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Evolution of Antioxidant Mechanisms: Thiol-Dependent Peroxidases and Thioltransferase among Procaryotes

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Summary. Glutathione peroxidase and glutathione S-transferase both utilize glutathione (GSH) to destroy organic hydroperoxides, and these enzymes are thought to serve an antioxidant function in mammalian cells by catalyzing the destruction of lipid hydroperoxides. Only two groups of procaryotes, the purple bacteria and the cyanobacteria, produce GSH, and we show in the present work that representatives from these two groups (*Escherichia coli, Beneckea alginolytica, Rhodospirillum rubrum, Chromatium vinosum,* and *Anabaena* sp. strain 7119) lack significant glutathione peroxidase and glutathione S-transferase activities. This finding, coupled with the general absence of polyunsaturated fatty acids in procaryotes, suggests that GSH-dependent peroxidases evolved in eucaryotes in response to the need to protect against polyunsaturated fatty acid oxidation. A second antioxidant function of GSH is mediated by glutathione thioltransferase, which catalyzes the reduction of various cellular disulfides by GSH. Two of the five GSH-producing bacteria studied (*E. coli* and *B. alginolytica*) produced higher levels of glutathione thioltransferase than found in rat liver, whereas the activity was absent in the other three species studied. The halobacteria produce γ-glutamylcysteine rather than GSH, and assays for γ-glutamylcysteine-dependent enzymes demonstrated an absence of peroxidase and S-transferase activities but the presence of significant thioltransferase activity. Based upon these results it appears that GSH and γ-glutamylcysteine do not function in bacteria as antioxidants directed against organic hydroperoxides but do play a significant, although not universal, role in maintaining disulfides in a reduced state. The function of GSH in the photosynthetic bacteria, aside from providing a form of cysteine resistant toward autoxidation, remains a puzzle, as none of the GSH-dependent enzymes tested other than glutathione reductase were present in these organisms.

Key words: Glutathione peroxidase — Glutathione S-transferase — Thioltransferase — Glutathione reductase — Glutathione — Bis-γ-glutamylcystine reductase — γ-glutamylcysteine — Procaryotes

Introduction

Eucaryotes utilize the low molecular weight thiol glutathione (GSH, γ-glutamylcysteinylglycine) as cofactor in the enzymatic reduction of toxic oxygen by-products. Two enzymes have been isolated from mammalian tissues that catalyze the destruction of peroxides by GSH: glutathione peroxidase (EC 1.11.1.19), a selenoprotein that is active with H_2O_2 and organic hydroperoxides (Flohé 1982; Mannervik 1985b), and glutathione S-transferase (EC 2.5.1.18), a group of selenium-independent enzymes that are active with organic hydroperoxides (Mannervik 1985a). Glutathione thioltransferase, which catalyzes the GSH-dependent reduction of protein disulfides and disulfides of low molecular weight thiols such as cysteine and CoA (Mannervik 1980), has been purified from several mammalian tissues (Axelsson et al. 1978; Larson et al. 1985; Gan and Wells 1987) and yeast (Nagai and Black 1968). Essential to the antioxidant functions of GSH is glutathione reductase (EC 1.6.4.2), which cycles GSSG, a product of the enzymatic processes mentioned above and of the autoxidation of GSH, back to GSH (Williams 1976).

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Aerobic and air-tolerant procaryotes also produce low molecular weight thiols that may function as cofactors in antioxidation processes. Three subgroups of purple bacteria (α, β, and γ groups) and the cyanobacteria produce millimolar levels of GSH (Fahey et al. 1987). Facultative and aerobic procaryotes that lack GSH have been found to produce other low molecular weight thiols and disulfide reductases. Thus, several groups of gram-positive eubacteria contain unusually high levels of CoA in the absence of GSH (Fahey and Newton 1983), and a disulfide reductase that is active with CoA disulfide was isolated from *Bacillus megaterium* and detected in several other species of *Bacillus* (Setlow and Setlow 1977; Swerdlow and Setlow 1983). The halobacteria are aerobic archaebacteria that lack GSH but produce millimolar levels of γ-glutamylcysteine (Newton and Javor 1985). Bis-γ-glutamylcystine reductase was purified recently from *Halobacterium halobium* (Sundquist and Fahey 1988) and found to maintain specifically γ-glutamylcysteine predominantly in its thiol form (Sundquist and Fahey 1989).

