# Purification by Affinity Chromatography of Glutathione Reductase (EC 1.6.4.2) from *Escherichia coli* and Characterization of such Enzyme

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The glutathione reductase from *Escherichia coli* strain S33 was purified to homogeneity by a simple and fast procedure consisting of two affinity chromatography steps. After 40–80% ammonium sulfate fractionation, the enzyme was adsorbed to an $N^6$-2′,5′-ADP-Sepharose affinity column from which it was specifically eluted by a 0–10 mM $NADP^+$ linear gradient. The enzyme was finally purified to homogeneity after a second affinity chromatography step in a $C^8$-ATPR-Sepharose column, from which it was eluted by means of the same $NADP^+$ gradient. Starting from 182 g of *E. coli* cells, 6.9 mg of pure enzyme was obtained after a 2632-fold purification, with a total yield of 63%.

The pure enzyme showed a specific activity of 361 U/mg, and its absorption spectrum was characteristic of a flavoprotein, with an $A_{272}/A_{450}$ of 7.84. The enzyme was a dimer with a molecular weight 109 000 and 40 Å hydrodynamic radius. The optimum pH were 7.5 and 4.5 with NADPH and NADH, respectively, as reductants. Apparent $K'_m$ values of 16, 377, and 66 μM were determined at pH 7.5 for NADPH, NADH, and GSSG, respectively. Upon storage the enzyme was stable at pH values ranging from 7.5 to 9.5, being additionally stabilized by FAD, $NADP^+$, dithiothreitol, or glycerol. The pure enzyme was quite heat stable, denaturing significantly only after 10 min at 70 °C. A marked activity loss was observed however, even at 0 °C, in the presence of 20 μM NADPH. The enzyme was inactivated by low concentrations of *para*-hydroxymercuribenzoate; the sensitivity towards such mercurial was greatly enhanced after reduction of the enzyme by NADPH.

## Introduction

Glutathione reductase (EC 1.6.4.2) is an ubiquitous flavoenzyme [1, 2] which catalyzes the reduction of glutathione disulfide by NADPH, according to the following equation:

$$GSSG + NADPH + H^+ \rightleftharpoons 2GSH + NADP^+ .$$

In accordance with the very important functions played by GSH in cellular metabolism [3], the enzyme has been purified and studied in a wide variety of organisms, including bacteria, animals, plants, and specially humans [1], where glutathione reductase deficiency has been found associated with some kinds of hemolytic anemias [3]. In fact, the three-dimensional structure of human erythrocyte glutathione reductase has been elucidated by X-ray crystallography at 2 Å resolution [4], and its complete aminoacid sequence [5–8] and catalytic mechanism [9] have been established.

Since the first study by Asnis in 1955 [10], the glutathione reductase from *Escherichia coli* has been previously purified by two different groups. Thus, in 1967 Williams *et al.* [11] described the purification of thioredoxin, thioredoxin reductase, lipoamide dehydrogenase, and glutathione reductase using gel filtration and ion-exchange chromatography, and reported an 80% purity for their final glutathione reductase preparation. Ten years later, Pigiet and Conley [12] devised a new purification scheme for thioredoxin, thioredoxin reductase, and glutathione reductase, based on affinity chromatography in $N^6$-2′,5′-ADP-Sepharose completed by

*Abbreviations:* TEMED, tetramethyl-ethylene-diamine; $N^6$-2′,5′-ADP-Sepharose, $N^6$-(6-aminohexyl)-amino-2′,5′-bisphosphate-Sepharose; $C^8$-ATPR-Sepharose, 8-(6-aminohexyl)-amino-phospho-adenosine diphosphoribose.

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several steps of hydrophobic, ion-exchange, and gel filtration chromatography. However since both purification procedures were designed for the simultaneous purification of several proteins, they were rather complicated and the yield was somewhat affected. Moreover, no extensive characterization of *E. coli* glutathione reductase has been previously published.

It has been recently reported that pure mouse-liver glutathione reductase can be regulated by redox interconversion, oscillating between two states, active and inactive, according to the levels of its reduced cofactor (NADPH) and oxidized substrate (GSSG) [13]. Similar regulatory behaviour has been observed with pure yeast glutathione reductase, which is reversibly inactivated by NADPH and other reduced compounds in a process dependent on time, temperature, concentration, and pH. The yeast enzyme can be protected or reactivated by oxidized glutathione, ferricyanide, and mono- or dithiols [14].

