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# Purification and Properties of Glutathione Reductase from the Cyanobacterium *Anabaena* sp. Strain 7119

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An NADPH-glutathione reductase (EC 1.6.4.2) has been purified 6,000-fold to electrophoretic homogeneity from the filamentous cyanobacterium *Anabaena* sp. strain 7119. The purified enzyme exhibits a specific activity of 249 U/mg and is characterized by being a dimeric flavin adenine dinucleotide-containing protein with a ratio of absorbance at 280 nm to absorbance at 462 nm of 5.8, a native molecular weight of 104,000, a Stokes radius of 4.13 nm, and a pI of 4.02. The enzyme activity is inhibited by sulfhydryl reagents and heavy-metal ions, especially in the presence of NADPH, with oxidized glutathione behaving as a protective agent. As is the case with the same enzyme from other sources, the kinetic data are consistent with a branched mechanism. Nevertheless, the cyanobacterial enzyme presents three distinctive features with respect to that isolated from non-photosynthetic organisms: (i) absolute specificity for NADPH, (ii) an alkaline optimum pH value of ca. 9.0, and (iii) strong acidic character of the protein, as estimated by column chromatofocusing. The kinetic parameters are very similar to those found for the chloroplast enzyme, but the molecular weight is lower, being comparable to that of non-photosynthetic microorganisms. A protective function, analogous to that assigned to the chloroplast enzyme, is suggested.

Glutathione, a ubiquitous thiol containing tripeptide, participates in its reduced state (GSH) in several functions of vital importance to the cell. In animal tissues and non-photosynthetic microorganisms, it maintains the thiol groups in the proper redox state and detoxifies a variety of potentially harmful electrophillic compounds. The oxidized disulfide form (GSSG) is reduced by the enzyme glutathione reductase (GR) (EC 1.6.4.2), which employs as a reductant NADPH generated by the hexose monophosphate pathway (1). GRs isolated from a variety of sources, from bacteria to mammalian cells, show great similarity both in physical and kinetic parameters (35).

Glutathione and GR are also present in chloroplast fractions (7, 28). The enzyme from spinach chloroplasts has been purified and found to be different from that of non-photosynthetic organisms, the catalytic properties, i.e., specificity for NADPH and alkaline optimum pH value, being in fairly good agreement with the protective role against $O_2^-$ and $H_2O_2$ (generated by reduction of oxygen by electron acceptors associated with photosystem I [7, 20]) assigned to GSH and GR activity in green tissues (10).

Although it has been recently reported that GSH is the most important nonprotein thiol both in vegetative cells and heterocysts of a nitrogen-fixing cyanobacterium (8), no detailed studies have been undertaken on the GR from these photosynthetic prokaryotes. In a previous paper (A. Serrano, J. Rivas and M. Losada, Proc. 6th Int. Congr. Photosynth., in press), it was shown that GR is present in both heterocysts and vegetative cells of the cyanobacterium *Anabaena* sp. strain 7119 with similar specific activity.

The aim of the present work was to purify and characterize GR from a cyanobacterium. Since this group of prokaryotes is the only one that, like higher plant chloroplasts, performs oxygenic photosynthesis, it was also of interest to compare the enzymes from both sources. The strong similarities observed in catalytic properties, otherwise quite different from those of the other GRs previously studied, support the distinctive metabolic function suggested for the enzyme in photosynthetic organisms.

## MATERIALS AND METHODS

**Chemicals.** Oxidized glutathione, $NADP^+$, and NADPH were purchased from Boehringer (Mannheim, Federal Republic of Germany); Polybuffer Exchanger 94, Polybuffer 74, Sephacryl S-300, Sephadex G-25, and protein standards for gel filtration and electrophoresis were from Pharmacia Fine Chemicals (Uppsala, Sweden); morpholinopropanesulfonic acid, 2-(*N*-morpholino)ethanesulfonic acid, oxidized coenzyme A, lipoic acid, L-cystine, *o*-phthalaldehyde, flavin adenine dinucleotide (FAD), flavin mononucleotide, riboflavin, Reactive Red-120, Reactive Red-120 agarose, bovine serum albumin, phenylmethylsulfonyl fluoride, Tris, *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, Triton X-100, and EDTA, were from Sigma Chemical Co. (St. Louis, Mo.); DEAE-cellulose DE-52 was from Whatman, Inc., (Maidstone, England); and ingredients for polyacrylamide gel electrophoresis (PAGE) were from Serva (Heidelberg, Federal Republic of Germany). All other chemicals were of analytical grade and were acquired from Merck (Darmstadt, Federal Republic of Germany).

**Growth of the organism.** *Nostoc muscorum* 7119—reclassified as *Anabaena* sp. strain 7119 (26)—was from the Department of Cell Physiology, Berkeley, Calif. (a gift of D. I. Arnon) and was grown photoautotrophically with 20 mM $KNO_3$ as the nitrogen source under the conditions previously described (30). The cells were harvested at the late exponential growth phase (about 4 days after inoculation) with a Szent-Gyorgyi-Blum continuous flow system (Ivan Sorvall, Inc., Norwalk, Conn.), washed twice in 50 mM Tris-hydrochloride buffer (pH 7.5) containing 0.5 mM EDTA (buffer A), and stored at −20°C before using. The yield of cells was about 3 g (wet weight) per liter.

**Analytical procedures.** GR activity was assayed by the decrease of absorbance at 340 nm as NADPH was oxidized

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in the presence of GSSG as electron acceptor. Reaction mixtures contained in a final volume of 1 ml: 100 μmol of Tris-hydrochloride buffer (pH 9.0), 2.5 μmol of GSSG, 0.5 μmol of EDTA, 0.2 μmol of NADPH, and an adequate quantity of enzyme. Reactions were started by adding enzyme, and the initial velocity was recorded. One unit of enzyme is defined as the amount which catalyzes the oxidation of 1 μmol of NADPH per min at 25°C.

