

per g of cells. The cell suspension was disrupted by sonic disintegration for two periods of 20 seconds with a Braun Labsonic apparatus, and the extracts centrifuged for 10 min at 12.000 × g. Cell disruption and all subsequent operations were carried out at 4° C.

Purification of S. cerevisiae glutathione reductase

Sigma type III suspension was dialyzed against 20 mM K-phosphate buffer (pH 7.0, 1 mM EDTA, 20 μM FAD, 1 mM DTT), adsorbed to a 2′-5′-ADP-Sepharose column (16 ml) equilibrated with the same buffer, and eluted with a 0–1 M linear NH_4Cl gradient. After dialysis with 0.2 M Tris-HCl buffer (pH 8.0), the enzyme was chromatographed in a Sephacryl S-300 column (1.6 × 80 cm) equilibrated with the same buffer. The final preparation had 250 U/mg specific activity and was homogeneous, as shown by electrophoresis [30]. All solutions were prepared with glass bidistilled water treated by active-charcoal adsorption, ion exchange, and organic matter removal using a Millipore (Milli-Q) apparatus to yield HPLC-grade quality.

Analytical methods

Glutathione reductase activity was determined at 30° C by the descent of NADPH absorbance at 340 nm due to GSSG reduction in 0.1 M K-phosphate buffer, pH 7.5, 1 mM EDTA, 2.5 mM GSSG, and 0.125 mM NADPH in 1 ml final volume [11]. The following activities were assayed as indicated: glucose-6-P dehydrogenase [31], 6-P-gluconate dehydrogenase [32], $NADP^+$- [33] and NAD^+-dependent isocitrate dehydrogenase [34], malate dehydrogenase [35] and L-lactate dehydrogenase [36]. One unit of enzymatic activity is defined as the amount of enzyme reducing one micromole of substrate per minute; specific activities are expressed as units of enzyme per mg of protein. The protein was measured by the biuret-phenol procedure [37], using bovine serum albumin as standard.

Standard conditions for glutathione reductase inactivation

The pure enzyme was inactivated by incubation at 30° C in 100 mM Tris-HCl buffer, pH 8.0, (buffer A) with 0.1 mM NADPH; all the solutions were

prepared with ultra-pure water. Inactivation of the enzyme in crude extracts was routinely performed by incubation at 30° C with 50 mM Tris-HCl buffer, pH 8.0, (buffer B) and 0.2 mM NADPH. To exclude uncontrolled redox inactivation during preparation of the experiments, all the incubations were iniciated by adding the enzyme, which in the control without additions ranged between 1 to 2 units per ml.

Anaerobic conditions

Anaerobic incubations were carried out in glass tubes (9 × 45 mm) with rubber stoppers connected to a manifold. The samples were subjected to five vacuum/N_2 cycles in the presence of 25 mM glucose, 0.2 mg/ml glucose oxidase and 0.2 mg/ml catalase. All additions and the collection of alliquots were carried out using gastight syringes, but the assays were performed in aerobiosis.

Results

Figure 1A shows that the inactivation of pure yeast glutathione reductase by 100 μM NADPH was fully protected by 5 mM EDTA, which also increased the activity of both control and NADPH-treated enzymes. Figure 1B shows protection against redox inactivation by several concentrations of two known metal chelators, EDTA and DETAPAC: 0.2 μM EDTA yielded 50% protection, while 10-fold higher DETAPAC concentrations were required for a similar effect; full protection was obtained beyond 3 μM EDTA and 10 μM DETAPAC. The full protection by EDTA from redox inactivation, and the low concentrations required of both cation chelators tested [38] indicated that metal cations could be involved in redox interconversion, directly or catalytically.

A search for metal cations potentiating NADPH-inactivation of GSSG reductase was undertaken. Table 1 shows the effect of different cations on redox inactivation of pure yeast glutathione reductase. The Ag^+, Zn^{2+}, and Cd^{2+} ions greatly increased the redox inactivation of glutathione reductase promoted by NADPH. No significant change in the inactivation promoted by NADPH alone was observed with most other cations, except for the slight potenciation by Pb^{2+} and the protection observed in the presence of Cr^{3+}, Fe^{2+}, Fe^{3+}, Cu^{2+} or Al^{3+}. Figure 2 shows the time-course of glutathione reductase inactivation by NADPH and several cations: 10 μM Zn^{2+} and Cd^{2+} drastically potentiated redox inactivation, with the activities in the presence of cation plus NADPH always more than 10-fold lower than with NADPH alone, while Cr^{3+} and Fe^{2+} protected the enzyme. No effect of the anion portion of the added chemicals was observed: thus, while the calcium or strontium nitrates did not significantly alter the inactivation promoted by NADPH, the copper or aluminium

Table 1. Effect of different cations on the redox inactivation of yeast glutathione reductase by NADPH

