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GLUTATION REDUCTASA EN PROCARIOTES

T E S I S

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INTRODUCCION

El glutatión, un compuesto de naturaleza peptídica, ha demostrado ser una molécula extremadamente versátil, desempeñando una gran variedad de funciones de vital importancia para el metabolismo celular. Dentro de dichas funciones destacan aquellas involucradas en la protección contra radiaciones ionizantes (1,2), eliminación de agentes electrofílicos nocivos a la célula (3), transporte de metabolitos a través de membrana (4,5), así como el mantenimiento de un estado redox adecuado para el buen funcionamiento de la célula (3).

En todos estos procesos, es quizá el sulfhidrilo del glutatión la parte más activamente involucrada en ellos (principalmente a través de intercambios tiol-disulfuro con otras moléculas), a tal grado que el glutatión reducido(GSH) es sustrato de un gran número de enzimas (Glutatión peroxidasa, Glutatión-S-transferasas, Glutatión-insulina transhidrogenasa, etc.). Ahora bien, como resultado de estas reacciones, el glutatión sufre una oxidación en la cual se convierte en su forma disulfuro (GSSG). Parecería entonces, de acuerdo con lo mencionado hasta ahora, que en ausencia de un sistema regenerador de GSH, la acumulación de su forma oxidada conduciría eventualmente a la total paralización de las actividades celulares. De este modo, la necesidad de tal sistema sugeriría, en principio, su distribución universal en todos los seres vivos.

En 1952, partiendo de extractos de tejidos animales, Rall y Lehninger (6) fueron capaces de obtener una actividad reductora del GSSG -dependiente de NADPH- que desde entonces ha sido denominada Glutatión reductasa (NADPH:GSSG oxidorreductasa E.C.1.6.4.2.). A la fecha, esta enzima se ha reportado y purificado a partir de un gran número de organismos, que van desde procariotes (7-10), hongos (11,12), plantas (13-15), invertebrados (16,17) hasta mamíferos (18-22).

En la Tabla I se resumen las propiedades de esta enzima en aquellos organismos donde ha sido caracterizada con detalle. Se incluyen también algunas fuentes en donde, si bien incompletamente purificadas y/o caracterizadas, exhiben ciertas propiedades que pueden considerarse poco usuales para esta enzima. El análisis cuidadoso de estos datos revela una notable similitud tanto en propiedades estructurales como cinéticas independientemente de la fuente de extracción. Así, en la gran mayoría de los casos, la enzima es un dímero constituido por subunidades idénticas - tanto en tamaño como en composición - con un peso molecular aproximado de 100 000. El pH óptimo, aunque varía en un rango amplio, oscila alrededor del valor neutro, y la presencia de FAD como grupo prostético le confiere a la enzima un espectro de absorción en el rango visible típico de una flavoproteína.

Sin embargo, es evidente que en algunos casos la enzima muestra propiedades que se desvían notablemente de los valores usuales. Así, en el caso de los procariotes estudiados, *Rhodospirillum rubrum* (10) parece poseer una glutatión reductasa monomérica, ya que el peso molecular reportado para el oligómero (63 000), cae en el rango observado para el protómero de la enzima en otras fuentes. Desafortunadamente no se conoce la composición de subunidades de la misma y la preparación obtenida no se purificó hasta homogeneidad. *Chromatium vinosum* (8), por otra parte, presenta una enzima con afinidad anormalmente baja por GSSG, ya que la *Km* obtenida para dicho sustrato cae en el rango milimolar y no micromolar como es usual para la misma.

En el caso de los eucariotes la enzima parece ser, en general, más homogénea que en los procariotes, si bien es posible observar algunas situaciones particulares. Oogus y Tezcan (26) reportan que en los leucocitos humanos la glutatión reductasa existe como un hexámero constituido por subunidades de 19 000, mientras que en cloroplastos de espinaca (13) la enzima, aunque de naturaleza dimérica, presenta subunidades con un peso molecular mucho mayor de lo esperado.

De particular interés, por otra parte, ha sido el estudio de la especificidad de esta enzima hacia el disulfuro, ya que si bien el GSSG es claramente el sustrato principal, existen evidencias de que la glutatión reductasa es también capaz de reducir otros compuestos, que incluyen a los disulfuros mixtos de glutatión y cisteína, glutatión y pantetina, así como el sulfonato de glutatión, cistina e incluso DTNB (32). A pesar de que la actividad relativa hacia estas sustancias es notablemente inferior cuando se le compara con aquella correspondiente al GSSG, es evidente que la enzima tiene el potencial de reconocer y reducir otros disulfuros con estructuras radicalmen

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TABLA I

te distintas al GSSG(como es el caso del DTNB). Dentro de la serie de compuestos ensayados, destaca el disulfuro mixto de Coenzima A y glutatión (CoASSG), ya que a pesar de ser un sustrato secundario para la enzima, los niveles de actividad exhibidos por esta última hacia el CoASSG son notablemente mayores que con respecto a cualquier otro disulfuro probado (32).

En relación con esto último, se ha propuesto que la actividad responsable de la reducción del CoASSG radica en una proteína físicamente distinta de la glutatión reductasa. Así, Ondarza et al (33) han reportado que en la levadura *Saccharomyces cerevisiae* las dos actividades son resueltas mediante la técnica de electroenfoque, presentando puntos isoeléctricos claramente distintos. Sin embargo, estas observaciones han sido refutadas por Carlberg et al (34), quienes sugieren que es la misma glutatión reductasa la responsable de reducir al CoASSG, ya que el cociente de actividades permanece constante durante todo el proceso de purificación, afirmando además la imposibilidad de separar ambas actividades en base a puntos isoeléctricos. Por otra parte, en extractos de hígado de rata ambas actividades muestran el mismo punto isoeléctrico (35). Desafortunadamente no se dispone de información parecida en ningún procariote, si bien los datos disponibles mencionados líneas arriba permiten plantear un modelo en el cual ambas actividades residían originalmente en proteínas diferentes (y por ende codificadas por genes independientes) que en el transcurso del proceso evolutivo se fusionaron en una proteína común.

De acuerdo con lo anterior se postula la siguiente hipótesis: si en tejidos de mamífero las dos reductasas parecen mostrar una proporción similar de residuos ácidos y básicos, imposibilitando así su separación, mientras que en levadura - un eucariote primitivo -se observan puntos isoeléctricos distintos, parecería entonces que mientras más primitivo sea el organismo, deberán existir mayores diferencias entre ambas enzimas (de acuerdo a lo señalado en el párrafo anterior) y por consiguiente su separación física debe facilitarse.

Así, durante la primera fase del trabajo, se procedió a rastrear la existencia de una actividad reductora del CoASSG en diversos procariotes a fin de disponer de una fuente adecuada de enzima con objeto de poner a prueba la hipótesis anterior. Fué precisamente en esta etapa cuando comenzó a observarse que en algunos organismos era practicamente imposible detectar, no ya la actividad de CoASSG reductasa, sino incluso la misma GSSG reductasa, enzima que supuestamente debía tener una distribución universal. Además, durante el proceso de purificación y caracterización de la enzima extraída de la cianobacteria *Spirulina maxima*, se puso de manifiesto que presentaba algunas propiedades poco comunes. De acuerdo con ello, fué necesario trabajar con dos hipótesis adicionales durante el desarrollo del presente trabajo:

a) Si la glutatión reductasa es una enzima cuya distribución depende de las particularidades metabólicas y/o ambientales de los distintos organismos, entonces, dependiendo de cuál sea la fuente de extracción, la actividad enzimática mencionada deberá estar ó no presente.

b) Si las propiedades estructurales y cinéticas de la enzima obtenida de procariotes, difieren en algún aspecto de aquellas de la enzima correspondiente a eucariotes, entonces, la enzima de *Spirulina maxima* deberá exhibir alguna característica inusual.

De este modo, aunque el objetivo inicial de este trabajo tenía como meta el dilucidar la posible existencia de una reductasa del CoASSG , físicamente independiente de la glutatión reductasa, se consideró interesante evaluar, simultáneamente, las dos hipótesis anteriores.

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Glutathione Reductase in Evolution

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Summary. The disulfide reducing activities of GSSG- and CoASSG-reductases were measured on partially purified extracts from a variety of prokaryotes and eukaryotes.