PAGE was carried out according to the method of Jovin et al. (15) on running columns (5 by 75 mm) at the concentrations specified in each case, with a stacking gel of 3.5% (wt/vol) acrylamide. Samples containing about 25 μg of protein were applied to the gels, and electrophoresis was performed at 4 mA per gel tube with 0.01% bromophenol blue as tracking dye. PAGE in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed according to the method of Laemmli (17) by using stacking and separating gel columns containing 3 and 10% (wt/vol) acrylamide, respectively. A current of 2 mA per gel tube was applied for 2 to 4 h. Proteins were stained with 1% (wt/vol) Coomassie brilliant blue in 7% (vol/vol) acetic acid for 4 h at room temperature. The NADPH-GR activity was located in the gels by the disappearance of NADPH fluorescence under UV light (Sylvania BLB 15 W). The gels were soaked for 20 min in buffer A plus 0.2 mM NADPH, washed with distilled water, and then placed in 50 mM Tris-hydrochloride buffer (pH 9.0) containing 0.5 mM EDTA and 2.5 mM GSSG. The enzyme could be immediately located as an obscure band when the gels were placed on a black background under lateral UV light illumination. Alternatively, gels were cut out in 4-mm slices from which the enzyme was extracted with 0.2 ml of buffer A, and the activity was subsequently assayed.

The flavin coenzyme was quantified by measuring the fluorescence of an enzyme sample treated with 0.1 N sulfuric acid in a boiling water bath, as described by Koziol (16). The coenzyme released from GR after trichloroacetic acid treatment was identified by thin-layer chromatography on silica gel plates by using purified flavin standards (6).

The Stokes radius of the purified enzyme was determined by gel filtration on a column (1.6 by 40 cm) of Sephacryl S-300 equilibrated at 4°C in buffer A plus 0.1 M NaCl, with catalase, aldolase, bovine serum albumin, ovoalbumin, chymotripsinogen A, and RNase A as protein standards. The sedimentation coefficient of the enzyme was determined by using 5 to 20% (wt/vol) sucrose gradients total volume, 4.5 ml in 5-ml Beckman cellulose nitrate tubes with the standards given above. Sedimentation was for 12 h at 4°C and 50,000 rpm with an SW65 Ti rotor in a Beckman ultracentrifuge model L2 65B. The native protein molecular weight was estimated by running adequate amounts of enzyme and protein standards on polyacrylamide gels of 5, 6, 7, and 8% (wt/vol) acrylamide concentration as described by Hedrick and Smith (11). The standards used were ovoalbumin, bovine serum albumin, lactate dehydrogenase, catalase, and ferritine. The subunit molecular weight was determined by SDS-PAGE as described above and by gel filtration under denaturing conditions on a column (1.6 by 35 cm) of Sephacryl S-300 equilibrated at 4°C in buffer A plus 6 M urea, with RNase A, chymotripsinogen A, ovoalbumin, bovine serum albumin, and phosphorylase *b* as protein standards.

A Waters Associates, Inc., (Milford, Mass.) high-pressure liquid chromatography system was used for the analysis of the amino acid mixture obtained by treating samples of the purified enzyme (about 50 μg of protein) with 6 N HCl in vacuum-sealed tubes at 105°C for 24 and 48 h. The high-pressure liquid chromatography system consisted of two M6000A solvent delivery units, an M720 gradient controller, a U6K universal liquid chromatography injector, and an M750 data module coupled to an M420AC fluorescence detector. Sample injections were made using a Microliter 810 syringe (The Hamilton Co., Reno, Nev.). Amino acids were reacted with *o*-phthalaldehyde before analysis to form a highly fluorescent isoindole product (27). Derivatized products were separated on a Waters μBondapak $C_{18}$ column (5 μm; 15-cm by 4-mm inner diameter) and detected by fluorescence according to the indications of the manufacturer. Tryptophan was determined spectrophotometrically in the presence of 6 M guanidine hydrochloride (5).

Reductive potentiometric titration of the purified enzyme was performed at 12°C under argon with a 3-ml anaerobic glass cuvette in an Aminco DW-2 spectrophotometer equipped with a magnetic stirrer accessory. The reaction mixture contained in a final volume of 2 ml: 50 mM buffer A, 3 μM purified GR, and 2 μM of each methyl and benzyl viologen. The changes in ambiental redox potential when sodium dithionite was added were measured by using a combined PtAg-AgCl electrode, and the absorbance changes in the range 400 to 600 nm were simultaneously recorded.

Protein was determined by a modification of the method of Lowry (40) when the enzyme preparation was in a crude stage. In latter stages of the purification procedure, however, protein was determined according to the method of Bradford (3) or by UV absorption (18). Bovine serum albumin was used as a standard.

Fluorescence measurements and absorbance spectra were made in an Aminco-Bowman spectrophotofluorimeter and an Aminco DW-2 spectrophotometer, respectively.

**Purification of *Anabaena* sp. strain 7119 GR.** Unless otherwise stated, all the operations were performed at 0 to 4°C. Centrifugations were carried out at 40,000 × *g* for 20 min in a Sorvall RC-2B centrifuge with an SS-34 rotor.