Group	Cation	Compound	Enzymatic activity [% ± SEM, (n)]
IA	Li^{1+}	$LiNO_3$	99 ± 5 (2)
	Na^{1+}	NaCl	145 ± 15 (2)
	K^{1+}	KCl	101 ± 7 (3)
	Rb^{1+}	RbCl	104 ± 16 (6)
IIA	Mg^{2+}	$MgCl_2$	132 ± 25 (3)
	Ca^{2+}	$Ca(NO_3)_2$	115 ± 15 (2)
	Sr^{2+}	$Sr(NO_3)_2$	111 ± 10 (2)
	Ba^{2+}	$Ba(NO_3)_2$	77 ± 9 (2)
IIIB	La^{3+}	$La(NO_3)_3$	64 ± 1 (2)
VIB	Cr^{3+}	$CrCl_3$	234 ± 6 (2)
VIIB	Mn^{2+}	$Mn(NO_3)_2$	62 ± 10 (3)
VIIIB	Fe^{2+}	$(NH_4)_2Fe(SO_4)_2$	189 ± 31 (3)
	Fe^{3+}	$NH_4Fe(SO_4)_2$	160 ± 30 (3)
	Co^{2+}	$Co(NO_3)_2$	138 ± 16 (4)
	Ni^{2+}	$Ni(NO_3)_2$	70 ± 21 (2)
IB	Cu^{1+}	CuCl	122 ± 21 (2)
	Cu^{2+}	$Cu(NO_3)_2$	203 ± 32 (3)
	Ag^{1+}	$AgNO_3$	3 ± 0.5 (2)
IIB	Zn^{2+}	$Zn(NO_3)_2$	7 ± 1 (4)
	Cd^{2+}	$Cd(NO_3)_2$	5 ± 3 (3)
IIIA	Al^{3+}	$Al(NO_3)_3$	165 ± 20 (2)
	Tl^{1+}	$TlNO_3$	91 ± 10 (2)
IVA	Sn^{2+}	$SnCl_2$	88 ± 31 (3)
	Pb^{2+}	$Pb(NO_3)_2$	43 ± 20 (3)
VA	Bi^{3+}	$Bi(NO_3)_2$	70 ± 30 (3)

The enzyme (2 μg) was incubated at 30° C in buffer B and 0.35 ml total volume with 0.1 mM NADPH and 10 μM of the cations shown. After 30 min the activities were determined and expressed as the percentage of the activity shown by the control with NADPH alone ± standard error of the mean of the number of experiments shown in parenthesis.

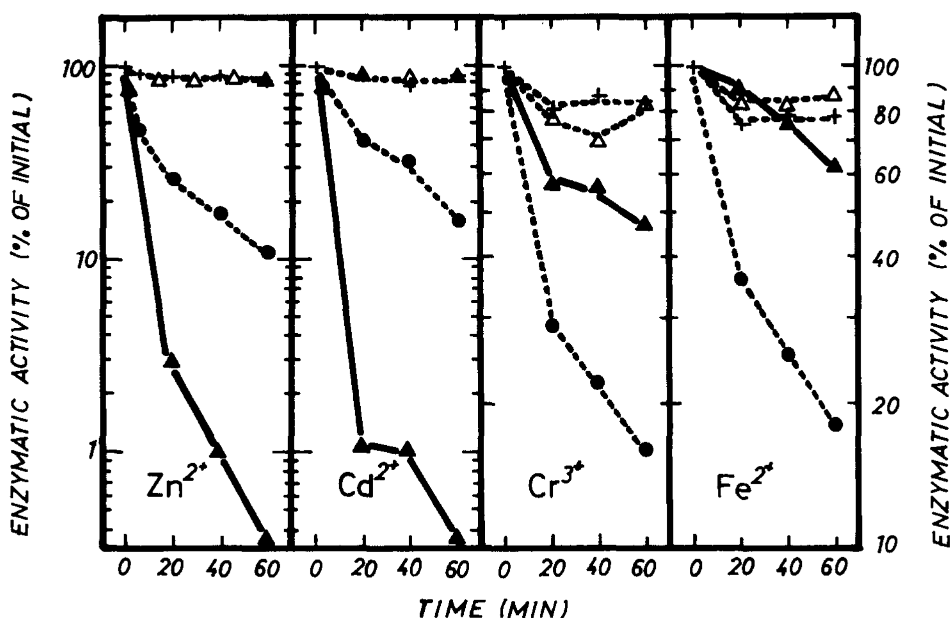

Fig. 2. Effect of several metal cations on the time-course of redox inactivation. The enzyme (1.5 μg) was incubated in buffer A without additions (+), with 10 μM cation (△), NADPH (●), or with cation plus NADPH (▲) in 0.35 ml. The activities were determined at the times shown and expressed as the percentage of the initial control without additions, 195 U/mg.

nitrates significantly protected the enzyme from redox inactivation, and the silver, zinc or cadmium nitrates greatly potenciated the redox inactivation.

The possible involvement of oxygen or active oxygen species was then studied with respect to the inactivation of yeast glutathione reductase by NADPH. Figure 3A shows that the enzyme was rapidly inactivated by 200 μM NADPH with or without O_2 (both protected by EDTA, not shown), the inactivation being even faster in anaerobiosis. Figure 3B shows that O_2 was not required either for the reactivation of the inactive enzyme promoted by GSSG, although it was slower without O_2. Figure 3 clearly indicates that glutathione reductase redox interconversion does not depend on the presence of O_2, excluding the participation of active oxygen species in the process.

To distinguish between the direct and catalytic roles of metal cations on the redox inactivation of glutathione reductase, the stoichiometry between enzyme and Zn^{2+} concentrations was studied. Figure 4A shows the inactivation of 11.3 μM glutathione reductase subunit by 100 μM NADPH and 1–30 μM Zn^{2+}: 10 μM of this cation was necessary for full inactivation, indicating that each reduced subunit required one Zn^{2+} to become completely inactivated. The direct involvement of Zn^{2+} (or another metal cation) on redox inactivation of glutathione reductase was confirmed by the experiment summarized in Fig. 4B: addition of 1 mM EDTA fully and rapidly reactivated an enzyme inactivated by 100 μM NADPH and 10 μM Zn^{2+}, suggesting that the cation was directly responsible for the modification suffered by the enzyme when incubated under reducing conditions. A similar reactivation by EDTA has been observed in the enzyme inactivated by NADPH without the addition of metals (results not shown).