The present paper reports a fast and very convenient procedure for the purification of *Escherichia coli* glutathione reductase based on two sequential steps of affinity chromatography, in $N^6$-2′,5′-ADP-Sepharose and $C^8$-ATPR-Sepharose, respectively. An extensive characterization of the *E. coli* enzyme, covering both the molecular and kinetic properties, is also included. The purification procedure here reported yields enough pure enzyme as to undertake the *in vitro* study of its redox interconversion, first step before the *in situ* and *in vivo* characterization of the same phenomenon, which at the present time are being carried out.

## Experimental

### *Reagents*

Bacto tryptone, bacto yeast extract, and bacto agar used for the preparation of liquid and solid growth media were supplied by DIFCO. Potassium phosphate, sodium citrate, citric acid, sodium chloride, EDTA, glycerol, sodium azide, copper sulfate, sodium carbonate, and the Folin-Ciocalteau reagent were purchased from MERCK, Darmstadt. NADPH, FAD, Trizma base, 2-mercaptoethanol, blue dextran, beef-liver catalase, rabbit-muscle lactate dehydrogenase, bovine serum albumin, ovoalbumin, α-chymotrypsinogen-A, bovine heart cytochrome-*c*, Sephadex G-150, CNBr-activated Sepharose 4B, acrylamide, N,N′-methylene-bis-acrylamide, TEMED, riboflavine, ammonium persulfate, para-hydroxymercuribenzoate, and Coomassie brilliant blue G-250 were purchased from SIGMA, London. NADH and dithiothreitol were supplied by BOEHRINGER, Mannheim. $N^6$-2′,5′-ADP-Sepharose was purchased from PHARMACIA, Uppsala. $C^8$-ATPR-Sepharose was prepared as previously described [13]. All other chemicals were of the highest purity and used without further purification.

### *Organism studied, growth conditions, and cell-free extract preparation*

The glutathione reductase was purified from *Escherichia coli* S33, Hfr, *phoA* which was a generous gift from the microorganisms collection of the Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Sevilla (Spain). The bacteria was grown for 6 hours at 37 °C in 5 l erlenmeyer flasks containing 4 l of Luria medium [15], after inoculating with 50 ml of an overnight culture in the same medium. The cultures were stirred by bubling air through them.

The cells were collected by centrifugation at 4 °C for 5 min at 15 000 × *g* and washed once with 20 mM potassium phosphate buffer pH 7.5, containing 1 mM dithiothreitol, 1 mM EDTA, and 10 μM FAD (buffer A). The packed cells were stored frozen at −18 °C for use later in enzyme purification. For cell-free extract preparation the cells were thawed, resuspended with 7 volumes of buffer A, and disrupted by sonic disintegration in 250 ml batches, at 4 °C during 30 min, in an apparatus MSE MK2. The broken cell suspension was subsequently cleared by centrifugation at 4 °C for 30 min at 15 000 × *g*.

### *Glutathione reductase assay*

The enzymatic activity was determined following at 340 nm the decrease of NADPH absorbance at 30 °C using a Spectronic-2000, uv-visible, double-beam spectrophotometer. The reaction mixture contained in 1 ml final volume 96 mM potassium phosphate buffer pH 7.5, 2.5 mM GSSG, and 0.125 mM NADPH. The assay was started by addition of 0.01 − 0.1 ml of enzyme solution. One unit of enzyme activity is defined as the amount of enzyme

which reduces one micromole of GSSG per minute under the conditions described above. Specific activities are expressed as units of enzyme per milligram of protein.

*Protein determination*

The protein concentration was estimated by the biuret-phenol method [16] using above serum albumin as standard.

*Polyacrylamide gel electrophoresis*

Analytical polyacrylamide disc gel electrophoresis was performed by the method of Davis [17], and the protein bands stained by the Coomassie blue G-250/perchloric acid procedure [18]. Electrophoresis under denaturing conditions and gel staining were performed according to the method of Weber *et al.* [19], using as molecular weight marker a mixture, supplied by BDH, formed by covalent cross-linking of a protein whose monomer molecular weight was 14 300.