**(i) Solubilization of the enzyme.** *Anabaena* sp. strain 7119 cell paste (50 g [wet weight]) was suspended in buffer A at the ratio of 10 ml/g (wet weight) of cells. Triton X-100 and phenylmethylsulfonyl fluoride were added to the cell suspension up to final concentrations of 3% (vol/vol) and 50 μM, respectively, and, after 6 to 8 h of gentle stirring in the dark, the extract was centrifuged to remove insoluble debris. The resulting supernatant was considered as the crude extract and used as such for the subsequent chromatographic procedures.

**(ii) DEAE-cellulose ion exchange chromatography and ammonium sulfate treatment.** The crude extract (about 540 ml) was applied to a DE-52 anion exchanger bed (2.5 by 60 cm) equilibrated with buffer A. The column was then washed with 5 bed volumes of buffer A, and the enzyme was eluted at a flow rate of 30 ml/h by using a linear gradient of NaCl (0 to 0.5 M; total volume, 1,200 ml) in the equilibration buffer. A saturated, ammonia-neutralized, $(NH_4)_2SO_4$ solution was added with stirring to the pooled active fractions (about 175 ml), up to 30% saturation. After 30 min, the protein solution was centrifuged, and the pellet was discarded. The supernatant was dialyzed three times against 12 liters of 6 mM Tris-hydrochloride buffer (pH 7.5) containing 0.2 mM EDTA. The dialyzed protein solution was eventually centrifuged, and the resulting supernatant was used for the next step.

**(iii) Reactive Red-120 agarose dye ligand chromatography.** The dialyzed protein solution (about 370 ml) was applied to a Reactive Red-120 agarose column (2.5 by 11 cm) equilibrated with 20 mM Tris-hydrochloride buffer (pH 7.5) containing 0.5 mM EDTA. The column was washed with 5 bed volumes

of the same buffer, and the enzyme was eluted by using a linear gradient of NaCl (0 to 1.5 M; total volume, 200 ml) in the equilibration buffer at a flow rate of 25 ml/h. The active fractions were collected and concentrated on a Diaflo PM-30 Amicon membrane to a final volume of 4 ml of 25 mM piperazine-hydrochloride buffer (pH 5.5) containing 0.5 mM EDTA (buffer B).

**(iv) Chromatofocusing.** Column chromatofocusing in the pH range of 5.0 to 3.5 was carried out on a Polybuffer Exchanger 94 column (1 by 20 cm) equilibrated with buffer B, according to the instructions of the manufacturer (Chromatofocusing; Pharmacia Fine Chemicals, Uppsala, Sweden, 1980). The concentrated enzyme solution was applied to the column, which was afterwards washed with 5 ml of buffer B, and the enzyme was eluted with the pH gradient created by washing the column with 12 bed volumes of 10-fold-diluted Polybuffer 74-hydrochloride (pH 3.5) at a flow rate of 12 ml/h. All buffers were previously degassed. The pH values of the fractions were measured by using a Beckman SS-2 pH meter with an expanded scale. The active fractions were collected and concentrated as described above to a final volume of 4 ml of buffer A plus 0.1 M NaCl.

**(v) Sephacryl S-300 gel filtration.** The concentrated enzyme solution was applied to a Sephacryl S-300 column (1.6 by 40 cm) equilibrated with buffer A plus 0.1 M NaCl and eluted at a flow rate of 30 ml/h. The active fractions were pooled, concentrated as above in buffer A, and stored at −25°C in the dark. This protein solution was used as the final enzyme preparation for the following experiments.

## RESULTS

**Enzyme purification.** In a previous work from this laboratory (A. Serrano, J. Rivas and M. Losada, in press), it was reported that *Anabaena* sp. strain 7119 cells, grown photoautotrophically with several nitrogen sources, contain an NADPH-GR. The enzyme was present (50 to 70 mU/mg of protein) in the soluble fractions of mechanically disrupted vegetative cells, grown with either dinitrogen or nitrate, and also in heterocysts. A milder procedure, with the nonionic detergent Triton X-100, previously employed successfully in the purification of another cyanobacterial flavoprotein (29), was also applied for the extraction of active GR from nitrate-grown cells. After centrifugation of the Triton X-100 extract, practically all the enzyme activity was recovered with the same specific activity in the resulting supernatant. Microscopic examination of the pellet showed only cell wall debris and "ghosts."

A representative purification procedure, summarized in Table 1, yielded the enzyme with a specific activity of about 249 U/mg of protein and a recovery rate of 67%. Both ion exchange and dye ligand chromatography represented by far the largest contribution to the overall purification (purification factor, 566), because during these steps hydrosoluble phycobiliproteins, which constitute the bulk of proteins in crude extracts of cyanobacteria, were removed from the enzyme preparation. Under the conditions used, GR remained tightly bound to the Procion Red agarose bed, whereas phycobiliproteins (otherwise difficult to completely remove by the usual techniques) were easily eluted by washing the column with the equilibration buffer. GR activity could afterwards be eluted as a symmetrical peak with about 0.75 M NaCl; however, $NADP^+$, even at 10 mM concentration, was completely ineffective in eluting the enzyme. Hence, although Procion Red behaved in free solution as a powerful competitive inhibitor with respect to NADPH (the estimated $K_i$ was 0.3 μM), in agreement with previous studies (34) ionic interactions with the ligand are also possible.

Chromatofocusing, a high-resolution technique of protein separation according to isoelectric point, was also employed, taking advantage of the very acidic character of the enzyme. As shown in Fig. 1, a close parallel between enzyme activity and the last protein peak eluted is evident; both peaks are, moreover, symmetrical, with maxima at a pH of ca. 4.02. This value can be considered as a good approximation to the isoelectric point of the cyanobacterial GR, thus revealing it as a very acidic protein. At this stage, the enzyme preparation was, as checked spectrophotometrically, contaminated with a hemoprotein, which was then separated from GR by gel filtration on Sephacryl S-300. Enzyme activity was finally eluted from the column as a symmetrical peak with constant specific activity, a fact which speaks in favor of only one protein in all these fractions.