The physiological significance of glutathione reductase interconversion has been studied with yeast cell-free extracts. Figure 5A shows the time-course of inactivation by NAD(P)H in fresh crude extracts: NADPH rapidly inactivated the enzyme ($t_{1/2}$ = 6 min), like NADH although at a slower pace ($t_{1/2}$ = 10 min) and not so completely; the enzyme was rapidly inactivated by $NADP^+$ ($t_{1/2}$ = 8 min), an unexpected result perhaps due to reduction of $NADP^+$ by the extracts. Figure 5B shows that this really was the case, since NADPH and NADH promoted fast and complete inactivations

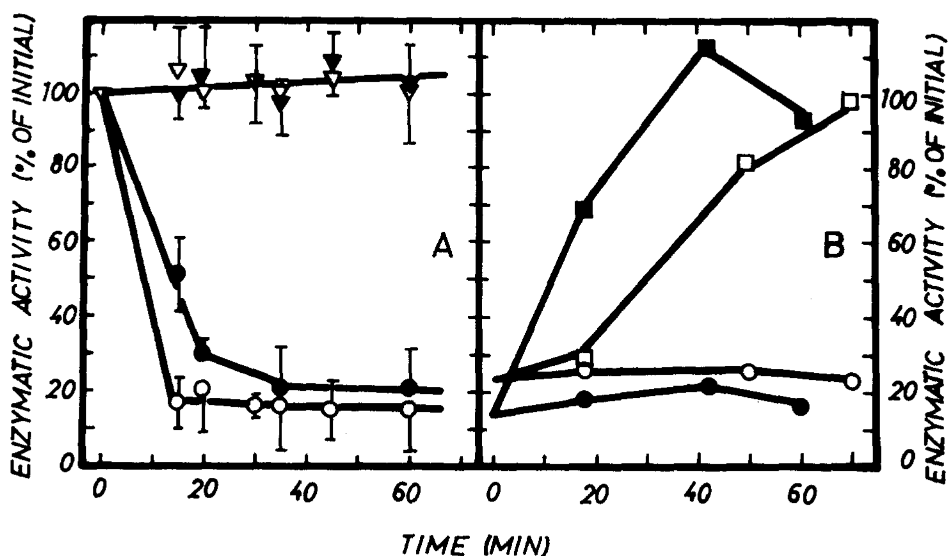

Fig. 3. Oxygen is not required for redox interconversion of glutathione reductase. A. – Inactivation: the enzyme (3.3 μg) was incubated in 20 mM Tris/HCl buffer (pH 8.5) in the absence (▽, ▼) or presence (○, ●) of 0.2 mM NADPH, both with (solid symbols) and without (open symbols) O_2 in 0.3 ml. At the times indicated the activities were determined and expressed as percentage of the initial aerobic control, 180 U/mg. Bars represent the standard error of at least three experiments. B. – Reactivation: a sample previously inactivated by NADPH without O_2, was divided in four aliquots and incubated at 30° C after addition of GSSG at 1 mM final concentration (□, ■) or buffer (○, ●), with (solid symbols) or without (open symbols) O_2. The activities were determined at the times shown and expressed (correcting for dilution) as the percentage of the initial aerobic control, 180 U/mg.

($t_{1/2}$ = 2 and 3 min respectively) after dialysis, while NAD^+ and $NADP^+$ left the enzyme unaltered. Figures 5A′ and 5B′ show the inactivation promoted by different concentrations of NAD(P)H on fresh and dialysed extracts: the fresh enzyme lost 50% activity after 30 min with 4 μM NADPH, 5 μM $NADP^+$, 350 μM NADH, and 6 mM NAD^+, while the dialysed enzyme required 1 μM NADPH or 3 μM NADH for a similar effect and no inactivation was produced by $NADP^+$ and NAD^+ up to 5 mM. The rapid inactivation observed in fresh extracts with $NADP^+$ should, then, be assigned to the NADPH produced by its reduction by different dehydrogenases and oxidizable substrates present in crude extracts. Although the inactivation by NADPH, NADH and $NADP^+$ was similar, whether or not it was reverted by GSSG, as previously shown [12–16] should be studied. Figure 6 shows the GSSG reactivation of an enzyme inactivated by each pyridine nucleotide: after full inactivation by NADPH or $NADP^+$ or partial by NADH, the enzyme was reactivated with very similar kinetics by GSSG, confirming that the inactivation by each nucleotide was similar.

Redox inactivation of glutathione reductase was also observed in yeast cell-free extracts incubated with diverse oxidizable substrates (Table 2). The glutathione reductase activity remained unaltered when fresh crude extracts were incubated without additions, with $NAD(P)^+$, or $NAD(P)^+$ plus glucose or citrate. Nevertheless, a nearly-complete inactivation was promoted by gluconate or glucose-6-P, an intermediate effect was observed with isocitrate or malate, and no inactivation was produced by lactate. The specific activity of several NAD(P)H-dependent dehydrogenases is also shown in Table 2: a direct correlation was observed between the degree of redox inactivation promoted by each oxidizable substrate and the activity of the corresponding dehydrogenase.