*Absorption spectrum*

The absorption spectrum of pure glutathione reductase was recorded after removing the contaminant $NADP^+$ and FAD by extensive dialysis of 0.12 mg of pure enzyme, dissolved in buffer A containing 10% glycerol and 5 mM $NADP^+$, against $28 \times 100$ ml of 20 mM potassium phosphate buffer pH 7.5, containing 1 mM dithiothreitol and 1 mM EDTA (buffer B).

*Molecular weight and hydrodynamic radius of the native enzyme*

The chromatographic behaviour of native *E. coli* glutathione reductase was studied using a Sephadex G-150 column ($1.6 \times 82$ cm) equilibrated with 100 mM potassium phosphate buffer pH 7.5, containing 5 mM 2-mercaptoethanol and 1 mM EDTA (buffer C). The molecular weight of the enzyme was estimated according to the procedure of Andrews [20], and its hydrodynamic radius measured by the procedure of Siegel and Monthy [21], using catalase, ovoalbumin, α-chymotrypsinogen-A, and cytochrome *c* as standards.

## Results

*Purification procedure for Escherichia coli glutathione reductase and criteria of purity*

In order to simplify the experimental conditions, all the operations during the purification were performed at 4 °C. In addition, centrifugations at $27000 \times g$ were employed whenever necessary.

The starting material for enzyme purification was 182 g of pelleted *E. coli* cells, grown in succesive batches up to a total of 100 liters of Luria broth, as described in Materials and Methods. The crude extract, containing 29 g of total protein, was then purified by ammonium sulfate fractionation between 40 and 80% saturation, maintaining the pH of the mixture at 7.5 by addition of 5 M $K_2HPO_4$ while the solid ammonium sulfate was being added. The pellet obtained after the second centrifugation was dissolved in buffer A and dialyzed for 18 hours against $2 \times 17$ liters of the same buffer.

The retentate was centrifuged, and 1250 ml of the clear supernatant loaded into a $N^6$-2′,5′-ADP-Sepharose column ($2.2 \times 12$ cm), equilibrated with buffer A containing 10% glycerol. As shown in Figure 1, the 3 436 units of glutathione reductase introduced into the column were efficiently retained by the affinity gel, while most of the contaminating

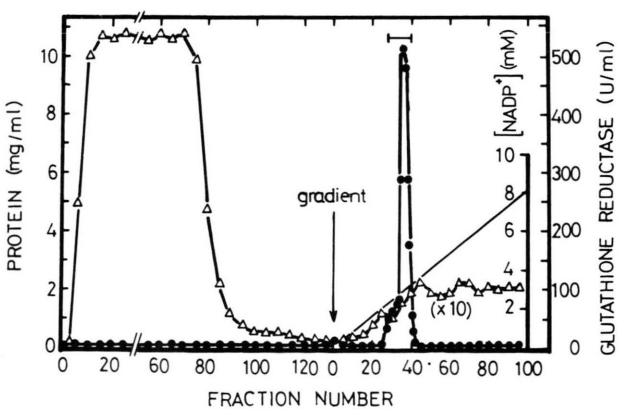


Fig. 1. Affinity chromatography of *E. coli* glutathione reductase in $N^6$-2′,5′-ADP-Sepharose. The enzyme, 3 436 units in 1250 ml total volume, was loaded into the affinity column previously equilibrated as described in the Results. After washing the gel with the same buffer, the enzyme was released by a $NADP^+$ linear gradient. Fractions containing 16.2 and 1.62 ml were collected during the adsorption and elution respectively (△–△, protein concentration; ●–●, glutathione reductase activity). The segment indicates the fractions pooled.