Homogeneity of the resulting enzyme preparation was checked by PAGE and by ultracentrifugation. The gel filtration eluate presented a single protein band on 7.5% polyacrylamide gels (Fig. 2). This protein band gave positive reaction for GR activity, as detected either in situ by GSSG-dependent quenching of NADPH fluorescence or, after extraction of the enzyme from gel slices, by assaying it according to the standard method. Moreover, the final enzyme preparation was also homogeneous when subjected to SDS-PAGE. Ultracentrifugation on sucrose gradients revealed only one protein peak containing the enzyme activity. The purified enzyme preparation had a ratio of absorbance at 280 nm to absorbance at 462 nm ($A_{280}/A_{462}$) of about 5.8; this characteristic index of purity for flavoproteins is better than those previously obtained for the enzyme from other sources.

TABLE 1. Purification of NADPH-GR from *Anabaena* sp. strain 7119

| Fraction | Vol (ml) | Total protein (mg) | Total activity (U) | Sp act (U/mg of protein) | Purification factor (fold) | Yield (%) |
|---|---|---|---|---|---|---|
| Triton X-100 extract (40,000 × *g* supernatant) | 541.0 | 8,064.1 | 346.9 | 0.04 | 1 | 100 |
| 30% $(NH_4)_2SO_4$ supernatant of DE-52 eluate | 369.0 | 335.0 | 343.4 | 1.02 | 25 | 99 |
| Reactive Red-120 agarose eluate | 64.2 | 11.3 | 274.1 | 24.33 | 566 | 79 |
| Chromatofocusing (pH 5.0 to 3.5) eluate | 23.1 | 2.1 | 263.7 | 142.04 | 3,303 | 76 |
| Sephacryl S-300 eluate | 16.8 | 0.93 | 232.4 | 249.20 | 5,795 | 67 |

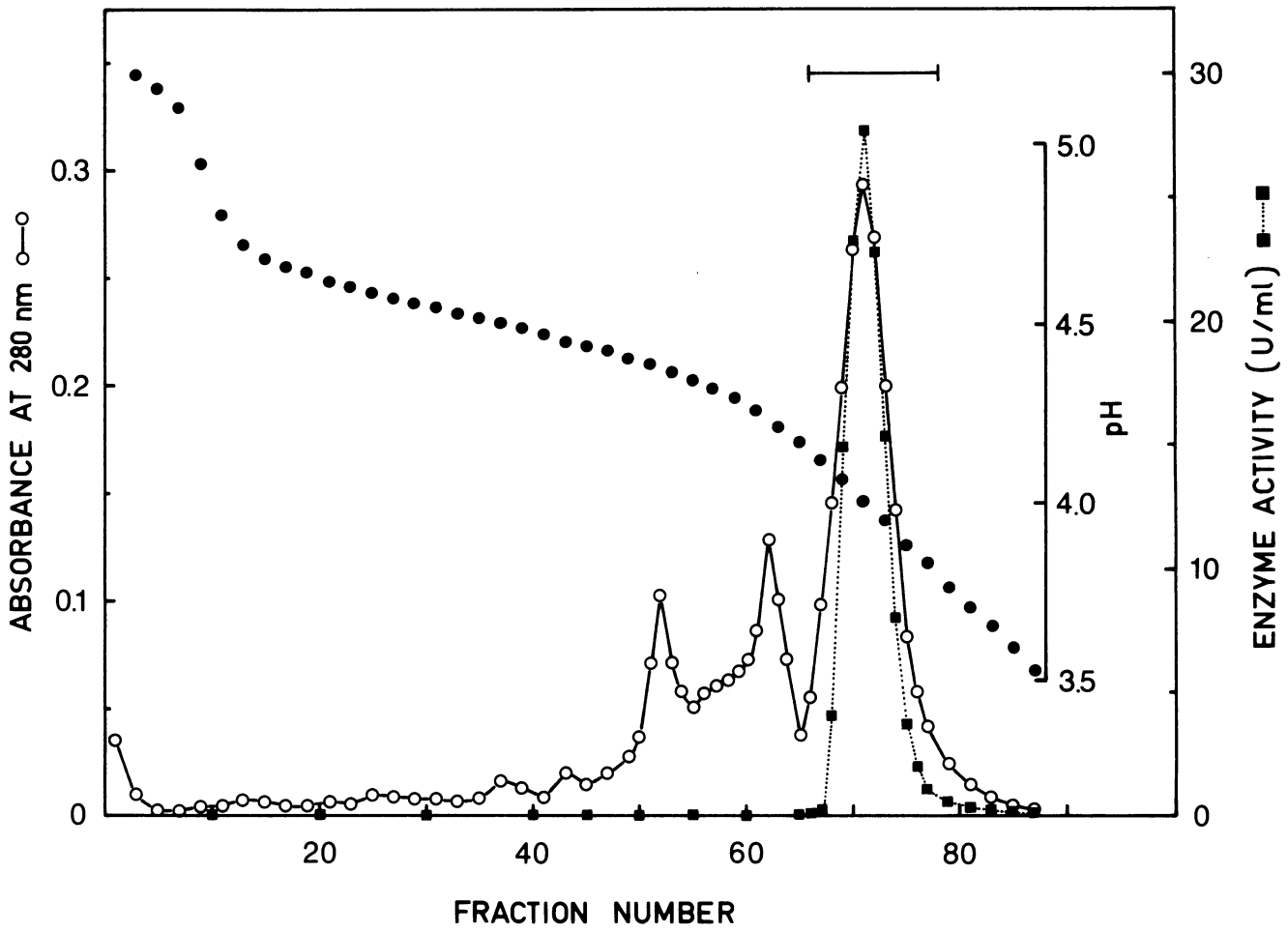


FIG. 1. Column chromatofocusing of GR from *Anabaena* sp. strain 7119 on Polybuffer Exchanger 94. A sample containing 11.3 mg of protein was applied to the Polybuffer Exchanger 94 column (1 by 20 cm), and the enzyme was eluted by using a pH gradient (●) generated by 12 bed volumes of Polybuffer 74-hydrochloride (pH 3.5) (0.0075 mmol per pH unit per ml). Two-milliliter-fractions were collected.

