Glutathione-coated Cadmium-Sulfide Crystallites in *Candida glabrata*

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Cadmium-sulfide crystallites form in the yeast *Candida glabrata* cultured in the presence of cadmium salts. The particles function to sequester and detoxify intracellular cadmium ions. The crystallites are peptide-coated, but the coating peptide varies with the nutrient conditions of the growth medium. When cultured in rich nutrient broth the yeast forms intracellular CdS particles coated with a mixture of glutathione and the γ-glutamylcysteine dipeptide. In contrast, cultures in synthetic minimal medium yield particles coated with polymerized γEC peptides of general structure (γ-Glu-Cys)$_x$-Gly. Glutathione/γ-glutamylcysteine peptides exhibit properties analogous to quantum, semiconductor-type crystallites. The optical properties are dependent on particle size, and irradiation results in photoluminescence and photoreduction not observed in bulk CdS mineral. Aerobic irradiation leads to particle decomposition presumably via oxidation of the sulfide ions within the crystallite.

Glutathione (GSH; γ-Glu-Cys-Gly) plays a critical role both in maintaining the redox status of the cell and in cellular detoxification reactions (1). The cellular glutathione concentration can be depleted by the inhibitory action of buthionine sulfoximine on the enzyme γ-glutamylcysteine synthetase (2). Glutathione depletion sensitizes cells to the cytotoxic effects of sulphydryl-reactive alkylating agents, radiation, thermal stress, reactive oxygen, and metal ions (3–8). Buthionine sulfoximine does not sensitizes cells to metal ions if appreciable levels of metallothionein are induced (6). Glutathione appears to be an initial defense against metal-induced cytotoxicity and functions in this role before the induction of metallothionein, a protein known to function in the detoxification of metal ions in many species (9). Exogenous GSH has also been shown to limit Cd-induced toxicity (10).

The mechanism by which GSH protects against metal toxicity is unclear but presumably involves chelation of the metal ions. Metal complexes with glutathione ligands have been formed in *vitro*, and the predominant species is the 1:1 metal-ligand bidentate complex at neutral pH (11–13). Biologically formed metal-glutathione complexes have not been characterized.

Glutathione-related peptides are synthesized instead of metallothionein in numerous species in the plantae and fungi kingdoms in response to metal ion stress (14–16). The peptides are related to glutathione (γ-Glu-Cys-Gly) in having the general structure (γ-Glu-Cys)$_x$-Gly (17, 18). The designation γEC peptides will be used in general reference to the peptides. Metal ions are ligated by cysteinyl thiolates to form a metal-peptide complex (19). Labile sulfide is an additional component in cadmium-peptide complexes (15, 19, 20). The content of bound sulfide in the metal-peptide complex varies depending on species and metal regulation of a metabolic pathway generating sulfide (21). Complexes with an S: Cd ratio of 0.7 have been isolated from *Candida glabrata* (16). These complexes contain a CdS crystallite core of 20 Å diameter and exhibit properties of a quantum semiconductor-type cluster (22). *C. glabrata* employs multiple metal resistance mechanisms. In response to excess copper the cells synthesize at least two metallothionein forms Cu(I) complexes (23).

We report here that different growth conditions dictate whether CdS crystallites in *C. glabrata* are coated with γEC peptides or an oligomeric mixture of glutathione and the dipeptide γ-Glu-Cys. This is the first demonstration of a biologically formed GSH-metal complex.