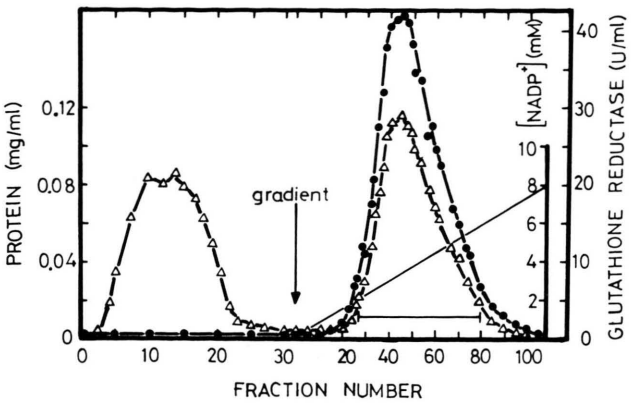


Fig. 2. Further purification of *E. coli* glutathione reductase by affinity chromatography in $C^8$-ATPR-Sepharose. The enzyme, 2 944 units in 21.5 ml total volume, was loaded into the affinity column previously equilibrated as described in the Results. After washing the gel with the same buffer, the enzyme was released by a $NADP^+$ linear gradient. Fractions containing 16.2 and 1.62 ml were collected during the adsorption and elution, respectively (△–△, protein concentration; ●–●, glutathione reductase activity). The segment indicates the fractions pooled.

proteins were eluted. After washing the column with 920 additional ml of the same buffer, a 0 – 10 mM $NADP^+$ linear gradient prepared in the equilibrating buffer (100 ml + 100 ml) was used to elute the enzyme. A very sharp peak of glutathione reductase activity could be observed, indicating that the elution had been also very specific. The fractions with activity were pooled and dialyzed against a total of 10 × 1 liters of the equilibrating buffer. When the dialysate was examined by polyacrylamide gel electrophoresis, one main protein band was observed, together with three additional contaminating proteins.

In order to further purify the preparation to homogeneity, the dialysate was submitted to a second affinity chromatography step in a different gel [13], as summarized in Figure 2. The 2 944 units of glutathione reductase still remaining (with 12.2 mg of total protein) wre loaded into a $C^8$-ATPR-Sepharose column (2.2 × 7 cm) equilibrated with buffer A containing 10% glycerol. Again the glutathione reductase activity was completely adsorbed onto the affinity gel, while the contaminating proteins which had coeluted with our enzyme in the previous step were eluted from the column, although somewhat retarded. After washing the gel with 450 additional ml of the same buffer, the glutathione reductase was eluted by means of a 0 – 10 mM $NADP^+$ linear gradient in the same buffer (100 ml + 100 ml). A broad peak of enzymatic activity could be detected, with a maximum centered around 3.5 mM $NADP^+$. The fractions with activity were then pooled and concentrated by ultrafiltration. At this stage the enzyme was homogeneous, as indicated by the single protein band observed upon analytical gel electrophoresis, either in non-denaturing conditions or in the presence of SDS.

Table I summarizes the purification procedure described in the present paper for the fast purification of glutathione reductase from the bacteria *Escherichia coli.*

### *Molecular and kinetic constants of E. coli glutathione reductase*

The absorption spectrum of pure *Escherichia coli* glutathione reductase free of contaminating nucleotides, shown in Figure 3, corresponds to that of a flavoprotein with absorption peaks at 272, 355, and 450 nm, minima at 320 and 392 nm, and a shoulder at 480 nm. The spectrum shows a somewhat high absorbance beyond 500 nm, probably due to the

Table I. Purification of glutathione reductase from *Escherichia coli.*

| Purification step | Volume [ml] | Total activity [U] | Total protein [mg] | Yield [%] | Specific activity [U/mg] | Purification (fold) |
|---|---|---|---|---|---|---|
| Crude extract | 1206 | 3 968 | 29 040 | 100 | 0.137 | 1 |
| 40 – 80% $(NH_4)_2SO_4$ | 1250 | 3 436 | 13 650 | 86 | 0.252 | 1.8 |
| $N^6$-2′,5′-ADP-Sepharose | 21.5 | 2 944 | 12.2 | 74 | 241.3 | 1 761.3 |
| $C^8$-ATPR-Sepharose | 80 | 2 488 | 6.9 | 63 | 360.6 | 2 632.1 |

In all steps the enzyme was dissolved in 20 mM potassium phosphate buffer pH 7.5 containing 1 mM dithiothreitol, 1 mM EDTA, and 10 μM FAD (buffer A) to which 10% glycerol had been added.