Glutathione-reductase was found in varying amounts in all eukaryotes and prokaryotes, used in this study, with the exception of the two strict anaerobes *Clostridium tartarivorum* and *Desulfovibrio vulgaris*, and the two primitive Archaebacteria *Methanosarcina barkeri* and *Halobacterium halobium*.

CoASSG-reductase was found in some eukaryotes and prokaryotes, but showed no clear pattern of distribution other than its absence whenever GSSG-reductase was not present.

The absence of GSSG-reductase activity in organisms lacking GSH, confirms that glutathione metabolism is not universal and suggests that this enzyme might be useful as a marker in classifying organisms. The data suggest that glutathione-reductase occurs as a result of the change from a reducing to a oxidizing atmosphere in the primitive Earth.

Key words: Disulfide reductase – Glutathione – Atmospheric oxygen – Microbial evolution – Prokaryotes-eukaryotes

Introduction

It is a generally accepted idea that the primitive Earth possessed a reducing atmosphere that slowly changed to an oxidizing one due to the oxigenic activity of the first photosynthetic organisms (Miller and Orgel 1974: Broda 1975). If we accept this hypothesis, it is of interest to consider the effect produced by this change on the equilibria between thiols and disulfides, which is dependent on the partial pressure of hydrogen. This value was calculated by Urey 1952 and Miller and Orgel 1974 for the primitive atmosphere to be approximately 10^{-3} atm and has since decreased to about 5×10^{-7} atm in the present atmosphere. Because of the instability and relatively facile reactivity of thiols towards oxygen, the accumulation of oxygen in the atmosphere must have had adverse consequences for the thiol dependent components of cells in particular for sulfhydryl enzymes (Fahey 1977). One of the mechanisms which may have played an important role to maintain a reduced state within cells adapting to more aerobic environments is the activity of the enzyme glutathione reductase which, in gram negative bacteria and various other prokaryotes and eukaryotes, maintains a high GSH/GSSG ratio through the following reaction:

$$\text{a) GSSG} + \text{NADPH} + \text{H}^+ \rightleftharpoons 2\ \text{GSH} + \text{NADP}^+.$$

Another potentially important system involves CoASSG reductase wich catalyzes the reaction:

$$\text{b) CoASSG} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{CoASH} + \text{GSH} + \text{NADP}^+$$

This enzyme and its mixed disulfide substrate have been the subject of a variety of studies in our laboratory (Ondarza et al. 1965, 1969, 1970 and 1974).

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It was recently reported that various aerobic and anaerobic gram positive bacteria and some anaerobic gram negative bacteria lack glutathione, whereas facultative and aerobic gram negative bacteria do produce this thiol (Fahey et al. 1978). These findings suggest that glutathione metabolism evolved after the divergence of gram positive and gram negative bacteria. Coenzyme A, on the other hand, as far as it is presently known, is a universal constituent of cells. Organisms lacking GSH should also lack glutathione reductase and CoASSG-reductase, but might exhibit disulfide reductase activity for other disulfides. To test this we undertook a survey of GSSG reductase and CoASSG reductase in a phylogenetically diverse group of organisms. The results of this survey are the subject of this paper.

Experimental Section

Organisms and Growth Conditions

Saccharomyces cerevisiae Aztec, provided by Dr. Antonio Peña was cultivated in our laboratory as previously described by Ondarza et al. 1969 and harvested during the logarithmic stage; *Spinacea oleracea* (Spinach) was purchased at the local super-market (only the green leaves were utilized); *Agave fourcroydes* (Henequen) which grows in the cultivated fields in Yucatan (Mexico), was kindly provided as a juice by Dr. Luis del Castillo-Mora; *Chlamydomonas reinhardtii* from the asexual logarithmic growth phase was kindly furnished by Dr. Manuel Robert; the marine diatoms, *Cylindrotheca fusiformis* (photosynthetic) and *Nitzschia alba* (non photosynthetic) were grown and collected from the logarithmic stage and kindly provided by Prof. Benjamin Volcani; *Dunaliella tertiolecta* grown in local sea water, sterilized by filtration and harvested at late exponential stage, was prepared through the collaboration of Prof. James A. Mathewson; *Codium sp.* was locally collected from the coast of California and kindly provided by Dr. Francis Knowles; *Rhodospirillum rubrum* supplied by Dr. Heliodoro Celis, was grown in our laboratory according to Cohen-Bazire and Sistrom 1957 and harvested during the logarithmic stage; *Rhodospirillum salexigens*, an obligatory halophilic synthetic organism was grown according to Golecki and Drews 1980 in a mineral acetate glutamic acid medium supplemented with 8% NaCl at early logarithmic stage and kindly supplied by Dr. T.E. Meyer; *Spirulina maxima* a blue-green bacteria which grows under alcaline conditions, was collected with a nytal nylon net from the open culture of the Sosa Texcoco Company at Lake Texcoco (Mexico)was kindly provided by Dr. Carlos Gomez-Lojero; *Desulfovibrio vulgaris* (chemosynthetic, strict anaerobic) and *Clostridium tartarivorum*, (fermentative, strict anaerobe, thermophile at 55° C) were grown in early stationary and kindly prepared by Dr. Robert Bartch; *Methanosarcina barkeri* strain Fusaro (strict anaerobe) was obtained during the logarithmic phase under liophilized form and kindly prepared by Dr. Claude Hatchikian; *Halobacterium halobium*, supplied by Dr. Walter Stockenius and Dr. Barbara Javor, was grown in our laboratory and at Scripps Institution of Oceanography at La Jolla, in the medium described by Brown 1963 with 1% peptone oxoid in the basal salt solution by Seghal and Gibbons (1960) and harvested during the stationary stage; *Escherichia coli* strains (X88 and KK1004) used in this study were obtained thanks to the courtesy of Dr. James A. Fuchs, and grown to the stationary stage, in liquid medium as described by Davis and Mingioli (1950).

Extraction Procedures

All microorganisms such as *Saccharomyces cerevisiae, Chlamydomonas reinhardtii, Rhodospirillum rubrum, Rhodospirillum salexigens, Spirulina maxima, Desulfovibrio vulgaris, Clostridium tartarivorum, Methanosarcina barkeri* and *Escherichia coli*, were broken in the cold by the Ribi cell fractionator at 16,000 psi in phosphate buffer 0.07 (pH 6.8) with a minute amount of DNAse with $MgCl_2$. *Cylindrotheca fusiformis* and *Nitzschia alba* were broken with the Yeda cell press at 3,500 psi.

The extraction procedure for *Halobacterium halobium* was done with the French Press (we are indebted to Dr. Lewin for the use of this) in an approximately isotonic solution of 20 mM TRIS-HCl (pH 7.5) containing 3 M KCl, 1.26 M NaCl, 81 mM $MgSO_4$. 7 H_2O. *Dunaliella tertiolecta* was broken by osmotic shock. *Spinacea oleracea* and the Algae *Codium sp.* were minced in a Waring blendor. *Agave fourcroydes* was received as a cold juice from the industry.

All extracts (except as indicated) were fractionated with ammonium sulfate between 40 to 60% saturation and dialyzed in buffer TRIS-HCl (pH 7.5) 1 mM EDTA, before use. *Cylindrotheca fusiformis, Nitzschia alba, Clostridium tartarivorum* and *Desulfovibrio vulgaris* extracts were measured in the crude form but previously dialyzed. *Escherichia coli* X88 and KK1004 were fractionated with ammonium sulphate between 60–100%, and dialyzed.

Enzymatic Assays

GSSG- and CoASSG-glutathione reducing activites were measured as reported by Ondarza 1970.

GSSG. A solution of GSSG (Sigma, free acid, grade III) 16 mM adjusted with 0.1 M NaOH (pH 7.0), was utilized for the estimation of GSSG-reductase activity.

CoASSG. A solution of CoASSG (Sigma, mixed disulfide sodium salt) (pH 5.5) 16 mM was utilized for the estimation of CoASSG reductase activity.

Menadione. (2-methyl-1, 4-NAPHTHOQUINONE) was a gift provided by Dr. James Mathewson.

GSSG-Reductase. It was measured in 0.1 M phosphate buffer (pH 7) with 1 mM EDTA and 150 μ moles of NADPH in a final volume of 1200 μl. The final concentration of GSSG in the cuvette was approximately 0.8 mM. The reactions were followed at 340 nm in a Pye Unicam SP 1800 spectrophotometer at room temperature.