**Protein structure and cofactor identity.** The molecular weight of the native enzyme was estimated to be 106,000 by PAGE at different acrylamide concentrations and 102,000 by Sephacryl S-300 gel filtration. However, the molecular weight values estimated by SDS-PAGE and gel filtration in the presence of urea were 53,000 and 55,000, respectively, thus suggesting that the enzyme is a dimer. The sedimentation coefficient of the pure enzyme was estimated to be 6.06S by sucrose density gradient centrifugation, and the Stokes radius was determined to be 4.13 nm by Sephacryl S-300 gel filtration. From these data a native molecular weight of 104,000, in fairly good agreement with the above mentioned values, and a frictional ratio of 1.33 were calculated (24, 31). From chromatofocusing data, a pI of about 4.0, showing a good constancy in different enzyme preparations, was estimated.

The amino acid composition of GR from *Anabaena* sp. strain 7119, estimated by high-pressure liquid chromatography of *o*-phthalaldehyde derivatives of amino acids, is presented in Table 2. The number of residues was calculated on the basis of a subunit molecular weight of 53,000.

The purified GR displayed a typical flavoprotein absorption spectrum (Fig. 3), with peaks at 274, 377, and 462 nm and with absorbance ratios of $A_{274}/A_{462}$, $A_{280}/A_{462}$, and $A_{377}/A_{462}$ of 6.36, 5.78, and 0.93, respectively. The extinction coefficients (1 mM, 1-cm path length) at 377 and 462 nm were calculated to be 9.6 and 11.3, based on a subunit molecular weight of 53,000.

The flavin cofactor was released from the enzyme and identified as FAD by thin-layer chromatography; its content was calculated to be 1.85 mol/mol of native enzyme. Therefore, the enzyme contains two identical subunits, each with one molecule of FAD. Flavin fluorescence was nearly completely quenched in the native enzyme, reaching only 1 to 2% of an equimolar solution of FAD.

From the reductive titration of the enzyme-bound FAD, an $E_m$ (pH 7.5) of about −217 mV, similar to that of the free flavin, was estimated. Nevertheless, the calculated $n$ was lower than the expected value of 2, thus suggesting the existence of interactions between the flavin and the disulfide group of the active center, as has been previously described for the similar enzyme thioredoxin reductase (41).

**Catalytic properties and substrate specificity.** As was previously shown for the enzyme in crude extracts, purified GR is highly specific for NADPH as the electron donor. No detectable activity was observed when NADPH was replaced by NADH, even at acidic pH values, at which the enzymes from mammalian cells and non-photosynthetic

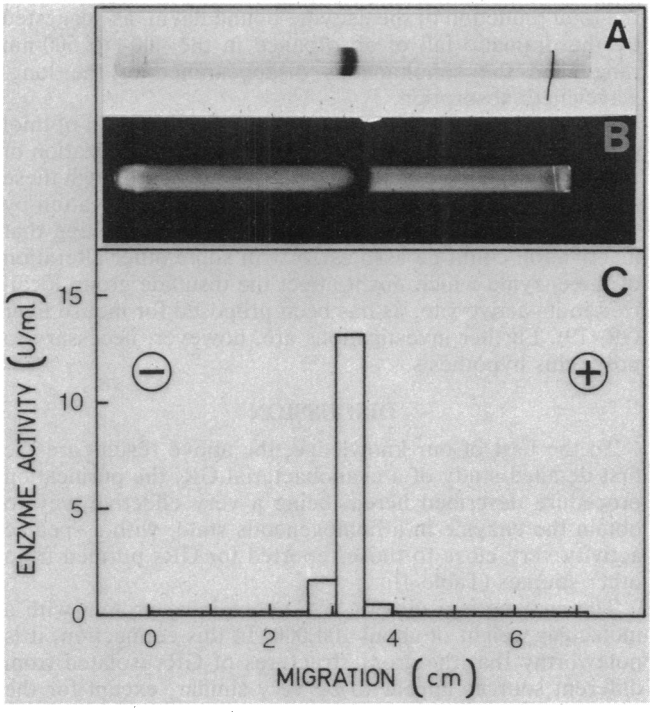


FIG. 2. PAGE of GR purified from *Anabaena* sp. strain 7119. Twenty-five micrograms of protein was run in 7.5% polyacrylamide gels. (A) Gel stained for protein. (B) Localization of NADPH-GR activity by the GSSG-dependent quenching of NADPH fluorescence under UV light. The photograph was done by using a Balzers interference filter DT (540 nm) to block blue and UV light. (C) Localization of GR by extraction of the enzyme from gel slices as described in the text.