Discussion

Redox inactivation of glutathione reductase has

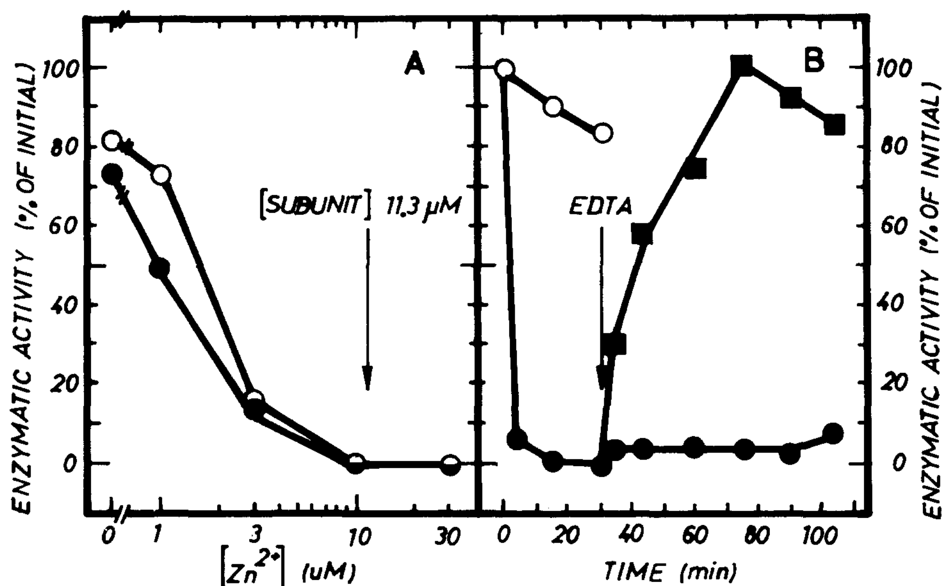

Fig. 4. Direct involvement of Zn^{2+} on the redox inactivation of glutathione reductase by NADPH. A. – Stoichiometry for Zn^{2+}: the enzyme (196 μg, 11.3 μM final subunit concentration) was incubated in buffer A with NADPH and the indicated Zn^{2+} concentrations in 0.35 ml. The activities were determined after 15 (○) and 30 (●) min incubation and expressed as percentage of the control without additions, 250 U/mg. B. – Reactivation by EDTA: the enzyme (196 μg) was incubated in buffer A in the absence (○) or the presence (●) of NADPH plus 10 μM Zn^{2+} in 0.35 ml. After 30 min incubation a 0.1 ml aliquot was made 1 mM on EDTA (■), while a inactive control was prepared with buffer (●). The activities were determined at the times shown and expressed (correcting for dilution) as the percentage of the initial control without additions, 185 U/mg.

been studied in recent years in several organisms with quite different results. While the enzyme from mouse, yeast, and *E. coli* is fast and completely inactivated by NAD(P)H [10–16], especially at very low concentrations [21, 22], the enzymes from human erythrocytes, *Anabaena*, and pea chloroplasts are only partially inactivated at a slow pace [9, 23, 24]. Their lack of sensitivity could be due to the high NADPH concentrations used, allowing the formation of $NADP^+$-EH_2 complexes reported to interfere in inactivation [21, 22], or to the presence of EDTA, absent in previous studies [10–16]. Since several metal cations are known to inhibit the reduced form of glutathione reductase [23–29], the effect of metal cations on redox inactivation was studied.

The complete protection against NADPH-inactivation of yeast glutathione reductase promoted by low concentrations of EDTA or DETAPAC (Fig. 1), with the enzyme and all solutions prepared with HPLC-grade water, indicated that metal cations could be involved in redox inactivation. The experiments summarized in Table 1 and Fig. 2 confirmed such an idea, in view of the specific potentiation by Zn^{2+}, Cd^{2+} and Ag^+ of the redox inactivation promoted by NADPH alone. It should be pointed out that the Zn^{2+} and Cd^{2+} effect (although not that of Ag^+) was not instantaneous but time-dependent (see Fig. 2), indicating that their effects were due to inactivation rather than to conventional inhibition [23–29]. In *S. thyphimurium* and *E. coli* cadmium chloride induces synthesis of adenylylated nucleotides, alarmones involved in defense against O_2 and heat shock [40, 41]; Zn^{2+} ions highly stimulate synthesis of ApppppA [40, 42], another alarmone signaling the level of oxidation in the cellular environment [40].

Several possible mechanisms could explain the role of metal cations in NADPH-inactivation of glutathione reductase: catalysis of a) thiol oxidation, or b) formation of reactive oxygen species, or c) blocking of some residues essential for the en-