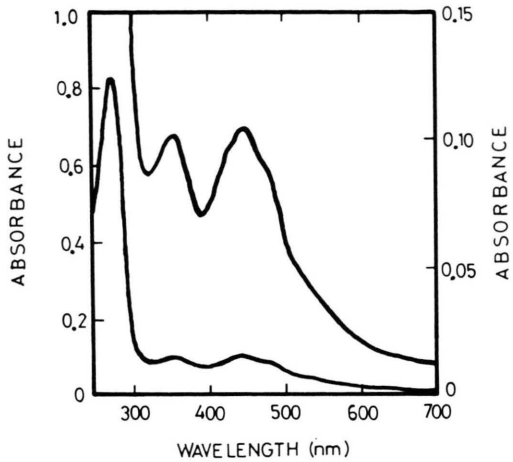


Fig. 3. Absorption spectrum of pure *E. coli* glutathione reductase. Protein concentration: 0.575 mg/ml dissolved in buffer B.

Table II. Molecular and kinetic constants of glutathione reductase from *Escherichia coli.*

| | |
|---|---|
| $A_{272}/A_{450}$ | 7.84 |
| $A_{355}/A_{450}$ | 0.97 |
| $A_{392}/A_{450}$ | 0.68 |
| Native molecular weight | 109 600 |
| Subunit molecular weight | 55 000 |
| Number of subunits | 2 |
| Hydrodynamic radius [Å] | 40 |
| Optimum pH (NADPH as reductant) | 7.5 |
| Optimum pH (NADH as reductant) | 4.5 |
| $K'_m$ for NADPH, pH 7.5 [μM] | 16 |
| $K''_m$ for NADH, pH 7.5 [μM] | 377 |
| $K'_m$ for GSSG, pH 7.5 [μM] | 66 |

presence of dithiothreitol in the buffer, which could have produced a partial reduction of the enzyme [1].

Table II summarizes the absorbance ratios characteristic of *E. coli* glutathione reductase spectrum. The table includes also some of the molecular and kinetic constants of the pure protein, as its molecular weight, number of subunits, hydrodynamic radius, optimum pH with different electron donors, and apparent $K_m$ values. Neither cysteamine nor cystine were effective as electron acceptors instead of GSSG, the physiological oxidant of the enzyme.

When assayed at the optimum pH of 7.5, a marked inhibition was found at high NADPH concentrations, as shown in Figure 4A. A significant substrate inhibition could be also observed at high GSSG concentrations under the above described conditions, as shown in Figure 4B.

The stability of glutathione reductase was also studied under different conditions. The enzyme was highly stable upon storage at 4 °C when dissolved in buffers of pH ranging between 7.5 and 9.5. A significant decrease of activity was however noticed when stored at pH values below 7.5. The enzymatic stability increased upon addition of FAD (the prosthetic group), $NADP^+$ (a product of the enzymatic reaction), dithiothreitol or glycerol to the storage medium. The enzyme was therefore stored frozen at −18 °C, in the dark, dissolved in buffer A containing 10% glycerol and 5 mM $NADP^+$. Under these conditions the enzyme was stable at least for one year. Before utilization, the enzyme was extensively dialyzed against the appropriate buffer.

Figure 5 shows the effect of temperature on the activity of *E. coli* glutathione reductase incubated under different conditions. While the enzyme in crude extracts was highly stable, loosing significantly activity only after 10 min incubation at 70 °C, the stability of the pure enzyme was markedly lower. This effect could be attributed to the lower protein concentration present in the experiment with pure

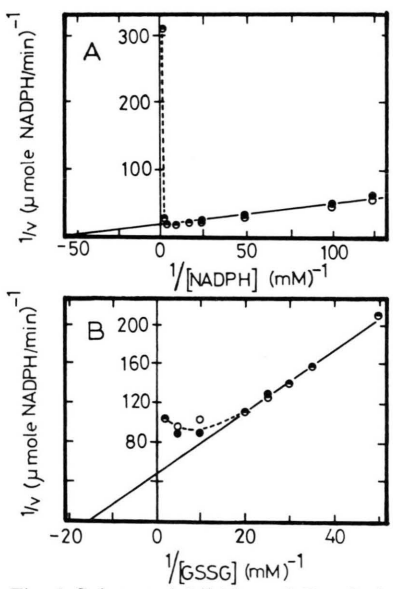


Fig. 4. Substrate inhibition of *E. coli* glutathione reductase by high NADPH and GSSG concentrations. A) The enzymatic activity of 0.13 μg of pure glutathione reductase was determined in the conditions described in the Experimental section at the NADPH concentrations indicated in the figure. B) The enzymatic activity of 0.06 μg of pure glutathione reductase was determined at the GSSG concentrations indicated in the figure (○ and ● correspond to different experiments).