**MATERIALS AND METHODS**

*C. glabrata* (ATCC strain 2001) was grown in either YTD culture medium (1% yeast extract, 2% tryptone, and 2% dextrose) or synthetic complete minimal media (1.25% yeast nitrogen base, 2% glucose, and a mixture of uracil and amino acids). The media components were sterilized by autoclaving for 20 min at 250 °C. Cadmium chloride (typically 0.5 mm) was added to the culture medium prior to cell inoculation. A 1% inoculum was used to initiate culture growth. Cell growth was monitored as turbidity at 600 nm. The yeasts were cultured for 16 h at 30 °C with aeration (180 rpm) in a Labline Orbit shaker. The cells (final optical density at 600 nm (5)) from six 1-liter YTD cultures were harvested by centrifugation at 5,000 × g for 5 min at 4 °C, and the 40–50 g of cell mass was washed twice with saline and once with distilled deionized water. The cell paste was diluted with an equal volume (v/v) of 10 mM Tris-Cl, pH 8, before processing in a Biox X-press. The frozen mass was pressed twice through the orifice, and the melted slurry was subsequently clarified by centrifugation at 20,000 × g for 10 min. The supernatant was diluted with deionized water to a conductivity of 2 mmho and applied to DE52 column (Whatman), 15-ml bed volume, equilibrated with 10 mM Tris-Cl, pH 8. The column was washed with approximately 500 ml of buffer until the absorbance at 280 nm was below 0.05. A linear gradient (400 ml) of total volume of 0–0.5 M KCl in the same buffer was used to elute the bound components, and 4-ml fractions were collected. Pooled fractions from the anion exchanger were concentrated to 6 ml by ultrafiltration at 5 °C on an Amicon YM 2 membrane and subsequently gel-filtered on Sephadex G-50 (2.5 × 70 cm) equilibrated with 10 mM Tris-Cl, pH 8. Characterization of components in the samples was accomplished by reverse phase high pressure liquid chromatography on a semi-preparative C$_8$ column (Vydac, 10 mm inner diameter × 25 cm long) followed by amino acid analysis. The solvent system used for the reverse phase chromatography was 0.1% triethylacetic acid and a 0–60% acetonitrile gradient at 1%/min. Components were detected in the eluent by absorbance at 214 nm. Retention times of elution from the C$_8$ column were compared with γEC peptides of known peptide length, glutathione and the γ-glutamylcysteine dipeptide. The γ-Glu-Cys dipeptide was prepared by digestion of glutathione with carboxypeptidase P at pH 4 at 25 °C in an
an anaerobic chamber followed by isolation of the digested components on C18 reverse phase HPLC under conditions mentioned. Amino acid analysis was performed on a Beckman 119 Cl analyzer after hydrolysis in 5.7 N HCl in vacuo at 110 °C. The total cysteine concentration was obtained by oxidation with performic acid prior to acid hydrolysis (24). The peptide concentration was assessed from amino acid analysis by quantitation of the cysteine concentration in peptides of known number of dipeptide repeats. The number of dipeptide repeats was determined by reverse phase HPLC calibrated with γEC standard peptides (16). Total glutathione (reduced and oxidized) was also quantified by a cyclic assay involving glutathione reductase and DTNB (25). This reaction quantifies glutathione but not γ-Glu-Cys dipeptides. Protein concentrations were estimated after prior digestion of cells with 1 N NaOH at 100 °C (26).

Since metal complexes contained multiple peptide components, metal binding stoichiometry was expressed with the total cysteine concentration as the denominator rather than the peptide concentration. Metal concentrations were quantified by analysis on a Perkin-Elmer 305A atomic absorption spectrometer. Metal analysis on whole cells was carried out after wet ashing using nitricperchloric acid in a 5:2 ratio. Sulfide was quantified by the methylene blue synthesis assay of King and Morris (27) calibrated as described previously (15). Routine luminescence measurements were conducted on a Perkin-Elmer 650-10S fluorimeter and ultraviolet/visible spectroscopy was carried out on a Beckman DU-65 spectrophotometer. Photo-induced electron transfer to methyl viologen was accomplished by anaerobically mixing the CdS complexes (0.57 mM in cadmium) with methyl viologen (100 μM) at pH 11 followed by irradiation of the anaerobic sample at 355 nm at 23 °C with the xenon source of the 650-10S fluorimeter using a 20-nm excitation slit. The ultraviolet/visible absorption spectrum was taken at 10-min intervals. Samples were irradiated aerobically under similar conditions except that methyl viologen was omitted.