The extent to which low molecular weight thiols function as cofactors in antioxidation processes in aerobic and air-tolerant procaryotes has not been well characterized, however. Glutathione thioltransferase activity was found at significant levels in crude extracts of *Escherichia coli* (Holmgren 1979a) but has not been investigated in other GSH-producing bacteria. Only low levels of glutathione S-transferase activity have been reported for *E. coli* (Lau et al. 1980; Shishido 1981), for *Pseudomonas aeruginosa* (Lau et al. 1980), and for several *Salmonella* strains (Meijer et al. 1980; Summer et al. 1980). Glutathione peroxidase activity was not detected in *E. coli* (Smith and Shrift 1978) or in three species of cyanobacteria (Tel-Or et al. 1986). No studies of these enzymes in photosynthetic purple bacteria have been reported, and nothing is known about γ-glutamylcysteine-dependent peroxidase, S-transferase, or thioltransferase activities in the halobacteria, where γ-glutamylcysteine is the main low molecular weight thiol. In the present study we report results of assays for GSH-dependent peroxidases and thioltransferase in representative species of eubacteria and for the corresponding γ-glutamylcysteine-dependent enzymes in *H. halobium*. These results indicate that bacteria do not utilize GSH and γ-glutamylcysteine for the destruction of peroxides and that these thiols initially must have served other functions in procaryotes.

Materials and Methods

Materials. Sodium phosphate was ACS grade from Mallinkrodt, Inc.; EDTA (disodium salt) was from EM Science. Sodium chloride and potassium chloride were certified ACS grade and H_2O_2 (3%) was certified grade from Fisher Scientific. Phenylmethylsulfonyl fluoride (PMSF), glutathione disulfide, and glutathione peroxidase (bovine erythrocyte) were from Calbiochem-Behring. Sodium azide was from Chemical Dynamics Corp. and NADPH was from United States Biochemical Corp. Reduced glutathione (crystalline), glutathione reductase (yeast, type IV), cystine, CoA disulfide (lithium salt), sodium tetrathionate, FAD (grade III), NADH (grade III), *t*-butyl hydroperoxide (70%, aqueous), and 1-chloro-2,4-dinitrobenzene were from Sigma Chemical Co. The preparation of bis-γ-glutamylcystine reductase (purified to electrophoretic homogeneity) and bis-γ-glutamylcystine (Sundquist and Fahey 1988), and of γ-glutamylcysteine (Newton and Javor 1985) were described previously. Sodium *S*-sulfocysteine was synthesized by the method of Segel and Johnson (1963).

Buffers. All buffers used in cell extraction and enzyme assays contained 50 mM sodium phosphate and 1 mM EDTA, and were prepared at the specified pH following the procedure described elsewhere (Sundquist and Fahey 1988). The buffers used to extract *H. halobium* cells and assay *H. halobium* extract in addition contained 3.0 M KCl and 1.3 M NaCl (Sundquist and Fahey 1988).

