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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:

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

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

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

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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