RESULTS

Purification and Properties of Metal-Glutathione Complexes—Exposure of C. glabrata to cadmium salts during growth in a synthetic minimal medium yielded a single cadmium-sulfide complex containing γEC peptides. The metal complex was purified by a combination of anionic exchange chromatography and gel filtration (16). The oligomeric peptide complex contained only Glu, Cys, and Gly. High pressure liquid chromatography on a C18 reverse phase column revealed peptides predominantly n2 and n2 desGly (Fig. 1A). These peptides eluted from C18 HPLC at 17.3 and 18.2% acetonitrile, respectively. We previously characterized the eluent γEC peptides by collecting the peaks and analyzing the Glu:Gly primary amine and terminal γ-Glu analysis. Liquid chromatography on a ClS reverse phase column revealed only minimal quantities of γEC peptides. The metal complexes revealed the usual three amino acids containing γEC peptides. The metal binding stoichiometry was expressed with the total cysteine concentration as the denominator rather than the peptide concentration. Metal concentrations were quantified by analysis on a Perkin-Elmer 305A atomic absorption spectrometer. Metal analysis on whole cells was carried out after wet ashing using nitric perchloric acid in a 5:2 ratio. Sulfide was quantified by the methylene blue synthesis assay of King and Morris (27) calibrated as described previously (15). Routine luminescence measurements were conducted on a Perkin-Elmer 650-10S fluorimeter and ultraviolet/visible spectroscopy was carried out on a Beckman DU-65 spectrophotometer. Photo-induced electron transfer to methyl viologen was accomplished by anaerobically mixing the CdS complexes (0.57 mM in cadmium) with methyl viologen (100 μM) at pH 11 followed by irradiation of the anaerobic sample at 355 nm at 23 °C with the xenon source of the 650-10S fluorimeter using a 20-nm excitation slit. The ultraviolet/visible absorption spectrum was taken at 10-min intervals. Samples were irradiated aerobically under similar conditions except that methyl viologen was omitted.

Fig. 1. Reverse phase HPLC chromatography of purified CdS complexes from C. glabrata cultured in synthetic minimal medium (panel A) and YTD medium (panels B and C). The samples chromatographed in panels B and C represent elution fractions 115 and 150 from Sephase G-50, respectively (see Fig. 2). Each sample containing 15 μg of Cd(II) was acidified to pH 2 and clarified by centrifugation prior to chromatography. The initial peak is the injection artifact.

n2 peptides were virtually absent in the HPLC profile based on elution position of standard γEC peptides. In an attempt to determine the identity of the components coating the cadmium-sulfide clusters, amino acid analysis was carried out on each component resolved by reverse phase HPLC. The Glu:Cys:Gly ratios of the two HPLC peaks in order of elution were 1:1:0.9 and 1:1:0.1, respectively. Glutathione was detected in HPLC fractions using the cyclic DTNB assay. The ratio of GSH detected in fraction 1 by the cyclic DTNB assay compared with Glu quantified by amino acid analysis was approximately 1. Since glutathione contains a single glutamyl residue per peptide, the concentration of Glu can be used to quantify glutathione. Minimal quantities of glutathione were detected in HPLC elution fraction 2. The nanomoles of GSH detected by the cyclic DTNB assay was equivalent to only 10% of the Glu concentration quantified by amino acid analysis. HPLC fractions 1 and 2 coeluted on reverse phase HPLC with reduced glutathione and γ-Glu-Cys, respectively. The HPLC fraction 2 therefore is predominantly γ-Glu-Cys with minimal contaminating glutathione.

The cycling DTNB assay verified that glutathione was present in both gel filtration fractions (Fig. 2B). The quantity of glutathione based on the cadmium concentration was higher in pool 2 fractions (Fig. 2B). In pool 2 the Cd:GSH molar ratio was nearly 1:1, whereas the Cd:Cys ratio was 0.5 suggestive of a mixture of glutathione and γ-glutamylcysteine. The Glu:Gly ratio of 2 observed by amino acid analysis is also indicative of equivalent concentrations of the two peptides. Thus, the CdS complexes isolated from YTD cultures appear to contain predominantly glutathione (γ-Glu-Cys-Gly) and

1 The abbreviations used are: HPLC, high pressure liquid chromatography; DTNB, 5,5'-dithiobis(nitrobenzoic acid).
the \( \gamma \)-Glu-Cys dipeptide. The ratio of GSH:\( \gamma \)-Glu-Cys is typically between 1 and 2.