Cell Culture and Preparation of Cell Extract. An *E. coli* B ATCC 11303 cell pellet was purchased from Grain Processing Corporation (cultured aerobically on Kornberg minimal medium). *Beneckea alginolytica* B-86, *Beneckea harveyi* B-392, and *Beneckea natriegens* B-108 were obtained from P. Baumann via K. Nealson and were cultured aerobically as described elsewhere (Fahey and Newton 1983). *Rhodospirillum rubrum* ATCC 11170 was cultured aerobically and heterotrophically on the medium described by Ormerod et al. (1961). *Chromatium vinosum* D ATCC 17899 was cultured photoautotrophically on the carbonate medium of Arnon et al. (1963). *Anabaena* sp. strain 7119 ATCC 29151 (formerly *Nostoc muscorum* 7119) was cultured photoautotrophically on the inorganic nutrient medium of Arnon et al. (1974). Cells were harvested by centrifugation (5–10 min at 4000 × *g* and 4°C), and cell pellets were stored at −70°C until used. Extracts of the above bacteria were prepared by combining the cell pellet with six parts buffer at pH 7.0 and containing 50 μM PMSF, and then subjecting the mixture to mechanical disruption by a Mini-beadbeater (Biospec Products; 0.15-mm zirconium beads) for 1 min at 4°C; the overlying liquid was then centrifuged (Eppendorf microcentrifuge 5414) for 20 min at 4°C and 13,000 × *g*, and the resulting supernatant was removed and kept on ice until assayed for enzyme activities. *Halobacterium halobium* R1 (Newton and Javor 1985) was cultured heterotrophically on the medium of Mevarech et al. (1976), harvested by centrifugation (15 min at 8000 × *g* and 4°C), and stored at −70°C prior to use. *Halobacterium halobium* extract was prepared by combining the cell pellet with seven parts of the high-salt buffer at pH 7.0 and containing 50 μM PMSF, and then homogenizing the mixture (Tekmar Tissumizer) on ice until of uniform consistency (~15 sec). The mixture then was centrifuged for 20 min at 4°C and 13,000 × *g*, and the entire supernatant was collected, vortexed, and kept on ice until used in the enzyme assays.

Enzyme Assays. All assays were conducted at 30°C and in a total assay volume of 1.0 ml. A 1–25-μl aliquot of cell extract was used in the assays.

Disulfide reductase assays were conducted in buffer at pH 7.5 with 1.0 mM disulfide and 0.17 mM NADPH. NADPH was the final addition to the assay and reaction progress was followed by continuously measuring the decrease in absorbance at 340 nm for the oxidation of NADPH [ϵ = 6.22 mM^{-1} (Horecker and Kornberg 1948)]. Assay rates were adjusted by subtracting the background rate determined by omitting disulfide from the assay.

Activity was expressed as the amount of NADPH oxidized per minute.

Glutathione thioltransferase activity was measured with a coupled-enzyme assay (Eriksson et al. 1974) that utilized the activity of glutathione reductase to monitor the oxidation of GSH by CoA disulfide:

$$2GSH + CoASSCoA = GSSG + 2CoASH$$
$$GSSG + NADPH + H^+ = 2GSH + NADP^+$$

These assays were conducted in buffer at pH 7.5 with 1.0 mM GSH, 1.0 mM CoA disulfide, 3 U glutathione reductase, and 0.17 mM NADPH. Glutathione peroxidase activity with hydroperoxides (ROOH) also was measured with a coupled-enzyme assay involving glutathione reductase (Paglia and Valentine 1967):

$$2GSH + ROOH = GSSG + H_2O + ROH$$
$$GSSG + NADPH + H^+ = 2GSH + NADP^+$$

These assays were conducted in buffer at pH 7.0 with 1.0 mM GSH, 0.20 mM H_2O_2 (or 1.5 mM *t*-butyl hydroperoxide), 1.0 mM sodium azide, 3 U of glutathione reductase, and 0.17 mM NADPH. In both coupled-enzyme assays, cell extract was incubated with the GSH for 10 min at 30°C prior to adding the remaining components of the assay, and reaction progress was followed by continuously measuring the decrease in absorbance at 340 nm for the oxidation of NADPH. Activity was expressed as the amount of NADPH oxidized per min. Glutathione S-transferase activity was measured as a conjugation reaction between 1-chloro-2,4-dinitrobenzene (CDNB) and GSH (Habig et al. 1974):

$$GSH + CDNB = GS\text{-}DNB + H^+ + Cl^-$$

These assays were conducted in buffer at pH 6.5 with 1.0 mM GSH and 1.0 mM CDNB. Extract was added last to the assay. Reaction progress was monitored by continuously measuring the increase in absorbance at 340 nm, which corresponds to the formation of S-conjugates of CDNB [$\epsilon = 9.6\ mM^{-1}$ (Habig et al. 1974)]. Activity was expressed as the amount of conjugate formed per min.