CoASSG-Reductase. Similar conditions as for GSSG reductase were used but with 5 mM sodium phosphate buffer (pH 5.5) and 1 mM EDTA. CoASSG was approximately 0.5 mM.

NADH-Reductase. Was measured according to Lanyi 1969. In the case of *Desulfovibrio vulgaris, Clostridium tartarivorum, Methanosarcina barkeri* and *Escherichia coli*, were used menadione as an electron acceptor, in 0.05 M TRIS-maleate buffer (pH 8) in the presence of KCN (2 μ moles/ml) and NADH (0.2 μ moles/ml).

Halobacterium halobium extracts were measured in the same conditions but with 4 M NaCl.

The initial velocities of the reactions were measured and calculated in units according to Webb 1964.

Protein Measurements

The protein content of the various fractions was estimated by the method of Lowry et al. 1951, using bovine serum albumin as standard.

Results and Discussion

Tables 1 and 2 show that Glutathione-reductase is present in meaningful amounts in all eukaryotes and prokaryotes, except in the strict anaerobes and the archaebacteria *Halobacterium halobium* and *Methanosarcina barkeri*. CoASSG is absent in some eukaryotes and prokaryotes, but we do not know up to now if this is due to the stage of growth, since in the case of *Rhodospirillum rubrum* we have found changes during cell growth in CoASSG-reductase which has the maximum activity at the logarithmic stage.

However, in accordance with our hypothesis, primitive organisms such as the extreme halophilic *Halobacterium halobium* or the methanogen *Methanosarcina barkeri* and the strict anaerobes such as *Clostridium tartarivorum* (thermophile 55° C) and *Desulfovibrio vulgaris* should lack the two disulfide-reductases. (See Table 2).

In order to rule out the possibility that the activities were not detectable due to unfavorable conditions during the extraction and measurement, we made extract of *Halobacterium halobium*, at different NaCl molarities, different pH's and used NADH as a cofactor, but in all cases, we were unable to detect any disulfide-reductase activities. As a control, we measured NADH-reductase activity using menadione as an electron acceptor, which has been already established in *Halobacterium cutirubrum* by Lanyi 1969. As can be seen in Table 2, the two strict anaerobes, and the two archaebacteria, have appreciable amounts of this enzyme.

The absence of the enzyme Glutathione-reductase in some organisms like the ones described above, is not surprising since other authors (Fuchs and Warner 1975; Apontoweil and Berends 1975) have found *Escherichia coli* glutathione-deficient mutants which can grow normally. As it has been mentioned, Fahey et al. 1978 also established the fact that some gram positive bacteria and strict anaerobes lack glutathione.

In the relation to this, Loewen 1981 has studied some of the *Escherichia coli* glutathione-deficient mutants, with a very low level of glutathione synthase (E.C.6.3.2.3.) and of γ Glutamyl-cysteine synthase (EC 6.3.2.2) and found that these mutants cannot form CoASSG but instead produce respectively a mixed disulfide formed by Coenzyme A and γ glutamyl cysteinil dipeptide and, in the second case, produces only a CoA dimer.

In order to see if these mutants also should lack GSSG-reductase and CoASSG-reductase, we decided to study one of these mutants. As can be seen in Table 2, the mutant X88 which cannot synthetize glutathione because it lacks glutathione synthase, has very low activity of Glutathione-reductase and CoASSG-reductase is not present, although NADH-reductase (measured as control) appears in a good amount. The other mutant, the KK1004, with normal content of glutathione synthase hase very high Glutathione-reductase and presents CoASSG-reductase and NADH-reductase activities.

With the above results we can conclude that neither the substrate GSSG, nor the enzyme GSSG-reductase, are any longer universal as it has been claimed, since some organisms can survive without these molecules, and may have some other systems in order to maintain the reduced state within the cell.

It is worthwhile to mention that Arscott et al. (1982) have found extensive homology between nine tryptic peptides obtained from pig heart lipoamide dehydrogenase and the sequence of human erythrocyte gluta-

Table 1. GSSG-reductase and CoASSG-reductase activities from various Eukaryotes

Source	GSSG-reductase mU/mg	CoASSG-reductase mU/mg
Rat liver (Ondarza et al. 1974)	3.36	0.77
Saccharomyces cerevisiae (ATCC) (Ondarza et al. 1969)	1.17	0.384
Saccharomyces cerevisiae (Aztec)	5.0	12.0
Spinacea oleracea (Spinach)	10.0	No activity detected
Agave fourcroydes (Henequen)	23.5	8.85
Chlamydomonas reinhardtii	104.5	37.1
Cylindrotheca fusiformis (photosynthetic marine diatom)	15.8	No activity detected
Nitzschia alba (non photosynthetic marine diatom)	77.4	No activity detected
Dunaliella tertiolecta (green algae moderate halophylic)	262.0	15.0
Codium sp. (marine green algae)	41.6	No activity detected

Table 2. GSSG-reductase, CoASSG-reductase and NADH-reductase from Prokaryotes

Source	GSSG-reductase mU/mg	CoASSG-reductase mU/mg	NADH-reductase mU/mg
Escherichia coli x 88	4.6	Measured but no activity detected	68
Escherichia coli KK 1004	113	6.0	94.2
Escherichia coli (Loewen 1977)	114.5	1.6	Not measured
Rhodospirillum rubrum (photosynthetic, purple bacteria, facultative anaerobic)	10.8	7.9	Not measured
Rhodospirillum salexigens (photosynthetic, purple bacteria, facultative anaerobic moderate halophilic)	12.0	No activity detected	Not measured
Spirulina maxima (photosynthetic, blue-green bacteria, moderate halophilic)	158.2 (crude)	No activity detected	Not measured
Desulfovibrio vulgaris (chemosynthetic, strict anaerobe)	No activity detected	No activity detected	54
Clostridium tartarivorum (thermophilic 55°C, strict anaerobe, fermentative)	No activity detected	No activity detected	163
Methanosarcina barkeri (strict anaerobe)	No activity detected	No activity detected	5.5
Halobacterium halobium (photosynthetic, extreme halophilic)	No activity detected	No activity detected	10.5

thione reductase; the average homology found is 40%. One of the lipoamide dehydrogenase peptides is homologous not only with Glutathione-reductase but also with the functional regions of the thioredoxin reductase, the aminoacid oxidase, the p-hydroxybenzoate hydroxylase and the lactate dehydrogenase family. Their findings offer strong evidence for an evolutionary relationship between lipoamide dehydrogenase and glutathione-reductase. They also suggest that glutathione reductase presumably diverged from lipoamide dehydrogenase during the oxygen build-up period.

According to our hypothesis, we think that disulfide reductases can serve as evolutionary markers, since the presence of glutathione-reductase in eukaryotes and aerobic prokaryotes indicate that these organisms could survive in the oxidizing atmosphere by synthesizing disulfide reductases. The absence of Glutathione-reductase and CoASSG-reductase in Archaebacteria, (a new kingdom proposed by Woese et al. 1978; which include methanogens, thermoacidophiles and extreme halophiles), so as in strict anaerobes, means that these organisms became adapted to microenvironments which resemble primitive Earth conditions.

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Purification, Properties, and Oligomeric Structure of Glutathione Reductase from the Cyanobacterium *Spirulina maxima*

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Glutathione reductase [NAD(P)H:GSSG oxidoreductase EC 1.6.4.2] from cyanobacterium *Spirulina maxima* was purified 1300-fold to homogeneity by a simple three-step procedure involving ammonium sulfate fractionation, ion exchange chromatography on DEAE-cellulose, and affinity chromatography on 2',5'-ADP-Sepharose 4B. Optimum pH was 7.0 and enzymatic activity was notably increased when the phosphate ion concentration was increased. The enzyme gave an absorption spectrum that was typical for a flavoprotein in that it had three peaks with maximal absorbance at 271, 370, and 460 nm and a $E^{1\%}_{271}$ of 23.3. K_m values were 120 ± 12 μM and 3.5 ± 0.9 μM for GSSG and NADPH, respectively. Mixed disulfide of CoA and GSH was also reduced by the enzyme under assay conditions, but the enzyme had a very low affinity (K_m 3.3 mM) for this substrate. The enzyme was specific for NADPH. The isoelectric point of the native enzyme at 4°C was 4.35 and the amino acid composition was very similar to that previously reported from other sources. The molecular weight of a subunit under denaturing conditions was 47,000 ± 1200. Analyses of pure enzyme by a variety of techniques for molecular weight determination revealed that, at pH 7.0, the enzyme existed predominantly as a tetrameric species in equilibrium with a minor dimer fraction. Dissociation into dimers was achieved at alkaline pH (9.5) or in 6 M urea. However, the equilibrium at neutral pH was not altered by NADPH or by disulfide reducing reagents. The M_r and $s_{20,w}$ of the oligomeric enzyme were estimated to be 177,000 ± 14,000 and 8.49 ± 0.5; for the dimer, 99,800 ± 7000 and 5.96 ± 0.4, respectively. Low concentrations of urea increased the enzymatic activity, but this increase was not due to changes in the proportions of both forms.