**Labile Sulfur in \( \text{Cd(II)} \) Complexes**—The two \( \text{Cd-GSH} \) complexes differed in the content of labile sulfur. Pool 1 \( \text{Cd(II)} \) complexes contained appreciable sulfide concentrations unlike pool 2 complexes (Fig. 2B). The sulfide-containing cadmium complex did not contain a uniform S:Cd ratio. Rather, a spectrum of complexes differing in their S:Cd ratios existed in the broad elution peak (Fig. 2B). The molar S:Cd ratio ranged from 0.49 to 0.13 in fractions across the elution profile encompassing pool 1. Sulfide was not present in pool 2 fractions. Properties associated with complexes having a high S:Cd ratio include larger apparent Stokes' radius, higher Cd:peptide ratio, and greater anionic character. A similar relationship of sulfide content with charge and size of the complex was observed in \( \text{Cd-Cy} \) EC peptide complexes (16, 19). The Cd:peptide ratio (peptide quantified as concentration of total Cys) varied from near 2 in the high sulfide fractions to 0.5 in pool 2 fractions. The quantitation of Cd:peptide stoichiometries was calculated from amino acid analysis data. The cyclic DTNB assay did not yield accurate quantitation of glutathione in high sulfide fractions of pool 1 due to interference of the sulfide anions. The presence of sulfide ions in the \( \text{Cd-GSH} \) complex depressed the rate of DTNB reduction.

The optical properties of the eluent fractions were also dependent on the S:Cd ratio (Fig. 3). The near UV transition (band gap transition) was red-shifted as the S:Cd ratio of the complex increased. The transition in eluent fraction 96 (S:Cd ratio = 0.49) was shifted to nearly 370 nm.

\( \text{Cd(II)} \)-glutathione complexes with a low S:Cd ratio could readily be converted to high sulfide complexes by the in vitro addition of sodium sulfide. Addition of sodium sulfide to a complex having a molar S:Cd ratio of 0.32 resulted in a red shift of the low energy optical transition near 329 nm. Sulfide was added as listed, and the samples were incubated for 16 h at 4 °C after which the molar S:Cd was measured. After 16 h less than 0.1% of the initial sulfide is present in control samples. The dashed line is after acidification of the 0.3 mol eq sample.

**Fig. 2.** Purification of glutathione-coated \( \text{CdS} \) particles. Panel A shows the elution profile from DEAE-cellulose in 10 mM Tris-Cl, pH 8, with a gradient of 0-0.5 M KCl. Panel B is the elution profile from Sephadex G-50 equilibrated and eluted with 10 mM Tris-Cl, pH 8. The glutathione concentration was determined by the cycling DTNB reaction. This assay does not quantify \( \gamma \)-glutamylcysteine. Peak fractions 112 and 150 have \( K_m \) values of 0.4 and 0.74, respectively.

**Fig. 3.** Ultraviolet absorption spectra of elution fractions from Sephadex G-50 (Fig. 2). Fractions as listed containing 15 \( \mu \)g of \( \text{Cd(II)} \) were scanned.

**Fig. 4.** Effect of added sulfide on the absorption spectrum of a sample of reduced glutathione-coated \( \text{CdS} \) crystallites. The starting sample exhibited a low energy transition near 329 nm. Sulfide was added as listed, and the samples were incubated for 16 h at 4 °C after which the molar S:Cd was measured. After 16 h less than 0.1% of the initial sulfide is present in control samples. The dashed line is after acidification of the 0.3 mol eq sample.
tions were absent in pool 2 fractions, sodium sulfide was titrated into mixtures of Cd(II) and glutathione varying in Cd:GSH ratios. Samples containing a Cd:GSH ratio below 0.1 exhibited discrete low energy transitions indicative of crystallites upon addition of 0.4 mol eq of sulfide (Fig. 5). Samples having a Cd:GSH ratio above 0.1 revealed only bulk CdS mineral formation upon sulfide addition. The optical transition red-shifted with the addition of 0.8 mol eq of sulfide (Fig. 5). Cysteine as a free amino acid is as effective as glutathione in serving as a matrix for controlled CdS crystallite formation at Cd:cysteine ratios below 0.1.

The C. glabrata Cd-glutathione complex exhibited properties similar to those of small semiconductor CdS clusters. Photoexcitation of synthetic CdS crystallites generates high potential electron-hole pairs capable of photoluminescence and photoreduction reactions (28-30). The CdS particles stabilized by the GSH/γ-Glu-Cys peptides luminesced when excited with ultraviolet radiation. The emission maximum was near 450 nm with an excitation maximum of 355 nm (Fig. 6). Volatilization of sulfide ions by acidification of the complex to pH 1.5 diminished the luminescence. Prolonged irradiation of the CdS-peptide complex aerobically led to a time-dependent diminution of the low energy optical transition of the complex and depletion of titratable sulfide ions (Fig. 7). After 40 min of irradiation 60% of the titratable sulfide was abolished. The increase in absorbance near 420 nm is consistent with formation of some bulk CdS mineral. The emission yield of the irradiated complex was likewise diminished. No photoinduced decomposition was observed when the irradiation was carried out anaerobically, although a definite red-shifting of the transitions was observed.