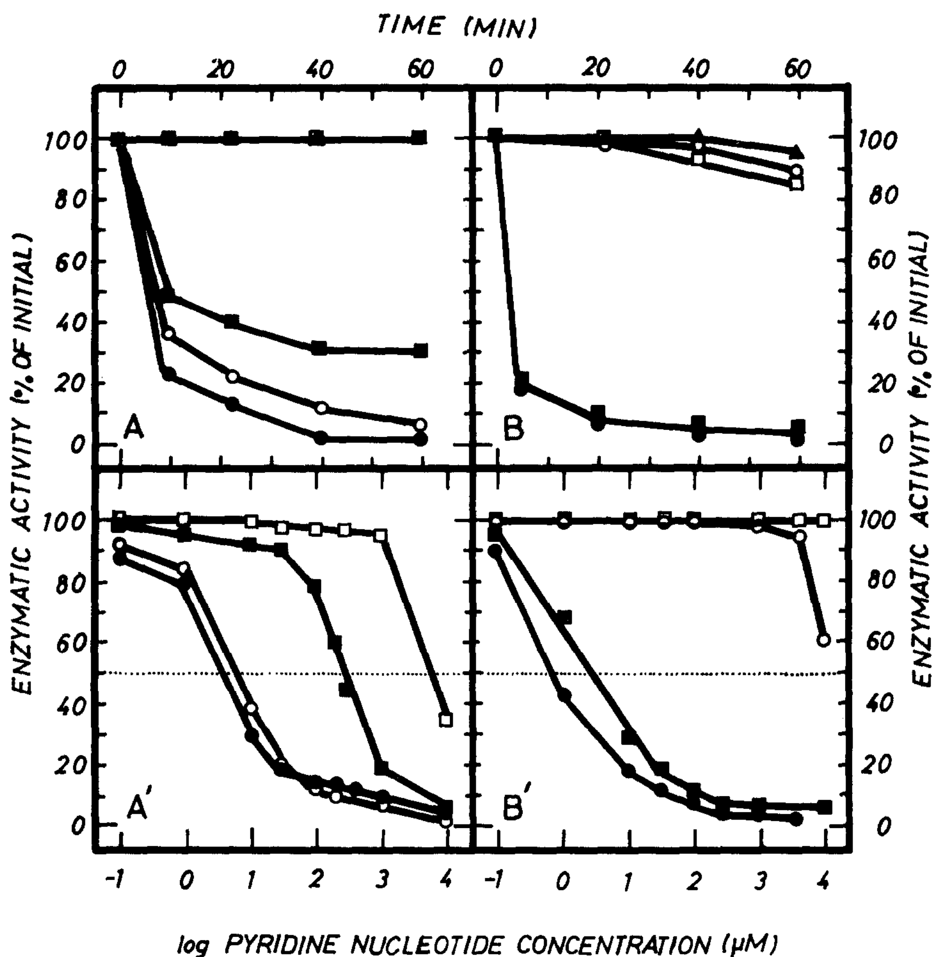

Fig. 5. Effect of different pyridine nucleotides on the redox inactivation of glutathione reductase in yeast cell-free extracts. *A:* a crude extract (132 μg prot) was incubated in buffer B without any addition (▲) or with 0.2 mM NAD^+ (□), NADH (■), $NADP^+$ (○), or NADPH (●) in 0.3 ml. Activities were determined and expressed as percentage of the initial control, 0.115 U/mg. *B:* a dialysed crude extract (96 μg prot) was treated as in A; 100% activity, 0.131 U/mg. *A':* a crude extract (36 μg prot) was incubated for 30 min in buffer B with several concentrations of NAD^+ (□), NADH (■), $NADP^+$ (○), or NADPH (●) in 0.1 ml; the activities were determined and expressed as the percentage of the initial control without additions, 0.25 U/mg. *B':* a dialysed crude extract (32 μg prot) was treated as in A'; 100% activity, 0.131 U/mg.

zyme. Thiol oxidation, the mechanism previously proposed by us for redox interconversion [15–16], is catalyzed by Fe^{2+} or Cu^{2+} [39]. However, Cu^{2+} and Fe^{2+} protected the enzyme against redox inactivation (Table 1 and Fig. 2), and DETAPAC (more selective for Fe^{2+} than EDTA) was less effective in protection against redox inactivation (Fig. 1B), excluding metal-catalyzed thiol oxidation as a possible mechanism. On the other hand, transition metals efficiently catalyze the formation of active oxygen species [43, 44] which could affect enzymatic activity [45]. In particular, Fe^{2+}-NAD(P)H complexes act as Fenton reagents converting $O_2\cdot^-$ and H_2O_2 into HO· radical [44]. If such were the base of redox inactivation, the process should disappear in anaerobiosis; the results summarized in Fig. 3 clearly exclude the participation of active oxygen species in NADPH-inactivation or in GSSG-reactivation, in agreement with our previous report [11]. The higher and faster inactivation promoted by NADPH in anaerobiosis (Fig. 3A)

has also been recently reported with the *E. coli* enzyme [21, 22].

The active site of reduced human glutathione reductase displays the two thiols corresponding to Cys 58 and Cys 63 plus the imidazole group of His 467′, which probably acts as a H^+ donor/acceptor in GSSG reduction, positionned by a H-bond to Glu 472′ [3, 4, 7, 19]. Such an arrangement could allow the binding of some metal cations, such as Zn^{2+}. The 1:1 stoichiometry between the concentrations of Zn^{2+} and glutathione reductase subunits required for complete inactivation in the presence of NADPH (Fig. 4A) suggests that those cations could block the dithiol formed at the active site of the reduced enzyme. The reactivation promoted by EDTA (Fig. 4B) also suggests a direct participation in the redox inactivation mechanism and excludes its catalytic role. In fact, many regulatory DNA-binding proteins have 'Zn fingers', with several repeating units about 30 aminoacids long folding around a Zn atom to form small independent domains interacting with DNA through several basic residues. Zinc coordinates to four highly conserved residues with sequences of either . . .CysCys. . .His His. . . or . . .CysCys. . .CysCys. . . [46].

The formation and disappearance of an erroneous disulfide at the active site of the reduced enzyme was previously proposed by us to explain the redox interconversion of glutathione reductase [10–16] based on the following findings: a) The enzyme remains inactive after dialysing the NADPH, and maintains the same MW as the active form [10, 11, 13]; b) GSSG and several thiols protect the enzyme from inactivation and reactivate [10–12]; c) The inactive enzyme retains full diaphorase activity [12]; d) A loss of the charge-transfer complex between proximal thiolate and FAD accompanies the inactivation by NADPH, while the reactivation parallels a full recovery of the 530 nm absorbance [12]. However, a close examination of the GSSG-binding site of the human enzyme does not reveal any additional Cys residue close enough to the redox-active dithiol to form the alleged erroneous disulfide [4, 6, 7]. Besides, the stoichiometric participation of Zn^{2+} or Cd^{2+} in the redox inactivation by NADPH as well as the lack of effect of Cu^{2+} or

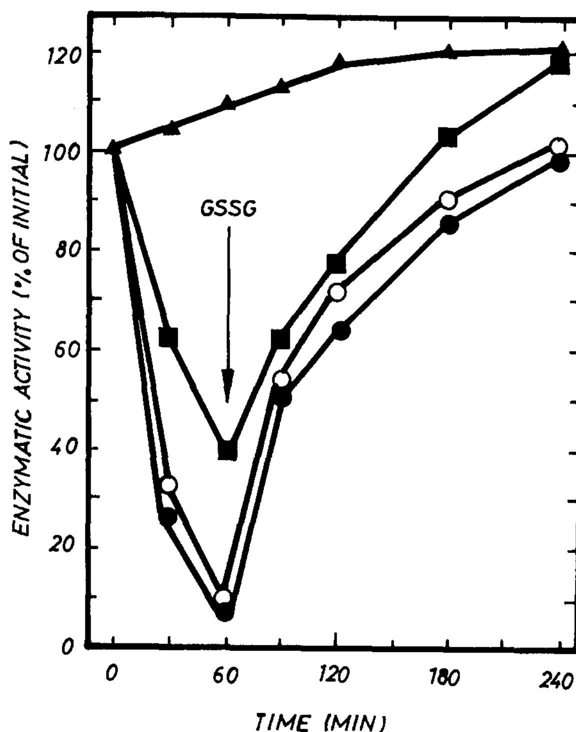

Fig. 6. Redox interconversion of glutathione reductase in yeast cell-free extracts. A yeast cell-free extract (132 μg prot) was incubated in buffer B without additions (▲) or in the presence of 0.2 mM NADH (■), $NADP^+$ (○), or NADPH (●) in 0.3 ml; after 60 min, 1 mM GSSG was added to each mixture. The activities were determined at the times indicated and expressed as the percentage of the initial control without additions, 0.115 U/mg.