Glutathione reductase [NAD(P)H:GSSG oxidoreductase, EC 1.6.4.2.], an enzyme widely distributed in nature, has been described in eubacteria (1-3), fungi (4, 5), plants (6, 7), and animals (1, 8–12). Despite its apparent ubiquity, we reported its absence in archaebacteria (13). GR[2] isolated from a variety of sources has shown remarkable similarity in molecular and kinetic properties. In reports where the subunit composition of different GR was given, the active form of the enzyme was always found to be a dimer of identical polypeptide chains (8, 10, 12). Nevertheless, several authors have reported the existence of a minor fraction of tetrameric protein as a consequence of the formation of interchain disulfide bonds (9, 14). Worthington and Rosemeyer (15) demonstrated that the inhibition of this enzyme from human erythrocytes by high NADPH concentrations can be explained by the aggregation of the dimer into a less active tetramer. Ii and

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[2] Abbreviations used: GR, glutathione reductase (EC 1.6.4.2); SDS, sodium dodecyl sulfate; CoASSG, the mixed disulfide of coenzyme A and glutathione.

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Sakai (16) reported that GR from cleaving sea urchin eggs becomes inactive after molecular aggregation. Serrano *et al.* (17) recently described the purification and properties of GR from a cyanobacterium of the genus *Anabaena*. The enzyme from this source is also a dimer, but diplays some unusual properties, mainly a very low isoelectric pH and an absolute specificity for NADPH.

In the course of our previous studies of GR from different sources (13), we observed that the enzyme from the cyanobacterium *Spirulina maxima* presents some distinctive properties, especially a high molecular weight. In this paper, we report the purification and characterization of GR from *S. maxima* and we demonstrate that this enzyme exists mainly as a stable tetramer that seems to be its active physiological form.

MATERIALS AND METHODS

Materials. All biochemicals were obtained from Sigma Chemical Company. Electrofocusing ampholytes (Pharmalytes) were products of Pharmacia Biotechnology, Sweden. All other chemicals were of analytical grade. *Spirulina maxima*, from an open culture taken from Lake Texcoco, México, was a gift of Sosa Texcoco Company, México. The harvested cells were thoroughly washed with distilled water in a 65-μm nylon net (Nytal, Switzerland) and stored frozen at −40°C. Microscopic examination revealed that this material was free of other microorganisms.

Assay of enzymatic activity. The activity of GR was determined spectrophotometrically in a double-beam recording spectrophotometer (Pye Unicam SP-1800). A typical reaction mixture contained 0.14 mM NADPH and 1.2 mM GSSG in 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM EDTA (final volume of 0.5 ml). The decrease in absorbance at 340 nm was recorded for 1 or 2 min in the absence of GSSG; thereafter, this substrate was added to start the reaction. A unit of enzyme activity was defined as the amount of enzyme required to oxydize 1 μmol of NADPH per min at pH 7.0 and 25°C. CoASSG reductase activity was measured under the same conditions by using varied amounts of CoASSG as substrate. Kinetic data were fitted to the Michaelis-Menten equation by using Cleland's Hyper program (18). Specific activities reported were calculated from true maximal velocities.

Protein assays. Protein was determined by the method of Lowry (19) or by the Coomassie G dye-binding technique of Bradford (20), with bovine serum albumin used as standard. The concentrations of the pure enzyme solutions were calculated from absorbance at 271 nm in a 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA.

Polyacrylamide gel electrophoreses. Electrophoreses under nondenaturing conditions were performed in 80 × 80 × 2.7-mm gel slabs with a linear pore gradient obtained by varying the concentration of the monomers from 4 to 30%, with a constant crosslinkage of 4% (21). Samples of six proteins with molecular weights from 29,000 to 240,000 were run simultaneously as standards. Electrophoreses were performed at 5°C either in 90 mM Tris-80 mM boric acid buffer (pH 8.35) or in 0.1 M sodium phosphate buffer (pH 7.0) at 125 V for 14 to 16 h. Electrophoreses in the presence of SDS were carried out in 160 × 160 × 0.75-mm gel slabs in phosphate-SDS buffer according to Weber and Osborn (22). Five proteins, from M_r 17,000 to 68,000, were run as molecular weight markers.

Isoelectric focusing. The isoelectric point of GR was determined by preparative isoelectric focusing in a sucrose density gradient in a 110-ml LKB column containing ampholytes, either pH range 3 to 10 or 4 to 6, both at a concentration of 2.2 g/100 ml according to Winter and Karlsson (23). Analytical isoelectric focusing was carried out in polyacrylamide gel (T = 6%, C = 2%) or in IEF-agarose in 125 × 125 × 2-mm gel slabs in a Multiphor apparatus (LKB, Sweden) with pH 3 to 9 or 4 to 6 ampholytes at a final dilution of 1:15.

Ultracentrifugation studies. Sedimentation coefficients, as well as molecular weights, were determined by ultracentrifugation in linear sucrose density gradients in a L5-65 Beckman preparative ultracentrifuge (SW 50.1 rotor). Sucrose density gradients containing the polyacrylamide monomers were also prepared according to the procedure described by Jolley *et al.* (24). These gradients were polymerized and stained by standard methods. Molecular weights were estimated as described elsewhere (25); sedimentation coefficients, according to Martin and Ames (26).

Amino acid analyses. Amino acid analyses were carried out in a Durrum D-500 amino acid analyzer. Samples from two different enzyme preparations were dialyzed 12 h against 500 vol of purified water, and hydrolyzed at 110°C in 6 M HCl in vacuum-sealed tubes for 20, 48, or 72 h. Cysteine and half-cystine were determined as cysteic acid by oxidation of the different enzyme samples with performic acid (27) and hydrolysis for 20 h at 110°C in 6 M HCl containing 0.5% phenol. Tryptophan was determined by hydrolysis with 2-mercaptoethanesulfonic acid, according to Penke *et al.* (28). All results reported are averages from two independent analyses.

RESULTS

GR purification. All operations described in this section were performed at 4°C.

The frozen cells were suspended in 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM EDTA (buffer A) in a ratio of 2 ml per gram of wet bacterial cells. The suspension was subjected to ultrasonic oscillations and centrifuged at 25,000*g* for 15 min, and the precipitate was discarded. Solid ammonium sulfate was added to the supernatant (pH 6.8) to reach a 50% saturation. The mixture was stirred 30 min and centrifuged at 20,000*g* for 20 min. The precipitate was discarded and solid ammonium sulfate was added to the supernatant with continuous stirring to give 90% saturation. After centrifugation, the precipitate was dissolved in a minimal volume of 25 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA (buffer B) and was dialyzed two times (6 h each) against 200 vol of the same buffer.

The dialyzed enzyme was applied to a DEAE-Sephacel column (2.6 × 30 cm for a preparation starting from 500 g bacteria) previously equilibrated with buffer B. After the column was washed, the enzyme was eluted by means of a linear NaCl concentration gradient (0 to 0.3 M) prepared in buffer B. The enzyme was recovered in a single peak of activity at 0.15 M NaCl. The pooled fractions containing the enzyme were dialyzed against buffer A (250 vol for 12 h) and applied to a column packed with adenosine 2',5'-bisphosphate Sepharose-4B (2.6 × 3 cm) equilibrated in the same buffer. After the column was washed, the enzyme was eluted by means of a liner concentration gradient (0 to 0.1 mM) of NADPH. Fractions containing the enzyme were pooled, dialyzed against 200 vol of buffer A for 12 h, and concentrated by immersion of the dialysis sack in a concentrated solution of polyethylene glycol compound (M_r 15,000 to 20,000; Sigma). The enzyme was stored frozen at −20°C. A typical purification procedure is summarized in Table I.