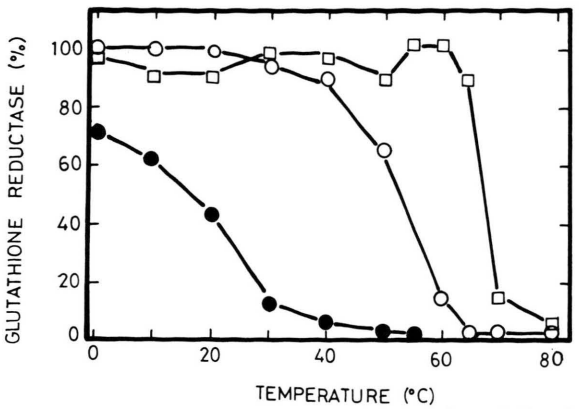


Fig. 5. Effect of temperature on the inactivation of *E. coli* glutathione reductase incubated under different conditions. One μg of pure glutathione reductase (○–○), or 2.4 mg of protein from a crude extract (□–□) were heated for 10 min at the temperatures indicated in 20 mM potassium phosphate buffer pH 7, containing 1 mM EDTA, 1 mM dithiothreitol, and 10 μM FAD, in 0.15 ml final volume. The samples were subsequently chilled at 0 °C and the remaining glutathione reductase activity assayed. The effect of NADPH (●–●) was studied using 0.24 μg of pure glutathione reductase dissolved in 50 mM Tris-HCl buffer pH 8, containing 1 mg/ml bovine serum albumin and 20 μM NADPH, in 0.4 ml final volume. One hundred per cent activity corresponded to 0.6, 1.6, and 0.52 U/ml for the pure enzyme, crude and extract, and pure enzyme plus NADPH, respectively.

enzyme, as confirmed when the incubation of the pure glutathione reductase was carried out in the presence of 1 mg/ml bovine serum albumin (results not shown). However, when the pure enzyme was heated in the presence of bovine serum albumin and 20 μM NADPH, the enzymatic activity was almost completely lost beyond 30 °C, and significantly reduced even at 0 °C, while neither the crude extract nor the pure enzyme were affected in the absence of reduced pyridine nucleotide.

The sensitivity of pure *E. coli* glutathione reductase to mercurials was also studied. The enzyme was totally inactivated after 10 min incubation at room temperature with 10 μM *para*-hydroxymercuribenzoate. Much lower concentrations of this mercurial were required, however, for complete inactivation if the enzyme had previously been reduced by incubation with its electron donor, NADPH.

## Discussion

The main objective of the present work was to establish a high-yield and fast procedure for the purification to homogeneity of *Escherichia coli* glutathione reductase in order to subsequently characterize its molecular and kinetic parameters and study its redox interconversion mechanism. For that purpose we chose the affinity chromatography as the most specific procedure currently in use for protein purification.

The first affinity chromatography support employed was the commercially available $N^6$-2′,5′-ADP-Sepharose [22], which had been previously used for the purification of glutathione reductase from different organisms [12–14]. Figure 1 and Table I illustrate the excellent results obtained with such affinity gel. The total purification of *E. coli* glutathione reductase was only obtained, however, after a second affinity chromatography step by means of a different gel, $C^8$-ATPR-Sepharose, which had been described as specific also for the $NADP^+$-dehydrogenases although showing a somewhat different affinity for such enzymes [13, 23]. In fact, the contaminating dehydrogenases did not show affinity for this second support, since they were easily removed from the column by extensively washing it with the equilibrating buffer (Fig. 2).

The present purification procedure closely resembles that described for mouse-liver glutathione reductase [13], although differs markedly from those reported for the purification of the *Escherichia coli* enzyme [11, 12]. In our opinion, the present procedure is much more convenient for the purification of the *E. coli* enzyme due to the following reasons: 1) its extreme simplicity, which compares favorably with the very complicated and much more time-consuming schemes previously described [11, 12]; 2) the final product of our purification was a pure protein, as compared with the 80% purity and eight protein bands of the purification described by Williams *et al.* [11], and the 95% purity of the purification devised by Pigiet and Conley [12]; and 3) the very high final yield, 63%, higher than those previously reported for the *E. coli* enzyme [11, 12]. It seems reasonable to attribute part of this succes to the use of 10% glycerol throughout the purification as stabilizing agent, as well as to having maintained control of the pH while performing the ammonium sulfate fractionation.