Fe^{2+} also seem to rule out thiol oxidation as a possible mechanism of redox inactivation.

The disulfide bridge connecting the Cys 90 of each subunit in human glutathione reductase has been also postulated to have regulatory significance: since both subunits contribute to the active center, the enzyme could regulate itself by a kind of negative feedback by disulfide opening and subsequent dissociation [3]. The lack of such intermolecular disulfide in *E. coli* glutathione reductase [17], highly sensitive to NADPH-inactivation, rules out its possible role in the redox interconversion mechanism. Similarly, the absence in the *E. coli* enzyme [17] of the N-terminal flexible arm as well as the Cys 2 residue present in the human enzyme argue against the regulatory role proposed for the disulfide bridge that could be formed be-

tween such residue and the active site dithiol of the reduced enzyme under certain circumstances [3, 4, 8].

The inactivation of *E. coli* glutathione reductase by NAD(P)H has been recently reexamined: the inverse relationship between the final level of inactivation by NADH and enzyme concentration suggested that the process is due to dissociation of the normally dimeric enzyme into inactive monomers by separation of the overlapping active site [21, 22]. NADPH-inactivation is much faster and complete, especially at very low NADPH concentrations; formation of complexes between EH_2 and NADPH or the $NADP^+$ generated by oxidation protect from inactivation at higher NADPH concentrations, precluding a detailed analysis of the inactivation process [22]. If dissociation were the real origin of inactivation, the MW of the inactive enzyme should be halved; however, the MW of the inactive enzyme does not differ from the dimeric MW of the active form, either upon low pressure or HPLC molecular exclusion chromatography with the mouse [10], yeast [11], or *E. coli* [13, 22] enzymes. In addition, the disulfide bridge engineered in the *E. coli* enzyme at the twofold axis, which blocks the enzyme in its dimeric form [20], does not decrease the sensitivity of the mutant enzyme towards NADPH inactivation, clearly indicating that redox inactivation can not be due to a simple dissociation of the dimer into inactive monomers [20].

Based on the results summarized in the present paper we now suggest that redox inactivation could arise from a metal cation (such as Zn^{2+} or Cd^{2+}) blocking the dithiol formed at the active site of the reduced dimeric enzyme [1, 3–8]. In such a case, removal of the blocking metal cation should reactivate the enzyme, as was clearly observed in the experiment summarized in Fig. 4B. The decrease of the 530 nm absorbance during redox inactivation [12] would not be due to disulfide formation, as initially proposed [12, 15, 16], but to its blocking by the cation. Reactivation by thiols could be explained by complexation of such a cation [39], parallel to the reactivation promoted by EDTA. Reactivation by GSSG could be due to reoxidation of active site dithiol while the cation was uncomplexed. The inverse relationship between redox inactivation and enzyme concentration could be assigned to a decrease in the ratio between the metal cation and the glutathione reductase subunit. Instead of other transition metals, Zn could have been chosen during evolution due to its absence of redox chemistry, that is, its inability to produce active damaging radicals [46].

It could be argued that the direct involvement of metal cations on the redox inactivation of glutath-

Table 2. Effect of different oxidizable sustrates on glutathione reductase activity in *S. cerevisiae* cell-free extracts

Addition	GSSG-rase activity (%)			Dehydrogenase activity	
Time (min)	0	30	60	Name	(U/mg)
None	100	116	102		
$NAD(P)^+$	105	109	85		
$NAD(P)^+$ + Citrate	115	119	116		
$NAD(P)^+$ + Glucose	97	111	114		
$NAD(P)^+$ + Glucose-6-P	105	45	6	G-6-PDH ($NADP^+$)	0.38
$NAD(P)^+$ + Gluconate	108	9	9	6-P-GDH ($NADP^+$)	0.28
$NAD(P)^+$ + Isocitrate	104	41	31	IDH ($NADP^+$)	0.11
				IDH (NAD^+)	0.08
$NAD(P)^+$ + Malate	102	88	33	MDH (NAD^+)	0.03
$NAD(P)^+$ + Lactate	98	109	87	LDH (NAD^+)	<0.01

A yeast cell-free extract (90 μg protein) was incubated at 30° C in 0.5 M Tris-HCl buffer (pH 8.0) and 0.2 ml total volume with 0.1 M of the oxidizable sustrates and 1 μM $NAD(P)^+$ as indicated. The glutathione reductase activity was determined at the times shown and expressed as percentage of the initial control without additions, 0.144 U/mg. the oxidizable sustrates were prepared as 0.25 M stock solutions and adjusted to pH 7.5. The different dehydrogenase activities were assayed as indicated in the experimental section.