Criteria of purity. The enzyme obtained was shown to be homogeneous by the following procedures: SDS-polyacrylamide gel electrophoresis, pore gradient polyacrylamide gel electrophoresis of the native protein at pH 9.5, electrofocusing in IEF-agarose, and density gradient ultracentrifugation. Some representative gels are shown in Fig. 1.

Stability. The pure enzyme, even at low concentrations (0.1 mg/ml), was stable up to one year when stored frozen at −20°C in buffers A or B; repeated freezing and thawing had no effect upon the enzyme activity. Heating the preparation at 55°C (pH 7.0) for 10 min did not produce any significant loss of activity, but the enzyme was readily inactivated at higher temperatures.

Kinetic parameters and substrate specificity. The enzyme reduced GSSG and also had some activity toward the mixed disulfide CoASSG, but neither CoASS nor cystine acted as substrates at all. The K_m for

TABLE I

PURIFICATION PROCEDURE FOR GLUTATHIONE REDUCTASE FROM *Spirulina maxima*

Procedure	Total volume (ml)	Total protein (mg)	Specific activity[a] (units mg^{-1})	Enrichment	Yield (%)
Crude extract	932	27,103.0	0.183	1.0	100.0
Crude extract (centrifuged)	817	21,468.0	0.245	1.3	106.0
Ammonium sulfate	152	623.0	2.530	13.8	31.6
DEAE-cellulose	219	166.0	6.430	35.0	21.5
Adenosine 2',5'-bisphosphate-Sepharose 4B	14	6.5[b]	238.000	1300.0	36.3

Note. Amounts rated for 300 g of biomass, wet weight.

[a] In each case, values were calculated from the corresponding V_{max}.

[b] Protein in this step was estimated from the $E^{1\%}_{1cm}$ for the pure enzyme.

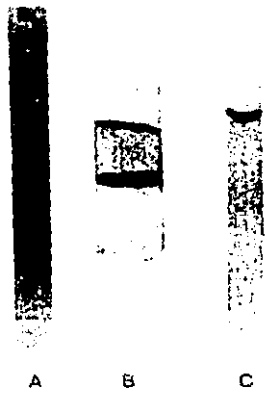

FIG. 1. Purity of GR preparation. (A) Polyacrylamide gel electrophoresis of denatured enzyme (10 μg) in the presence of SDS (T = 10%, C = 2.6%). (B) Ultracentrifugation in acrylamide-containing sucrose density gradient (T = 8%, C = 3.6%). Protein samples (15 μg) were subjected to 290,000*g* for 14 h at 4°C. The gradient material was polymerized and then stained. (C) Electrofocusing in 2% IEF-agarose with Pharmalytes 3 to 9 (diluted 1:15). Gels were run at 5°C for 2500 V h. An amount of 5 μg were applied. In all cases, gels were stained by standard methods using Coomassie brilliant blue R dye.

GSSG at a high fixed NADPH concentration in buffer A (20 times K_m) was 121 ± 12 μM (SE) at pH 7.0 and 25°C. Under the same conditions, a K_m of 3.3 ± 0.9 mM (SE) was calculated for CoASSG. The specific activities for GSSG and for CoASSG, respectively, were 238 ± 8 and 3.0 ± 0.4 μmol min^{-1} mg^{-1} (SE), giving a V_{max} ratio of 79. At a high fixed concentration of GSSG (20 times K_m), the K_m for NADPH was 3.5 ± 0.9 μM (SE) in the same buffer. NADH could not substitute for the reducing substrate NADPH. The order in which substrates were added had no effect on the initial velocities.

When V_{max} was studied as a function of pH, a sharp maximal value was obtained at pH 7.0 when either GSSG or CoASSG was used as substrate. V_{max} also increased with increased phosphate buffer concentration; the resulting curve (data not shown) was hyperbolic; an apparent activation constant for phosphate was calculated to be 15 mM.

Amino acid composition and spectral properties. The amino acid composition of GR from *S. maxima* is shown in Table II. From these results and according to the method of McMeekin and Marshall (29), a partial specific volume of 0.734 cm^3 g^{-1} was estimated, without taking into account the flavine coenzyme. The ultraviolet-visible spectrum of the enzyme in buffer B was typical for a flavoprotein, with maxima at 271, 370, and 460 nm. From the spectral data and the mass of the enzyme calculated from the amino acid analyses, the value of $E^{1\%}_{271}$ at pH 7.5 was estimated to be 23.3. The molar absorptivity coefficient, assuming a molecular weight of 192,000, was 4.46×10^5 M^{-1} cm^{-1}. Assuming that the flavine coenzyme is FAD and taking into account the molar absorptivity coefficient reported for protein-bound FAD at 460 nm (30), a ratio of 1.06 mol flavine/mol enzyme subunit was calculated.

Isoelectric point. The isoelectric focusing of the native enzyme gave an homogeneous peak with maximal activity at pH 4.35 (average of two determinations at two different pH ranges, 3 to 10 and 3 to 6).

Molecular weight and oligomeric structure. The subunit molecular weight, as determined by SDS-polyacrylamide gel electrophoresis, was 47,000 ± 1200 (SD). The molecular weight of the native protein, estimated by pore gradient gel electrophoresis was 92,000 ± 5000 (SD) at pH 9.5 (Fig. 3A). Approximately the same value (95,700 ± 9000) was obtained by ultracentrifugation of the enzyme in acrylamide-containing sucrose density gradients at pH values of 5.2, 7.0, and 8.8 which were polymerized and stained after the run. Sedimentation of the enzyme in sucrose density gradients prepared in 0.1 M Tris-HCl buffer (pH 9.5) without acrylamide also gave a single protein peak for which an $s_{20,w}$ of 5.96 ± 0.4 (SD) and a molecular weight of 98,000 ± 7000 (SD) were calculated (Fig. 2A). Gel filtration chromatography of the enzyme in a calibrated column packed with Ultrogel AcA 34 (0.1 M Tris-HCl; pH 9.5;

TABLE II

AMINO ACID COMPOSITION OF GLUTATHIONE REDUCTASE FROM *Spirulina maxima*

Amino acid	Residues per subunit of molecular weight 47,400[a]				
	20 h	48 h	72 h	Average[b]	Nearest integer
Asx	41.54	41.85	41.25	41.55	42
Thr	29.96	27.90	28.08	31.33[c]	31
Ser	27.71	25.27	22.62	29.35[c]	29
Glx	46.96	46.85	46.03	46.61	47
Pro	7.72	6.96	6.29	6.99	7
Gly	46.59	47.36	46.76	46.90	47
Ala	44.40	42.95	43.81	43.72	44
Val	32.53	33.26	34.52	33.44	33
Met	11.13	10.38	10.53	10.68	11
Ile	25.98	29.03	29.25	28.09	28
Leu	31.96	34.70	34.60	33.75	34
Tyr	7.85	7.01	6.74	7.20	7
Phe	18.75	18.31	17.90	18.32	18
His	10.79	11.37	11.47	11.21	11
Lys	22.81	23.89	24.05	23.58	24
Arg	18.34	18.24	18.27	18.28	18
Cys[d]	6.40	—	—	6.40	6
Trp[e]	3.59	—	—	3.59	4
Total number of amino acids: 441					

[a] Excluding flavine.
[b] Unless otherwise indicated.
[c] Extrapolated to zero time.
[d] Determined in a separate hydrolysis as cysteic acid, with leucine used as internal reference.
[e] Determined in a separate hydrolysis with leucine used as internal reference.

4°C) also gave a single symmetrical enzyme peak with similar molecular weight (data not shown). However, when the enzyme was analyzed by sedimentation in the ultracentrifuge in sucrose density gradients at lower pH values, a second sedimenting zone appeared which had approximately twice the previously determined molecular weight. This peak was the predominant enzyme species at neutral pH (Fig. 2B-D). The sedimentation coefficient of this heavier component was estimated to be 8.49 ± 0.5 (SD) and the corresponding molecular weight to be 177,000 ± 14,000 (SD). This sedimentation pattern was not changed by the addition of 0.1 mM NADPH, 5 mM 1-mercaptoethanol, or 2 mM dithiothreitol. A similar pattern was obtained in pore gradient polyacrylamide gel electrophoreses in buffer A (Fig. 3B) and in gel filtration chromatography in Ultrogel AcA 34 in the same buffer (data not shown).