microorganisms present their maximum NADH-dependent activity. Figure 4 shows the effect of pH on the activity of the cyanobacterial enzyme. The optimum pH value was 9.0, with considerable activity in the range of 7.5 to 10.0. The enzyme was also very specific for the disulfide substrate at different pH values, with no significative NADPH oxidation observed when GSSG was replaced by L-cystine, lipoic acid, or oxidized coenzyme A, even at the millimolar concentration range (data not shown). GR activity is strongly affected by the ionic strength, so the concentration of Tris-hydrochloride buffer in the reaction mixture was adjusted to give the maximum reaction rate. From reciprocal plots of reaction rates in the presence of a saturating concentration of the other substrate, apparent $K_m$ values for GSSG and NADPH were estimated to be 210 and 9.4 μM, respectively. Steady state studies, performed with various concentrations of NADPH and GSSG in the assay mixture, presented a series of parallel lines both in the plots of $1/v$ versus 1/[GSSG] and 1/[NADPH]. From these data, an apparent $V_{max}$ of 23,000 μmol/min μmol of enzyme was determined. Nevertheless, a deviation from the general pattern of parallel lines was obtained at high concentrations of NADPH (>0.5 mM) or GSSG (>5 mM). On the other hand, both products, $NADP^+$ and GSH, behaved as inhibitors of enzyme activity. The inhibition by $NADP^+$ was competitive with respect to NADPH (the estimated $K_i$ was about 70 μM) and noncompetitive with respect to GSSG. GSH was, however, a noncompetitive inhibitor with respect to both substrates, but only at very high concentrations (>5 mM).

TABLE 2. Amino acid composition of *Anabaena* sp. strain 7119 GR[a]

| Amino acid | Occurrence in GR (residues/53,000-mol-wt subunit) | |
|---|---|---|
| | As found | To the nearest integer |
| Aspartic acid | 40.64 | 41 |
| Glutamic acid | 44.26 | 44 |
| Serine[b] | 43.44 | 43 |
| Histidine | 7.81 | 8 |
| Glycine | 58.73 | 59 |
| Threonine[b] | 39.35 | 39 |
| Arginine | 29.42 | 29 |
| Alanine | 32.62 | 33 |
| Tyrosine | 8.23 | 8 |
| Methionine | 8.81 | 9 |
| Valine | 49.36 | 49 |
| Phenylalanine | 14.92 | 15 |
| Isoleucine | 30.67 | 31 |
| Leucine | 34.41 | 34 |
| Lysine | 19.28 | 19 |
| Proline[c] | 23.10 | 23 |
| Tryptophan[d] | 3.28 | 3 |
| Half-cystine[e] | 5.79 | 6 |

[a] The results are calculated as relative molar ratios with respect to the total yield of amino acids.
[b] Determined by extrapolation at zero time.
[c] Determined after treatment with sodium hypochlorite.
[d] Determined spectrophotometrically (5).
[e] Determined as cysteic acid after performic acid oxidation.

The cyanobacterial enzyme showed a marked inhibition by sulfhydryl reagents and heavy-metal ions (Table 3). The sensitivity of the enzyme to thiol reagents was increased if NADPH was present in the preincubation mixture; in contrast, GSSG behaved as a protective agent. The arsenite anion, a reactive of vicinal dithiols, inhibited GR activity only in the presence of NADPH. In the presence of heavy-metal ions, such as $Zn^{2+}$ or $Cu^{2+}$—known to form chelates with dithiol groups (14)—NADPH and GSSG behaved again

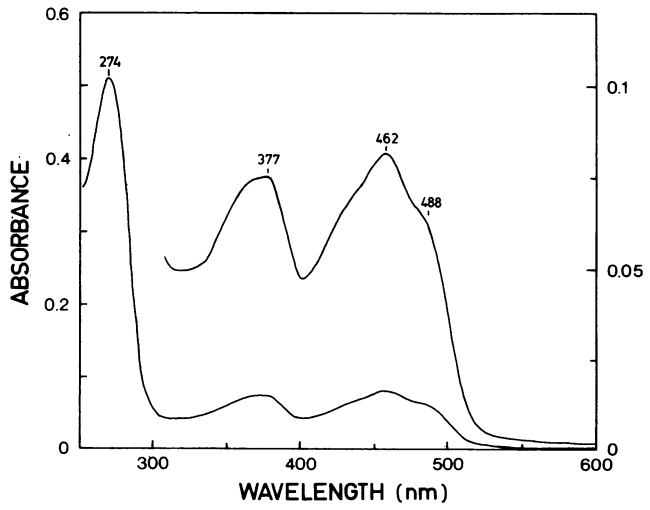


FIG. 3. Absorption spectrum of GR purified from *Anabaena* sp. strain 7119. Protein, about 370 μg/ml, was in 50 mM Tris-hydrochloride buffer (pH 7.5) containing 0.5 mM EDTA.

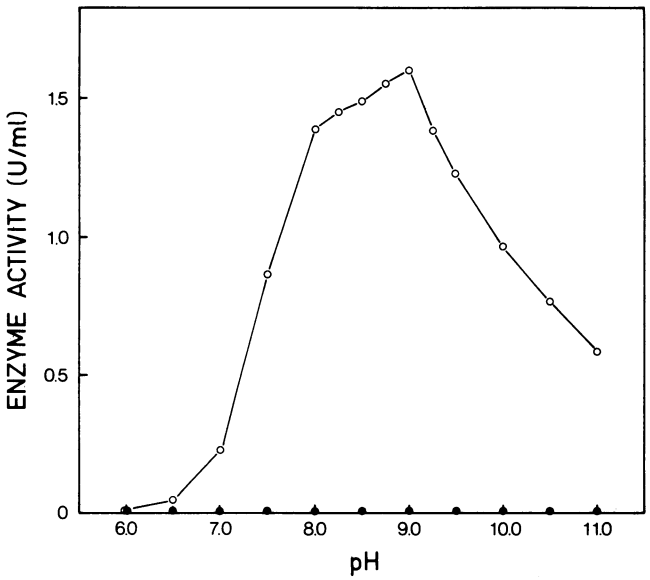


FIG. 4. Effect of pH on the GSSG-dependent NADPH (○) and NADH (●) oxidation by GR purified from *Anabaena* sp. strain 7119. The buffer used throughout the whole pH range consisted of an equimolar mixture of 2-(*N*-morpholino)ethanesulfonic acid, morpholinopropanesulfonic acid, Tris, and glycine (total concentration, 0.1 M), adjusted by additions of NaOH to the indicated pH values. Protein concentration in reaction mixture was about 6 μg/ml.

as they did with sulfhydryl reagents. EDTA, even at the millimolar concentration range, did not reverse heavy-metal inhibition. Cyanobacterial GR is not, therefore, different from GR from other sources in that it has a disulfide group reducible by NADPH in the catalytic center for GSSG (35).