ione reductase could be a side effect observed only with pure enzymes, but not *in vivo* where a wide variety of metal chelators (nucleic acids, proteins, nucleotides, ... etc.) could exert a protective role similar to that of EDTA. The physiological significance of the redox interconversion of glutathione reductase has been confirmed in the present work with *S. cerevisiae* cell-free extracts: reduced pyridine nucleotides rapidly inactivate the enzyme both in freshly prepared or dialyzed crude extracts (Fig. 5), although in the latter case NADPH and NADH behaved similarly; the fast inactivation by $NADP^+$ observed in fresh crude extracts is due to the NADPH produced after its reduction by different dehydrogenases and oxidizable substrates present in the extracts, since such inactivation disappeared after extensive dialysis (Figs 5B and 5B′). Redox inactivation of glutathione reductase has been also observed in yeast cell-free extracts incubated with different oxidizable substrates, particularly with those as glucose-6-P, gluconate or isocitrate which upon oxidation yield mainly NADPH (Table 2); the direct correlation found between the extent of inactivation promoted by each substrate and the specific activity of its corresponding dehydrogenase confirms that the redox inactivation is due to the NADPH formed *in vitro*. In summary, redox interconversion of glutathione reductase can be also observed in the highly complex situation represented by the cell-free extracts, and whatever their chelating ability may be, there is still enough concentration of metal cations (as Zn^{2+} or Cd^{2+}) available to account for the interconversion. The physiological significance of redox interconversion has been previously established with the *E. coli* glutathione reductase, in cell-free extracts [13], permeabilized, or intact cells [14], where the enzyme is rapidly inactivated *in vivo* upon incubation with malate and other NADPH-producing carbon sources, being protected by diamide, a compound which oxidizes glutathione [14–16].

The physiological significance of glutathione reductase interconversion can be better understood when related to oxidative stress. Glutathione reductase and the NADPH-generating pathway are central to antioxidant defense and tightly coordinated [45, 47]. In normal conditions (high |NADPH| and low |GSSG|), glutathione reductase would be partially inactive [15, 16]. Actually, several cellular functions are activated by GSSG making necessary the maintenance of higher than threshold concentrations [48], incompatible with a fully active enzyme; under such circumstances the activity of the oxidative phase of the pentose phosphate pathway is rather low [15, 16, 47]. When oxidative stress develops, the GSSG concentration rapidly increases reactivating glutathione reductase which subsequently increases the $NADP^+$ concentration and deinhibits the pentose-phosphate pathway, thus producing more NADPH to counteract oxidative stress [15, 16, 45, 47].

Acknowledgements

This work was supported by grant number PB86-0146 from the Comisión Interministerial de Ciencia y Tecnología, and by another from the Plan Andaluz de Investigación, España.

References

1. Williams CH Jr: Flavin-containing dehydrogenases and oxidases. In: PD Boyer (ed) The Enzymes vol *XIII*, Academic Press, New York. 1976, pp 89–173
2. Krauth-Siegel RL, Blatterspiel R, Saleh M, Schiltz E, Schirmer RH, Untücht-Grau R: Glutathione reductase from human erythrocytes. The sequences of the NADPH domain and of the interface domain. Eur J Biochem 121: 259–267, 1982
3. Thieme R, Pai EF, Schirmer RH, Schulz GE: Three-dimensional structure of glutathione reductase at 2 Å resolution. J Mol Biol 152: 763–782, 1981
4. Karplus PA, Schulz GE: Refined structure of glutathione reductase at 1.54 Å resolution. J Mol Biol 195: 701–729, 1987
5. Pai EF, Karplus PA, Schulz GE: Crystallographic analysis of the binding of NADPH, NADPH fragments, and NADPH analogues to glutathione reductase. Biochemistry 27: 4465–4474, 1988
6. Karplus PA, Pai EF, Schultz GE: A crystallographic study of the glutathione binding site of glutathione reductase at 0.3 nm resolution. Eur J Biochem 178: 693–703, 1989
7. Karplus PA, Schulz GE: Substrate binding and catalysis by glutathione reductase as derived from refined enzyme: sub-