In order to demonstrate the reversible conversion of these two forms of aggregation of the enzyme when the pH was changed, the following experiment was performed. A 200-μg sample of enzyme which had been stored at pH 7.0 was dialyzed for 24 h against 200 vol of 0.1 M Tris-HCl buffer (pH 9.5) containing 1 mM EDTA. A 100-μg aliquot was then removed and ultracentrifuged at pH 9.5; a sedimentation pattern was obtained as already described, the resultant profile being identical to that of Fig. 2A. The remaining 100 μg of the dialyzed enzyme sample was again dialyzed for 24 h against buffer A (pH 7.0) before being analyzed by ultracentrifugation by the same procedure. This sample gave a two-peak pattern similar to that in Fig. 2D.

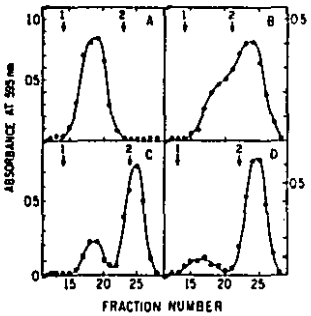

FIG. 2. Sedimentation patterns of GR at various pH values. Protein samples (90 to 100 μg) were layered on 5 ml of a 5 to 20% linear sucrose density gradient and ultracentrifuged for 14 h at 290,000*g* and 4°C. Then tubes were punctured at the bottom and fractions of four drops (about 125 μl) were collected. Molecular weight markers were run simultaneously and the protein profile was determined by the procedure of Bradford (20). Standard proteins were myoglobin (not shown), ovalbumin (1), and IgG (2). All gradients were made in 0.1 M Tris-HCl buffer containing 1 mM EDTA, with the exception of D, which was made in 0.1 M sodium phosphate buffer. The bottom of the tubes is shown at the right. (A) pH 9.5; (B) pH 8.9; (C) pH 7.9; (D) pH 7.0.

Effect of urea. The enzyme activity was assayed in the presence of different urea concentrations (Fig. 4A). At 0.4 to 0.6 M urea, the enzyme activity was nearly doubled. Sedimentation experiments performed at these same urea concentrations and pH values did not demonstrate changes in the relative proportions of the two-peak pattern shown in Fig. 2D. At higher urea concentrations, the enzyme was progressively inactivated and was completely inactivated in 4.0 M urea. Nevertheless, this inactivation did not correlate with changes in the sedimentation pattern since, by ultracentrifugation analysis under the same conditions, the two-peak pattern with the same molecular weights as already determined was obtained (Fig. 4B). However, a slight increase in the fraction of the lighter component was evidenced, as compared with the sedimentation profile shown in Fig. 2D. At intermediate (2.5 and 3.2 M) urea concentrations the sedimentation pattern was almost identical to the one shown in Fig. 2D in spite of the evident changes in the observed enzyme activity. This activity profile did not change after a 24-h period of incubation in the presence of different urea concentrations. The effect of 6 M urea was assayed by gel filtration chromatography in Sephadex G-150 equilibrated with the urea solution prepared in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA. Under these conditions, the

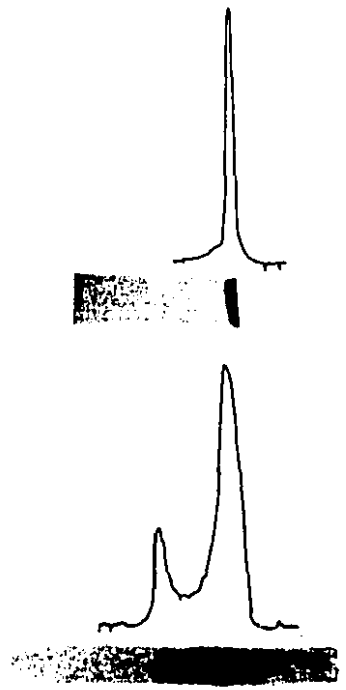

FIG. 3. Pore gradient electrophoreses of GR in polyacrylamide and the corresponding densitometry. (A) Gel precquilibrated and run in Tris-HCl buffer (pH 9.5) containing 1 mM EDTA at 5°C. (B) Gel preequilibrated and run in 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM EDTA at 5°C. In both cases an amount of 15 μg protein was applied.

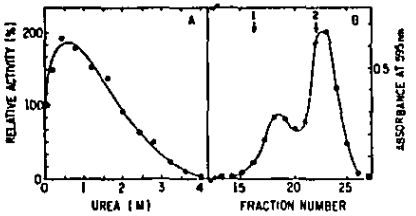

FIG. 4. (A) Effect of urea on enzyme activity. The enzyme was incubated for 1 to 2 min in the presence of NADPH and buffer A containing urea at the indicated concentrations; the reaction was started by addition of GSSG. The activity is shown as percentages of the value obtained in control experiments without urea. Other conditions were performed as described under Materials and Methods. Each point represents the average of four independent assays. (B) Effect of urea on enzyme dissociation. Glutathione reductase (100 μg) was placed on 5 ml of a 5 to 30% sucrose density gradient prepared in 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM EDTA and 4 M urea. Ultracentrifugation was run for 24 h at 290,000*g* and 4°C. Other conditions and protein standards were the same as described in Fig. 2.

enzyme eluted as a single peak of M_r 92,000 (average of two determinations).

DISCUSSION

In this paper, we describe a three-step procedure for the total purification of GR from the cyanobacterium *S. maxima*. The enzyme was shown to be homogeneous according to several criteria of purity and to have a specific activity of 238 μmol min^{-1} mg^{-1}. The enzyme had an absolute specificity for NADPH, in contrast with GR from other sources that can also oxidize NADH (5, 6, 10). GR from another cyanobacterium, *Anabaena* sp., is also absolutely specific for NADPH (17). The specificity of GR for disulfide substrate has been studied for the enzyme from baker's yeast (31, 32) and rat liver (10, 33). Both enzymes are also able to reduce CoASSG, a mixed disulfide of unknown function, the existence of which was first reported by Ondarza (34). The GSSG/CoASSG activity ratio for baker's yeast was reported to be 9.1 by Carlberg and Mennervik (31); Ondarza (33) and Carlberg and Mannervik (10) reported 12.2 and 7.45, respectively, for the enzyme isolated from rat liver. For these enzymes, the optimum pH for the reduction of CoASSG is more acidic than is the corresponding optimum for reduction of GSSG. In *S. maxima*, both activities were maximal at neutral pH, and the activity toward CoASSG is minimal. Throughout the purification, there was no evidence for the presence of a separate reductase in *S. maxima* which was specific for CoASSG. Unfortunately, since there are no reports of CoASSG reductase activity in GR purified from other procaryotes, we cannot be sure that this low activity is a general characteristic of these organisms.

The amino acid composition of our preparation was very similar to those reported for the enzyme from *Anabaena* sp. (17), *Escherichia coli* (1), yeast (1), and animal sources (9, 12). Except for low content of proline and tyrosine residues (Table II), there were no great variations. The acidic isoelectric pH (4.35) of the GR from *S. maxima* is an unusual characteristic that it shares with GR from *Anabaena* sp. (4.02). To date, these values are the lowest reported for any GR. However, the most striking characteristic of the *Spirulina* enzyme was its oligomeric structure. Although the subunit molecular weight was in the range expected for a glutathione reductase, our results suggest that the oligomeric enzyme existed mainly as a tetramer throughout a wide range of pH. This tetramer seems to be in equilibrium with a small dimeric component, with the transition between these aggregation states sufficiently slow to allow their separation by different methods. The sedimentation pattern at pH 7.0 did not change in the presence of NADPH or thiol reducing reagents. This excluded the possibility of an aggregation of dimers mediated by NADPH as described by Worthington and Rosemeyer for GR from human erythrocytes (15) and also ruled out the association of dimers by interchain disulfide bridges (9, 14).