γ-glutamylcysteine thioltransferase and γ-glutamylcysteine peroxidase activities in *H. halobium* extract were measured with coupled assays identical to those used for the corresponding GSH-dependent enzymes except that 1.0 mM γ-glutamylcysteine and 3 U bis-γ-glutamylcystine reductase were substituted for GSH and glutathione reductase, and high-salt buffers (see above) were used. The glutathione S-transferase assay described above was modified to measure γ-glutamylcysteine S-transferase activity in *H. halobium* extract by replacing GSH with 1.0 mM γ-glutamylcysteine and using a high-salt buffer (see above).

Protein Determination. The protein concentration of the extracts was estimated with the assay of Lowry et al. (1951) using bovine serum albumin as the standard. Protein in the *H. halobium* extract was precipitated with trichloroacetic acid prior to adding the assay reagents in order to remove salt present in the extraction buffer (Sundquist and Fahey 1988).

Results

Disulfide Reductase Activity

Glutathione reductase activity was found in all five GSH-producing bacteria included in the present study (Table 1). The specific activities were 40–70% higher than previously reported for *E. coli* (Mata and Pinto 1984), *R. rubrum* (Boll 1969), and *Anabaena* sp. strain 7119 (Serrano et al. 1984). Glutathione reductase purified from *C. vinosum* has been shown to be specific for NADH rather than NADPH (Chung and Hurlbert 1975), and, in accord with this result, we found that it was ninefold more active with NADH than NADPH in reducing GSSG. Bis-γ-glutamylcysteine reductase was present in *H. halobium* extract (Table 1) at a level comparable to what had been measured earlier (Newton and Javor 1985; Sundquist and Fahey 1988).

Thiol-Dependent Peroxidase Activity

Glutathione peroxidase activity was measured in the eubacterial extracts using the coupled-enzyme assay of Paglia and Valentine (1967) with H_2O_2 as substrate. As illustrated for *E. coli*, the rate of nonenzymatic oxidation was estimated by omitting extract from the assay, and addition of extract failed to produce an increase in this rate (Table 2). The absence of detectable glutathione peroxidase activity was not due to an inhibitor in the extract, as the addition of purified glutathione peroxidase to the complete assay mixture resulted in the expected activity (data not shown). Analogous assays of extracts of the other GSH-producing bacteria resulted in a similar failure to detect activity (Table 1). Because glutathione S-transferase can exhibit peroxidase activity with organic hydroperoxides but not with H_2O_2, the assays were repeated using *t*-butyl hydroperoxide as substrate. In the absence of extract a rate of 1.0 nmol/min was observed, and this rate did not increase measurably upon addition of extract from any of the five eubacteria examined (data not shown). Similarly, no γ-glutamylcysteine peroxidase activity could be detected in *H. halobium* extracts with H_2O_2 (Table 2) or *t*-butyl hydroperoxide (data not shown) as substrate. Thus, as summarized in Table 1, no significant thiol-dependent peroxidase activity towards H_2O_2 or *t*-butyl hydroperoxide could be detected for any of the bacteria studied.

S-Transferase Activity

Thiol S-transferase activity was also assayed with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Only *E. coli* showed measurable activity, but this activity was only twice the background level (Table 2) and constitutes only 1% of the activity found in rat liver (Table 1). None of the other bacteria examined showed measurable S-transferase activity (Table 1).