It has been reported that the catalytic activity of the mammalian enzyme exhibits a redox interconversion mediated by the NADPH-$NADP^+$ couple (19). The cyanobacterial GR was also inactivated when preincubated with an excess of NADPH. At neutral pH values, the inhibition process was fairly slow, taking about 9 h at 25°C with 0.5 mM NADPH to reach about 60% inactivation. At 1 mM concentration, either $NADP^+$ or GSSG behaved as a protective agent of enzyme activity. Once inactivated, GR remained inactive even after removal of the excess of NADPH by gel filtration on Sephadex G-25, but addition of GSSG or potassium ferricyanide reactivated the dialyzed enzyme, requiring about 9 h at 25°C with 2 mM GSSG or potassium ferricyanide to reach 80 to 90% of the control activity value—about 300 mU/ml—used in these experiments.

No significant changes in physical parameters, i.e., the Stokes radius and sedimentation coefficient, were observed for the partially inactive enzyme, thus ruling out the possibility of aggregation states upon inactivation. Nevertheless, several spectral changes were observed when GR was incubated in the presence of an excess of NADPH; namely, a decrease of absorbance in the range of 400 to 500 nm, the 462-nm peak of the oxidized enzyme being moved to 453 nm, as well as the appearance of new absorbance at longer wavelengths, up to 670 to 680 nm. Similar changes in GR from other sources have been ascribed to the formation of an electron paramagnetic resonance-silent charge-transfer complex when the enzyme was reduced by the pyridine nucleotide (35). Subsequent treatment with a thiol reagent, such as *p*-hydroxymercuribenzoate or sodium arsenite, produced the total reduction of the enzyme-bound flavin, as suggested by the dramatic fall of absorbance in the 400- to 500-nm range and the simultaneous disappearance of the long-wavelength absorption.

These results are consistent with the participation of thiol groups, probably the catalytic dithiol, in the stabilization of the spectral changes observed. Nevertheless, although these spectral changes are instantaneous, enzyme inactivation by NADPH is slow and time dependent, thus suggesting that inactivation could be associated with some other alteration of the enzyme which might affect the disulfide group localized in its active site, as has been proposed for mouse liver GR (19). Further investigations are, however, necessary to prove this hypothesis.

## DISCUSSION

To the best of our knowledge, the above results are the first detailed study of a cyanobacterial GR, the purification procedure described herein being a very effective way to obtain the enzyme in a homogeneous state, with a specific activity very close to those reported for GRs purified from other sources (Table 4).

The enzyme is a dimeric FAD-containing protein with a molecular weight of about 100,000. In this connection, it is noteworthy that the gross structures of GRs isolated from different sources appear to be very similar, except for the otherwise poorly purified enzyme from the photosynthetic bacterium *Rhodospirillum rubrum*, which seems to be a monomeric protein (Table 4). Notwithstanding, although no significative differences have been observed in physical parameters between GRs from cyanobacteria and non-photosynthetic organisms, the stronger acidic character of the first one in comparison with the enzyme from mouse liver (pIs of 4.02 and 6.46, respectively) is a sign of disparity (see Table 4).

The mechanism of cyanobacterial GR seems to be a binary complex or two-site ping-pong mechanism, although anomalous behavior was observed at a high concentration of either GSSG or NADPH. These facts are consistent with the branched mechanism proposed by Mannervik for the yeast enzyme (21).

TABLE 3. Inhibition of *Anabaena* sp. strain 7119 GR by sulphydryl reagents and heavy-metal ions[a]

| Additions | Relative activity (%) |
|---|---|
| None | 100[b] |
| GSSG (2.5 mM) | 100 |
| NADPH (0.5 mM) | 98 |
| NEM (5 mM) | 32 |
| NEM (5 mM), GSSG (2.5 mM) | 81 |
| NEM (5 mM), NADPH (0.5 mM) | 0 |
| Sodium arsenite (5 mM) | 97 |
| Sodium arsenite (5 mM), GSSG (2.5 mM) | 101 |
| Sodium arsenite (5 mM), NADPH (0.5 mM) | 10 |
| $ZnSO_4$ (0.5 mM) | 51 |
| $ZnSO_4$ (0.5 mM), GSSG (2.5 mM) | 96 |
| $ZnSO_4$ (0.5 mM), NADPH (0.5 mM) | 0 |

[a] The preincubation mixtures (5 min, 25°C) included in a final volume of 1 ml: 100 μmol of Tris-hydrochloride buffer (pH 9.0), the above indicated additions, and an adequate amount of purified enzyme. NEM, *n*-ethylmaleimide.

[b] One hundred percent activity corresponded to 643 mU.