The tetramer was fairly stable since up to 6 M urea was needed to completely dis-

sociate it into dimers. It is worth noting that the activation of the enzyme at low urea concentrations did not correlate with changes in the quaternary structure of the protein, suggesting that this effect was merely conformational. The existence of a major tetrameric component and a minor dimeric fraction was observed in ultracentrifugation experiments, in gel permeation chromatography, and in pore gradient gel electrophoreses. Because the patterns obtained with these different techniques were quite similar, the possibility of artifact due to the well-known dissociating effect of proteins by the hydrostatic pressure (tenths of kilobars) during ultracentrifugation (35) can be discarded. The dissociation of GR from *S. maxima* was strongly dependent upon pH, as was demonstrated by experiments at different pH values. These results indicated that charged groups on the protein may be involved in the interaction between dimers and that these groups may change their protonation state at pH 8–10.

An unexpected finding was the sedimentation of the enzyme as a single form with a M_r of 95,700 when it was analyzed at pH 7.0 in the presence of acrylamide monomers. However, under standard conditions at this same pH value, the GR from *S. maxima* predominantly existed as tetramer (Fig. 2D), suggesting a dissociating effect of acrylamide upon the enzyme. In experiments performed in the presence of acrylamide without polymerizing agents, a notable increase in the dimer fraction was observed.

The experiments reported here did not clarify the question about the activity of the enzyme in each state of aggregation. Both forms assayed immediately after their separation by sedimentation or by gel filtration chromatography appeared active in the standard enzyme assays, but it should be noted that under these conditions the equilibrium between both species may be distinct. The kinetics of the conversion between the dimeric and tetrameric forms of the enzyme should give additional information about this structural transition and its possible significance on the regulation of GR from *S. maxima*.

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DISCUSION

La existencia de una actividad enzimática asociada con la reducción del glutatión fué considerada durante mucho tiempo como un requisito esencial para el buen funcionamiento del metabolismo celular. De hecho, se llegó a pensar que la vida misma no sería posible sin el mantenimiento de un cociente elevado GSH/GSSG. Sin embargo, en 1975 Apontoweil et al (36) reportan el aislamiento de mutantes de *Escherichia coli* que, a pesar de ser deficientes en la biosíntesis del glutatión, presentan cinéticas de crecimiento normales, si bien muestran una mayor susceptibilidad a ciertos agentes químicos. Por otra parte, Fahey et al (37) analizan el contenido del tripéptido en todo un espectro de bacterias Gram-positivas y Gram-negativas, encontrando diferencias significativas entre ambos conjuntos, con el grupo Gram-nagativo exhibiendo las mayores concentraciones. Sin embargo, el anaerobio *Desulfovibrio vulgaris*, a pesar de incluirse en la categoría Gram-negativo, muestra niveles muy bajos de glutatión. Es interesante señalar que incluso dentro de una misma especie bacteriana los niveles de glutatión parecen variar dependiendo de las condiciones de crecimiento, siendo más abundantes durante la aerobiosis. Así, en *Escherichia coli* la concentración de glutatión se incrementa cuatro veces (37) al pasar de anaerobiosis a aerobiosis (de 7 a 27 µmol/g, respectivamente).

En lo que respecta a los eucariotes, aunque la participación del glutatión en una gran variedad de procesos ha quedado claramente demostrada, se han reportado casos en donde el tripéptido parece estar completamente ausente del metabolismo. En *Entamoeba histolytica*, por ejemplo, Fahey et al (38) han reportado la ausencia total del compuesto así como de enzimas relacionadas, y los autores sugieren que , debido a que este eucariote carece también de mitocondrias, la adquisición de ambos - tanto del metabolismo del glutatión como de mitocondrias - durante el proceso evolutivo se llevó a cabo posiblemente de manera simultánea. En este sentido, Meredith y Reed (39) reportan que las mitocondrias de hepatocito de rata son incapaces de sobrevivir a la depleción de la reserva de glutatión mitocondrial.

En base a todo lo anterior, los resultados presentados en nuestro trabajo, acerca de la ausencia de glutatión reductasa en el grupo de bacterias conocido colectivamente como arqueobacterias (Archaebacteria, Woese 1978 (40)), parecen reforzar la idea de que la participación del GSH en el metabolismo no es un factor crítico para la sobrevivencia de la célula.

La posibilidad de que nuestra falla para detectar actividad de glutatión reductasa en estas bacterias se debiera a condiciones de cultivo inadecuadas parece poco probable, ya que en el caso particular de *Halobacterium halobium*, los cultivos fueron crecidos con burbujeo constante de oxígeno - condiciones que deberían favorecer la presencia de dicha enzima - e incluso la cosecha fué realizada en diferentes estadíos del crecimiento.

En este sentido existen evidencias de que la enzima no es inducible, ya que en aquellos microorganismos en donde se ha medido, la glutatión reductasa parece estar en todas las fases de crecimiento de un cultivo axénico. Loewen (46) ha reportado que en cultivos de *Escherichia coli* crecidos aeróbicamente, la actividad específica de la enzima se incrementa gradualmente hacia la fase estacionaria, llegando a ser el doble (121.6 mU/mg) que al inicio de la fase logarítmica (60.6 mU/mg); sin embargo, a pesar del evidente incremento en la actividad, la enzima siempre está presente. Es interesante mencionar que el mismo autor observó que al cambiar un cultivo de la misma bacteria de condiciones aerobias a anaerobias, la enzima permanece en un nivel constante, aproximadamente igual al que tenía al momento del cambio.

Es posible generalizar, entonces, que la presencia de glutatión reductasa siempre se verá favorecida en organismos que vivan en ambientes donde las presiones parciales de oxígeno sean apreciables. En el caso de los tres grupos de arqueobacterias estudiadas (metanógenas, termoacidófilas y halófilas), podemos afirmar que una característica común a todas ellas lo es el habitar lugares en donde dichas presiones parciales son extremadamente bajas, siendo innecesaria, por lo tanto, la presencia de dicha enzima.

Ahora bien, aunque en el presente trabajo no se evaluó el nivel de concentración del glutatión en ninguna arqueobacteria, Newton et al (41) han encontrado recientemente que dicho compuesto está totalmente ausente en seis especies de halobacterias; en su

lugar, los autores encuentran niveles apreciables de γ glutamil-cisteína, el dipéptido precursor del glutatión. Además, en éstas mismas bacterias parece existir una enzima cuya actividad de reductasa hacia el dipéptido mencionado es casi ocho veces mayor que la correspondiente actividad sobre GSSG, reforzando así los resultados de nuestro trabajo.

Cabe mencionar que los equivalentes reductores utilizados por la enzima durante el ciclo catalítico son suministrados por la vía del fosfogluconato bajo la forma de NADPH. Dicha vía ha sido bien caracterizada en eucariotes heterótrofos y fotosintéticos funcionando en estos últimos en sentido inverso durante la fijación de CO_2. Desafortunadamente no se dispone de información sobre la existencia de la vía mencionada en arqueobacterias, ya que sería interesante conocer la disponibilidad de poder reductor en estos procariotes así como el posible grado de control ejercido sobre la glutatión reductasa.

Dentro de los procariotes, el grupo de las cianobacterias constituye un caso particularmente interesante, ya que son las únicas bacterias fotosintéticas que generan oxígeno durante el proceso de fijación del CO_2 y, por consiguiente, la existencia de sistemas antioxidantes en estos organismos parece una necesidad. En este sentido, la presencia de glutatión ha sido reportada en varias cianobacterias. En *Nostoc muscorum*, Karni et al (42) encuentran una concentración de 0.6 - 0.7 mM, mientras que en *Synechococcus* sp. Tel-or et al (43) reportan 3.2 mM.

A pesar de que la presencia del glutatión en estos procariotes es un hecho bien establecido, poco se sabe acerca de las enzimas participantes. Karni et al (42) reportan la existencia de glutatión reductasa en células vegetativas y heterocistos de *Nostoc muscorum*, adjudicando propiedades diferentes a la enzima de ambas estructuras, esencialmente en lo que respecta a su afinidad por GSSG y sensibilidad a iones Zn^{++} ; sin embargo, los mismos autores declaran su incapacidad para detectar glutatión peroxidasa. Por otra parte Serrano et al (9) han publicado recientemente la purificación y propiedades de la glutatión reductasa aislada de la cianobacteria *Anabaena* sp.. La enzima comparada con aquella de otras fuentes (ver Tabla I), no muestra mayores particularidades a excepción de una especificidad absoluta por NADPH y un punto isoeléctrico notablemente ácido.