Thioltransferase Activity

Measurement of thioltransferase activity was made initially with *E. coli* extract using both S-sulfocys-

Table 1. Enzyme activities in cell extracts (nmol min^{-1} mg^{-1})

Cell extract	Thiol	Disulfide reductase[a]	Thiol-dependent enzyme[b]		
			Peroxidase	S-transferase	Thioltransferase
Nonphotosynthetic purple bacteria					
Escherichia coli	GSH	190	nd[c]	6.4	98
Beneckea alginolytica	GSH	1600	nd	nd	45
Photosynthetic purple bacteria					
Rhodospirillum rubrum	GSH	57	nd	nd	nd
Chromatium vinosum	GSH	53[d]	nd	nd	nd
Cyanobacteria					
Anabaena sp. strain 7119	GSH	73	nd	nd	nd
Halobacteria					
Halobacterium halobium	γ-Glu-Cys	9.6	nd	nd	15
Eucaryote					
Rat liver[e]	GSH	57	180	570	16

[a] Activity was determined with NADPH and the disulfide of the listed thiol as described in Materials and Methods unless noted otherwise

[b] Activities in the bacterial extracts were determined with the listed thiol and H_2O_2 in peroxidase assays, CDNB in S-transferase assays, and CoA disulfide in thioltransferase assays, as described in Materials and Methods. The amount of extract (μg protein) used in the thiol-dependent enzyme assays was 60, *E. coli*; 130, *B. alginolytica*; 140, *R. rubrum*; 82, *C. vinosum*; 92, *Anabaena*; 300, *H. halobium*

[c] nd, not detectable. The level of detectability was set at a net activity ≥30% of the background activities (see Table 2)

[d] Determined with 0.16 mM NADH, 12.5 mM GSSG, and 14 μM FAD (see Chung and Hurlbert 1975)

[e] Values given for glutathione-dependent enzymes in rat liver are for comparison and were taken from the literature: reductase and S-transferase (CDNB), Moron et al. 1979; peroxidase (H_2O_2), Nakamura et al. 1974; thioltransferase (S-sulfocysteine), Axelsson et al. 1978

Table 2. Thiol-dependent enzyme assay rates[a]

Extract	Thiol	Assay	Peroxidase assay (H_2O_2) (nmol min^{-1})	S-transferase assay (CDNB) (nmol min^{-1})	Thiol-transferase assay (CoA disulfide) (nmol min^{-1})
Escherichia coli	GSH	Complete	2.9	0.83	11
		Minus extract	3.0	0.44	4.4
		Minus substrate	0.55[b]	<0.09[c]	0.39[d]
		Net assay rate:	Not detectable	0.39	6.2
Halobacterium halobium	γ-Glu-Cys	Complete	5.8	0.83	7.6
		Minus extract	2.7	0.94	0.96
		Minus substrate	3.4[b]	ND[e]	2.1[d]
		Net assay rate:	Not detectable	Not detectable	4.5

[a] Assays were conducted with the specified thiol as described in Materials and Methods using 10 μl *E. coli* extract (60 μg protein) or 25 μl *H. halobium* extract (300 μg protein). The rates of the control assays omitting substrate that were observed with *E. coli* extract were typical of the corresponding assays with the other eubacterial extracts (see Table 1)

[b] Determined by omitting H_2O_2 from the assay

[c] Determined by omitting CDNB or GSH from the assay

[d] Determined by omitting CoA disulfide from the assay

[e] ND, not determined

teine (Eriksson et al. 1974) and CoA disulfide as substrate, but the latter was selected for further assays, as it provided a 50% lower assay rate in the absence of extract, yet yielded a comparable net thioltransferase activity. With *E. coli* extract the activity increased slightly during the first several minutes and then remained constant for an additional 7 min at the level shown in Table 2 (complete assay). To establish that the observed activities were not due to an NADPH-dependent CoA reductase, the assays were conducted in the absence of GSH, and only a small activity (0.5 nmol/min) was observed. *Beneckea alginolytica* and *H. halobium* also showed levels of thioltransferase activity comparable to or

greater than found for rat liver, but none of the three photosynthetic bacteria examined demonstrated detectable activity (Table 1). The absence of activity with the photosynthetic bacteria was not due to an inhibitor in the extracts, as addition of *E. coli* extract to the complete assays gave the expected activity in each case (data not shown).