TABLE 4. Comparison of the properties of GRs from different sources

| Source (reference) | Mol wt (native) ($10^3$) | Subunit mol wt ($10^3$) | Electron donor | Apparent $K_m$ (μM) for: | | Sp act[a] (U/mg) | Optimum pH | pI |
|---|---|---|---|---|---|---|---|---|
| | | | | NADPH | GSSG | | | |
| Human erythrocytes (38, 39) | 100 | 50 | NAD(P)H | 8 | 65 | 240 | 6.8 | ND[b] |
| Mouse liver (19) | 105 | 55 | NAD(P)H | 6 | 107 | 158 | 7.2 | 6.46 |
| *Penicillium chrysogenum* (37) | 109 | ND | NAD(P)H | 10 | 55 | 460 | 6.8–7.6 | ND |
| Yeast (4, 22) | 118 | 56.5 | NAD(P)H | 4 | 55 | 270 | 6.6–7.6 | ND |
| *Escherichia coli* (25, 36) | 105 | 51 | NAD(P)H | 16[c] | 66[c] | 505 | 7.0–7.6 | ND |
| Rice embryos (13) | 106 | 52 | NADPH | 13 | 34 | 150 | 7.9 | ND |
| *Rhodospirillum rubrum* (2) | 63 | 63 | NADPH | 8 | 58 | 3 | 7.5–8.2 | ND |
| Spinach chloroplasts (10) | 145 | 72 | NADPH | 4 | 195 | 246 | 8.5–9.0 | ND |
| *Anabaena* sp. strain 7119[d] | 104 | 53 | NADPH | 9 | 210 | 249 | 9.0 | 4.02 |

[a] Specific activity of purified enzyme.
[b] ND, Not determined.
[c] A. M. Mata, M. C. Pinto, and J. López-Barea, Abstr. 2nd Meet. Spanish Fed. Soc. Exp. Biol. 1981, abstr. no. 471, p. 269.
[d] Present work.

The reductive inactivation of cyanobacterial GR, as well as the subsequent reactivation of the inactive enzyme by oxidizing agents, can not be attributed to a simple inhibition-activation effect by the reducing-oxidizing substrates. Actually, whereas the spectral changes promoted by NADPH are instantaneous, the resulting GR inactivation is slow and time dependent. This kind of interconversion by oxidation reduction is also exhibited by another outstanding photosynthetic flavoprotein, ferredoxin-$NADP^+$oxidoreductase, although, in this later case, the time required for the inactivation process to occur is rather shorter, about 20 min under the same experimental conditions. Moreover, in contrast with GR, the spectral changes promoted by NADPH in ferredoxin-$NADP^+$oxidoreductase indicate that the neutral blue semiquinone of the flavin cofactor is generated upon reduction, a fact that has been confirmed by electron paramagnetic resonance studies (A. Serrano, J. Rivas, and M. Losada, Abstr. 15th FEBS Meeting, 1983, S-09 TU-175, p. 258). Hence, although both NADPH flavoproteins can undergo redox interconversion, some differences seem to exist between them. The possible metabolic role proposed for the redox interconversion of the mouse liver GR (19) remains to be investigated in cyanobacteria.

GR has been extensively studied in mammalian cells and yeast (22, 35) and was early claimed to be a ubiquitous enzyme in higher plants (33), but it is only recently that the chloroplast enzyme has been purified and characterized (10). The spinach chloroplast enzyme, as well as that from cyanobacteria, exhibits a more alkaline optimum pH value than the GR isolated from non-photosynthetic sources (Table 4). Assuming that protection against the activated species of oxygen generated by photosynthetic activity is an important function of GSH in green tissues (9), GR could certainly be more active around the thylakoid membrane, where strong alkalinization and high generation rates of toxic $H_2O_2$ and $O_2^-$ can be expected after the onset of illumination. Moreover, also as shown in Table 4, GR from photosynthetic organisms—including purple bacteria—presents high specificity for NADPH, the main reduced product of the photosynthetic electron transport path. These catalytic properties of the enzyme suggest, therefore, an adaptation to the cellular environment in which it works.

The strong similarities found in catalytic properties of GR isolated from spinach chloroplasts and cyanobacteria, despite slight differences in physical parameters, are consistent with the expected similar function of the GR-GSH system in these organisms, since cyanobacteria are the only prokaryote group that performs oxygenic photosynthesis. An additional observation that also points in favor of the important role played by the GR-GSH system in vivo is the high rate of GSSG-dependent oxygen evolution found in reconstituted illuminated systems containing thylakoid membranes and purified enzymes from *Anabaena* sp. strain 7119, with GSSG behaving in consequence as a very effective physiological Hill reagent (A. Serrano, J. Rivas and M. Losada, in press). By photosynthetically generating GSH in high amounts, several cyanobacteria, which under defined culture conditions show an intense light-dependent production of $H_2O_2$ (32), may protect themselves against this toxic compound.

Although a catalase activity has been described in filamentous strains of cyanobacteria (12), it is unlikely to be involved in the detoxification of low concentrations of $H_2O_2$, given its low affinity for the substrate. Thus, as has been described for higher plant chloroplasts (7), the ascorbate-GSH cycle could be also the main detoxification mechanism for low $H_2O_2$ concentrations in cyanobacteria.

Finally, it has been reported that several strains of cyanobacteria carry out anoxygenic photosynthesis, independent of photosystem II and driven by photosystem I, with sulfide as an electron donor (23). Whether the cyanobacterial cells would also be able to use thiol-containing compounds, such as GSH, as electron donors under defined physiological conditions, i.e., in nitrogen-fixing heterocysts, is an exciting possibility that needs further investigation. Although more work is obviously necessary to elucidate the redox metabolism of glutathione in cyanobacteria, it seems clear that these prokaryotes represent a simple and appropriate system to study the relationships between glutathione metabolism and photosynthetic processes.

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