strate crystal structures at 2 Å resolution. J Mol Biol 210: 163–180, 1989
8. Pai EF, Schulz GE: The catalytic mechanism of glutathione reductase as derived from X-ray diffraction analyses of reaction intermediates. J Biol Chem 258: 1752–1757, 1983
9. Worthington DJ, Rosemeyer MA: Glutathione reductase from human erythrocytes. Catalytic properties and aggregation. Eur J Biochem 67: 231–238, 1976
10. López-Barea J, Lee, C-Y: Mouse-liver glutathione reductase. Purification, kinetics and regulation. Eur J Biochem 98: 487–499, 1979
11. Pinto MC, Mata AM, López-Barea J: Reversible inactivation of *S. cerevisiae* glutathione reductase under reducing conditions. Arch Bioch Biophys 228: 1–12, 1984
12. Pinto MC, Mata AM, López-Barea J: The redox interconversion mechanism of *Saccharomyces cerevisiae* glutathione reductase. Eur J Biochem 151: 275–281, 1985
13. Mata AM, Pinto MC, López-Barea J: Redox interconversion of glutathione reductase from *Escherichia coli*. A study with pure enzyme and cell-free extracts. Mol Cell Biochem 67: 65–76, 1985
14. Mata AM, Pinto MC, López-Barea J: Redox interconversion of *Escherichia coli* glutathione reductase. A study with permeabilized and intact cells. Mol Cell Biochem 68: 121–130, 1985
15. López-Barea J, Bárcena JA: Redox control of glutathione and thioredoxin reductases. In: FL Crane, DJ Morre, HA Low (eds) Plasma membrane oxidoreductases in control of animal and plant growth. Plenum Publishing Corp, New York, 1988, pp 349–358
16. López-Barea J, Bárcena JA, Bocanegra JA, Florindo J, García-Alfonso C, López-Ruiz A, Martínez-Galisteo E, Peinado J: Structure, mechanism, functions, and regulatory properties of glutathione reductase. In: J Viña (ed) in CRC Handbook of Glutathione: Metabolism and Physiological Functions. CRC Press, Boca Raton, in press
17. Greer S, Perham RN: Glutathione reductase from *Escherichia coli*: cloning and sequence analysis of the gene and relationship to other flavoprotein disulphide oxidoreductases. Biochemistry 25: 2736–2742, 1986
18. Berry A, Scrutton NS, Perham RN: Switching kinetic mechanism and putative proton donor by directed mutagenesis of glutathione reductase. Biochemistry 28: 1264–1269, 1989
19. Deonarain MP, Berry A, Scrutton NS, Perham RN: Alternative proton donors-acceptors in the catalytic mechanism of the glutathione reductase of *Escherichia coli*. The role of histidine-439 and tyrosine-99. Biochemistry 28: 9602–9607, 1989
20. Scrutton NS, Berry A, Perham RN: Engineering of an intersubunit disulphide bridge in glutathione reductase from *Escherichia coli*. FEBS Lett 241: 46–50, 1988
21. Arscott LD, Drake DM, Williams CH Jr: Inactivation of two-electron reduced *Escherichia coli* glutathione reductase by NADPH and NADH. In: DE Edmonson, DB McCormick (eds) Flavins and Flavoproteins 1987. Walter de Gruyter, Berlin, 1987, pp 75–78
22. Arscott LD, Drake DM, Williams CH Jr: Inactivation-reactivation of two-electron reduced *Escherichia coli* glutathione reductase involving a dimer-monomer equilibrium. Biochemistry 28: 3591–3598, 1989
23. Serrano A, Rivas J, Losada M: Purification and properties of glutathione reductase from the cyanobacterium *Anabaena* sp. Strain 7119. J Bacteriol 158: 317–324, 1984
24. Connell JP, Mullet JE: Pea chloroplast glutathione reductase: purification and characterization. Plant Physiol 82: 351–356, 1986
25. Mize CE, Langdom RG: Hepatic glutathione reductase. I. Purification and general kinetic properties. J Biol Chem 237: 1589–1595, 1962
26. Chung YC, Hurlbert RE: Purification and properties of glutathione reductase of *Chromatium vinosum*. J Bacteriol 123: 203–211, 1975
27. Halliwell B, Foyer CH: Properties and physiological function of a glutathione reductase purified from spinach leaves by affinity chromatography. Planta 139: 9–17, 1978
28. Rafter GW: Copper inhibition of glutathione reductase and its reversal with gold thiolates, thiol, and disulfide compounds. Biochem Med 27: 381–391, 1982
29. Shigeoka S, Onishi T, Nakano Y, Kitaoka S: Characterization and physiological function of glutathione reductase in *Euglena gracilis* z. Biochem J 242: 511–515, 1987
30. Davis BJ: Disc electrophoresis II. Method and application to human serum proteins. Ann NY Acad Sci 121: 404–427, 1964
31. Lohor GW, Waller HD: Glucose-6-phosphate dehydrogenase. In: HU Bergmeyer (ed) Methods of Enzymatic Analysis 2nd ed. Academic Press, New York, 1974, pp 636–643
32. King J: 6-Phosphogluconate dehydrogenase. In: HU Bergmeyer (ed) Methods of Enzymatic Analysis 2nd ed. Academic Press, New York, 1974, pp 632–635
33. Bergmeyer HU, Bernt E: Isocitrate dehydrogenase. UV-assay. In: HU Bergmeyer (ed) Methods of Enzymatic Analysis 2nd ed. Academic Press, New York, 1974, pp 625–627
34. Kornberg A, Pricer WE Jr: Di- and triphosphopyridine nucleotide isocitric dehydrogenase in yeast. J Biol Chem 189: 123–136, 1951
35. Bergmeyer HU, Bernt E: Malate dehydrogenase. UV-assay. In: HU Bergmeyer (ed) Methods of Enzymatic Analysis 2nd ed. Academic Press, New York, 1974, pp 613–617
36. Bergmeyer HU, Bernt E: Lactate dehydrogenase. UV-assay with pyruvate and NADH. In: HU Bergmeyer (ed) Methods of Enzymatic Analysis 2nd ed. Academic Press, New York, 1974, pp 574–579
37. Lowry OH, Rosebrough MJ, Farr AL, Randall RJ: Protein measurements with the Folin-phenol reagent. J Biol Chem 193: 265–275, 1951
38. Buettner GR, Doherty TP, Patterson LK: The kinetics of the reaction of superoxide radical with Fe(III) complexes of

EDTA, DETAPAC and HEDTA. FEBS Lett 158: 143–146, 1983
39. Jocelyn PC: Biochemistry of the SH Group. Academic Press, London, 1972, pp 94–100
40. Bochner BR, Lee PC, Wilson SW, Cutler CW, Ames BN: ApppppA and related adenylylated nucleotides are synthesized as a consequence of oxidation stress. Cell 37: 225–232, 1984
41. Morgan RW, Christman MF, Jacobson FS, Storz G, Ames BN: Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. Proc Natl Acad Sci USA 83: 8059–8063, 1986
42. Zamecnik P: Diadenosine 5′,5′′′-P^1,P^4-tetraphosphate (Ap_4A): its role in cellular metabolism. Anal Biochem 134: 1–10, 1983
43. Malmström BG: Enzymology of oxygen. Ann Rev Biochem 51: 21–59, 1982
44. Dunford HB: Free radicals in iron-containing systems. Free Rad Biol 3: 405–421, 1987
45. Sies H: Biochemistry of oxidative stress. Angew Chem Int Ed Engl 25: 1058–1071, 1986
46. Klug A, Rhodes D: 'Zinc fingers': a novel protein motif for nucleic acid recognition. Trends Biochem Sci 12: 464–469, 1987
47. Llobell A, López-Ruiz A, Peinado J, López-Barea J: Glutathione reductase directly mediates the stimulation of yeast glucose-6-phosphate dehydrogenase by GSSG. Biochem J 249: 293–296, 1988
48. Kosower NS, Kosower EM: The glutathione status of the cell. Int Rev Cytol 54: 109–160, 1978

Address for offprints: J. López-Barea, Departamento de Bioquímica y Biología Molecular, (Unidad de Veterinaria), Universidad de Córdoba, Avda. de Medina Azahara s/n, 14071 Córdoba, Spain