The specific activity of the pure *Escherichia coli* glutathione reductase, 361 U/mg, was in fact higher than those previously reported with the *E. coli* enzyme [11, 12] or with that of yeast [14, 24], mouse

[13], human erythrocytes [25], plants [26], sea urchin eggs [27], or rabbits [28]. The purity of our final preparation was also confirmed by the absorbance ratios shown by its absorption spectrum. Thus, the value of 7.84 for the $A_{272}/A_{450}$ shown in Table II was lower than that described previously for other analogous enzymes, such as those from rat-liver [29], yeast [30], sea urchin eggs [27], or even *E. coli* [11, 12]. In connection with the absorption spectrum (see Fig. 3) it should be noticed a considerable absorbance beyond 520 nm, characteristic of half-reduced flavoproteins [1]. This could be directly attributed to the presence of 1 mM dithiothreitol while the spectrum was recorded, in order to prevent the possible denaturation of the enzyme.

As for most glutathione reductases previously studied [1], the enzyme from *E. coli* turned out to be a dimer with a subunit molecular weight of 55 000 and a native molecular weight of 109 000 (see Table II). Our results were very close to those previously reported for the *E. coli* enzyme, for which a native molecular weight of 111 400 [11], and 105 000 [12] have been reported. Zanetti has described, however, that rabbit-liver glutathione reductase is a monomer with a molecular weight of 67,000 [28]. This results should be taken cautiously since the crystallographic studies of the human erythrocyte enzyme have shown that both subunits must be present for the enzyme to be active because the GSSG-binding site is formed by residues from the A and B monomers [4, 9]. The hydrodynamic radius of the *E. coli* glutathione reductase, 40 Å, is very similar to the 41.8 Å previously described for the mouse-liver enzyme [13], or the 43 Å of the porcine erythrocytes enzyme [31].

With respect to the optimum pH for activity with both reduced pyridine nucleotides as electron donors (see Table II), it should be noticed that while 7.5 is within the normal range reported for NADPH [1], the optimum pH of 4.5 for NADH should be considered as rather low. In fact, the usual differences between the two values of optimum pH lie within 1–2 units of pH for the analogous enzymes [13, 29]. As for the apparent $K_m$ values for NADPH, NADH, and GSSG (16, 377 and 66 μM respectively), they are within the normal range of those previously reported for the enzyme from other sources [1, 2]. The only previous determination of kinetic parameters of the glutathione reductase from *E. coli* was that of Asnis in 1955 [10] which reported an apparent $K_m$ of 37 μM for NADPH (twice that found in the present paper), and an apparent $K_m$ of 1400 μM for GSSG (21 times higher than the one described in the present paper). Perhaps the strong inhibition observed with both high NADPH and GSSG concentrations (see Fig. 4) could explain such significant discrepancies.

An important loss of enzymatic activity was observed when pure *Escherichia coli* glutathione reductase was incubated with NADPH (see Fig. 5). Such result clearly indicates that the enzyme can also be subjected to a redox interconversion mechanism similar to that described for the mouse-liver or yeast glutathione reductases [13, 14]. In fact, experiments currently under way indicate that *in vitro* (with pure enzyme or crude extracts), *in situ* (with permeabilized *E. coli* cells), or even *in vivo* the enzyme readily interconverts between two states, active and inactive, due to covalent redox modification [32].

Finally, the extreme sensitivity shown by glutathione reductase from *E. coli* against *para*-hydroxymercuribenzoate after reduction by NADPH could be interpreted assuming that some very reactive thiol groups should appear in the reduced enzyme. In fact, a redox-active cystine group located at the GSSG-binding site has been implicated in the catalytic mechanism of human erythrocyte glutathione reductase [9]. Such redox-active disulfide, which is converted to a dithiol upon reduction by NADPH, could easily explain the behaviour of the *E. coli* enzyme against mercurials under reducing conditions. It should be also noticed that one of these two active site thiols has been considered to participate in the redox interconversion mechanism of yeast glutathione reductase [14].

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