Discussion

The failure to find significant peroxidase or S-transferase activity among procaryotes (Table 1) indicates that GSH and γ-glutamylcysteine are not utilized to control peroxide levels in procaryotes as is GSH in eucaryotes. At first this might seem surprising, but there are reasons why this is plausible. First, there are alternate routes for control of H_2O_2 in bacteria. Thus, catalase is present in purple bacteria (Nadler et al. 1985) and cyanobacteria (Tel-Or et al. 1986), and cyanobacteria also may utilize ascorbate peroxidase in the destruction of H_2O_2 (Tel-Or et al. 1986). Secondly, procaryotes possibly are less susceptible to the deleterious effects of organic hydroperoxides than are eucaryotes. It is the polyunsaturated fatty acids of eucaryotic membranes that represent the most sensitive sites for peroxidation, and membranes of both the eubacteria (Margulis 1970) and the halobacteria (De Rosa et al. 1986) are free of polyunsaturated lipids. These considerations lead us to suggest that GSH-dependent peroxidases evolved in eucaryotes as a mechanism to protect the polyunsaturated fatty acids of membranes from uncontrolled peroxidation. Furthermore, because glutathione S-transferase activity is distributed widely among eucaryotes, whereas glutathione peroxidase seems to be restricted to higher animals (Jakoby and Habig 1980; Mannervik 1985a), the peroxidase activity associated with glutathione S-transferase appears to have evolved as the earliest GSH-dependent protection mechanism against lipid peroxidation.

Aside from disulfide reductase activity, only thioltransferase activity was detected in the bacteria included in this study, and this only in three out of the six species examined (Table 1). Thioredoxin, which has been isolated from purple bacteria (Johnson et al. 1984, 1988) and cyanobacteria (Gleason and Holmgren 1981), and glutaredoxin also display thioltransferase activity (Holmgren 1979b, 1985) and may provide an alternative or additional path for the reduction of cellular disulfides. Although GSH and γ-glutamylcysteine clearly can function as cofactor in thioltransferase-mediated disulfide reduction, it would appear that this is not the primary function of these thiols in bacteria. The presence of glutathione thioltransferase in some purple bacteria suggests that this enzyme may have been incorporated into eucaryotes via the endosymbiotic process (Margulis 1981) giving rise to mitochondria (Villanueva et al. 1985; Yang et al. 1985), as appears to be the case for the enzymes responsible for GSH synthesis (Fahey et al. 1984).

The absence of GSH-dependent enzymes other than glutathione reductase from photosynthetic bacteria emphasizes that our understanding of the primary function of GSH in the procaryotes remains limited. The synthesis of GSH appears to have evolved early in the evolution of photosynthetic bacteria (Newton and Fahey 1989). Comparison of the autoxidation rate of GSH and γ-glutamylcysteine with that of cysteine indicates that one of the simplest roles these thiols can play is to serve as a slowly autoxidizable form of cysteine for the cell, which is due in part to the decreased capacity of GSH and γ-glutamylcysteine for binding metals (Sundquist and Fahey 1989). Although resistance of the cellular thiol pool to autoxidation may be an important function of GSH in the photosynthetic bacteria that grow in air, it is more difficult to accept this as the primary role of GSH in *C. vinosum*, a phototroph that tolerates but does not grow in air. Moreover, the glutathione reductase of *C. vinosum* is unique in having a high K_m (5 mM) for GSSG and in utilizing NADH rather than NADPH (Chung and Hurlbert 1975). These observations suggest that the original function of GSH in the photosynthetic bacteria may not have been associated with resistance to oxygen toxicity. What the function might have been is unclear, but it would appear that *C. vinosum* represents a key organism for exploring the role played by GSH prior to its recruitment as an antioxidant coenzyme.

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