La enzima de *Spirulina maxima*, cuya purificación y propiedades se reportan en el presente trabajo, comparte con aquella de *Anabaena* las dos características señaladas, aunque definitivamente el rasgo más inusual de la glutatión reductasa de *Spirulina maxima* reside en su estructura cuaternaria. De hecho, durante el transcurso de la caracterización preliminar de la enzima, existieron resultados radicalmente opuestos, ya que mientras el peso molecular obtenido por electroforésis en gradiente de poro sugería una estructura dimérica, los experimentos de filtración en gel apoyaban la idea de una molécula más compleja. La imposibilidad de conciliar ambos resultados se debió esencialmente a que la posición de la enzima en los cromatogramas se rastreaba por su actividad reductora y no por el perfil de proteína, de tal manera que el dímero, menos abundante, pasó inadvertido durante mucho tiempo. Por otro lado, la inexistencia de tetrámero en los geles de gradiente de poro, se debió a que el pH al cuál eran corridos (alrededor de 9.0) favorece completamente la aparición de la forma dimérica. La coexistencia simultánea de ambos oligómeros se puso de manifiesto en los experimentos de sedimentación en gradiente de sacarosa a pH neutro, en donde, al determinar el perfil de proteína, fué evidente que la enzima sedimentaba bajo dos formas de agregación diferentes, siendo más abundante la molécula tetramérica.

A primera vista, la existencia de glutatión reductasa tetramérica no parece representar un hallazgo inesperado; de hecho, éste ha sido reportado en otros casos. Así, Worthington y Rosemeyer (44) Demuestran que la inhibición producida por NADPH sobre la enzima obtenida de eritrocito humano es debida a la agregación de la forma dimérica en un tetrámero menos activo, mientras que Boggaram et al (27) reportan la agregación de la enzima de eritrocito porcino debido a la ausencia de tioles en el medio. Un resultado similar a este último es mencionado por Carlberg et al (29) para la enzima de hígado de ternera. Sin embargo, en el caso de la glutatión reductasa de *Spirulina maxima*, ni la adición de NADPH ni la presencia de ditiotreitol lograron cambiar el perfil de sedimentación obtenido sin estos agentes, minimizando la posibilidad de que el tetrámero observado fuese un artefacto.

Además, la notable estabilidad de la enzima frente a un agente disociante como la urea parece reforzar esta última idea, ya que se requiere urea 6 M para disociar el tetrámero en dímeros, mientras que con sólo 4 M la actividad enzimática es abolida completamente, no habiendo una correlación entre el grado de disociación y la pérdida de actividad. En este sentido, cabe señalar que la glutatión reductasa de *Anabaena* sp. es disociada a monómeros en presencia de urea 6 M (9), mientras que Zanetti (22) reporta una inhibición de sólo el 2% para la enzima de hígado de conejo en presencia de urea 8 M.

Los resultados reportados demuestran además, que ambas formas son fácilmente interconvertibles por ajuste al pH adecuado, sugiriendo que en el fenómeno de asociación-disociación estan en juego grupos ionizables. Queda por esclarecer la naturaleza química de dichos grupos, sus pK_a así como su posible dependencia de la temperatura. Con relación a esto último, es interesante mencionar el hecho de que los experimentos de sedimentación fueron realizados a 4°C, mientras que los ensayos de actividad se verificaron a temperatura ambiente. Será importante dilucidar el perfil de sedimentación de la enzima en este último rango de temperatura, ya que podría proporcionar información sobre cuál de los dos oligómeros es la forma activa. Resultados preliminares en este sentido permiten sugerir que es el tetrámero la forma más activa, si bien no excluyen la posibilidad de que el dímero participe también en la reducción del glutatión.

Ahora bien, aunque ya se mencionó la interconversión reversible de las dos formas de la enzima al pasar de un pH neutro a uno alcalino y viceversa, en el presente trabajo no se muestran evidencias definitivas de que exista un equilibrio entre ambos oligómeros al mismo pH. Sin embargo, la posibilidad de resolver con bastante efectividad dímero y tetrámero mediante experimentos de transporte - sea electroforésis ó sedimentación - sugiere, en principio, que de existir interconversión a un pH dado, la velocidad con la cuál se lleva a cabo es baja (45). La cuantificación de la cinética de interconversión es una meta importante si se desea esclarecer el problema de la actividad de ambas formas y, por ende, el posible significado fisiológico del fenómeno.

Si analizamos las características de la glutatión reductasa de procariotes mostradas en la Tabla I, conjuntamente con los resultados del presente trabajo, curiosamente observamos que, a excepción de *Escherichia coli*, las otras tres bacterias incluidas - al igual que *Spirulina maxima* - exhiben alguna propiedad que se desvía del valor "promedio" conocido para esta enzima. Esta observación, aunque aparentemente irrelevante, apoya una de las hipótesis planteadas al inicio del trabajo, es decir, que la glutatión reductasa de procariotes presenta una mayor variabilidad que su contraparte eucariótica, si bien por el momento somos incapaces de dar una explicación a dicho fenómeno. En principio, la única cosa en común que presentan estas bacterias es la de ser fotosintéticas.

Finalmente, con respecto al reconocimiento del disulfuro mixto CoASSG por parte de la enzima, los resultados reportados parecen sugerir que la glutatión reductasa de *Spirulina maxima* muestra la mayor especificidad hacia este compuesto en comparación con la enzima de otras fuentes en las cuales se ha estudiado esta característica.

Así, en la levadura *Saccharomyces cerevisiae*, se han reportado cocientes de actividad GSSG reductasa/CoASSG reductasa que van desde tres (33) hasta casi diez(34), mientras que en hígado de rata dicho cociente oscila entre 7.5 (18) y 12 (35). Desafortunadamente el elevado costo del CoASSG, aunado a la baja afinidad de la enzima por el mismo (en comparación con el GSSG), no han permitido realizar estudios de este tipo utilizando concentraciones saturantes del disulfuro mixto. En *Spirulina maxima*, el cociente de actividades enzimáticas es tan alto como 79, y esto es particularmente significativo considerando que se trata de una relación de velocidades máximas. Parecería entonces que en esta cianobacteria la glutatión reductasa es notablemente específica por su sustrato. La comparación con la enzima de otros procariotes en este sentido no es posible, ya que además de que solo se ha purificado hasta homogeneidad en *Escherichia coli* y *Anabaena* sp., en ningún caso se da información respecto a su actividad sobre CoASSG.

Cabe señalar asimismo, que en *Spirulina maxima* ambas actividades presentan un máximo a pH 7.0, contrastando con reportes previos donde se observó un desfasamiento de las dos actividades reductoras, de tal manera que mientras el óptimo para la reductasa del GSSG es de pH 7.0 (tanto en *Saccharomyces cerevisiae* como en hígado de rata), el máximo correspondiente a la CoASSG reductasa

en estas mismas fuentes oscila alrededor de pH 5.5 (18,33).

Durante la determinación del punto isoeléctrico de la enzima, se rastreó la supuesta CoASSG reductasa independiente. Sin embargo, con los niveles de sustrato que se utilizaron, no fué posible ubicarla ni siquiera en la zona correspondiente a la GSSG reductasa. Esto último, aunado a la ausencia de actividad en extractos crudos, parecen descartar la hipótesis planteada al comienzo de este trabajo.

Los resultados anteriores, junto a la falta de evidencia en favor de una reductasa para el CoASSG - independiente de la GSSG reductasa - sugieren que en *Spirulina maxima* hay una sola proteína (posiblemente con un único sitio activo) involucrada en la reducción de ambos disulfuros, con una afinidad y eficiencia catalítica notablemente mayor hacia el glutatión oxidado. Queda por aclarar la posible existencia del disulfuro mixto en cianobacterias, a fin de esclarecer si la actividad reductora presente en la glutatión reductasa desempeña un papel fisiológico ó es simplemente carencia de especificidad absoluta por el sustrato.

Es interesante mencionar que la enzima aislada a partir de una célula anucleada como el eritrocito, es incapaz de reconocer al mencionado disulfuro mixto, siendo que la enzima de eucariotes es, en promedio, sólo un orden de magnitud más activa sobre GSSG respecto al CoASSG. Esto, aunado a la poca actividad de CoASSG reductasa del procariote *Spirulina maxima*, plantea una posibilidad interesante, a saber, que el metabolismo de dicho compuesto esté involucrado en algún proceso nuclear.

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