Irradiation of an anaerobic sample of the native CdS-glutathione complex with ultraviolet light in the presence of methyl viologen resulted in photo-induced reduction of the dye (Fig. 8). The reduction of methyl viologen was dependent on anaerobic conditions and the presence of the CdS complex. Acidification of the complex to volatilize the sulfide as H2S followed by reneutralization abolished the ability of the complex to mediate the photo-induced electron transfer.

**Fig. 5.** CdS crystallite formation *in vitro* with stabilization by glutathione. Cadmium sulfate in 0.01 N HCl (44 nmol) was mixed with 880 nmol of reduced glutathione (0.01 N HCl), and the mixture was neutralized to pH 8 with 0.1 M potassium phosphate. Sodium sulfate was added to give a S:Cd molar ratio of 0.4 (---) and 0.8 (----). The samples were incubated at 25 °C for 18 h and then scanned. Sulfide (0.4 mol eq) was also added to a sample containing only cadmium sulfate (no glutathione) followed by neutralization (----).

**Fig. 6.** Relative luminescence of the glutathione-coated CdS complex. A native colloid sample (64 µg of cadmium) exhibiting a low energy transition near 320 nm was irradiated at 355 nm for emission scanning (line a). Curve c represents the luminescence for the sample after acidification to pH 1.5 for a brief period and curve b the sample after neutralization to pH 7.0. Residual sulfide remained in this sample. The peak near 400 nm is the Raman water peak. The inset shows the excitation spectrum from 220 to 400 nm with emission monitored at 440 nm.

**Fig. 7.** Photo-induced decomposition of the glutathione CdS colloid. The native GSH-coated CdS particle (64 µg of cadmium) described in Fig. 6 was irradiated at 355 nm in a cuvette aerobically for 10, 20, and 40 min prior to scanning absorption spectra (solid lines). The dashed line shows the 40-min time point for an identical sample irradiated anaerobically.

**Regulation of CdS Crystallite Coating by Growth Condi-
Yeasts cultured in YTD or synthetic minimal media in the absence or presence of Cd(II) had similar total glutathione concentrations, 0.1-0.2 μmol of total GSH/mg of protein. The concentration of γ-Glu-Cys dipeptide was not monitored.

**DISCUSSION**

*C. glabrata* cells cultured in the presence of cadmium sulfate form CdS crystallites coated with peptides. The nature of the coating peptide is dictated by the culture medium. Cells grown in synthetic minimal medium synthesize a mixture of polymerized γEC peptides as coating material, whereas cells grown in YTD broth coat the particles with a mixture of glutathione and γ-glutamylcysteine. This effect of the culture medium is not apparent with *Schizosaccharomyces pombe*, an organism that synthesizes γEC peptides in both minimal and YTD media (19). Supplementation experiments suggested that the yeast extract component of YTD was partially responsible for the effect. The basis of the regulation of coating material is unclear.

The glutathione-coated CdS complex exhibits properties analogous to small quantum CdS crystallites formed in *C. glabrata* and coated with γEC peptides (22). The γEC peptide particles exhibit size-dependent excited electronic states (22). Crystallites of 20 Å diameter exhibit a band gap electronic transition near 313 nm. Smaller sulfide-deficient complexes in *S. pombe* have transitions shifted to higher energies (22). The glutathione-CdS particles also have optical properties that vary with the size of the complex. Particles with the most red-shifted low energy transitions have the highest Stokes’ radius on gel filtration. This heterogeneity is related to the sulfide content of the particles which in turn is related to the cadmium-stimulated pathway yielding sulfide ions.

In addition to the quantum effects, photoexcitation of the particles results in luminescence and the ability to mediate electron transfer to methyl viologen. Luminescence in CdS semiconductor clusters has been attributed to photogenerated trapped electron tunneling to a trapped hole (28). The emission arises from a state lying below the excited states and one not detected in the absorption spectrum (28). A 22 Å CdS synthetic cluster prepared by Chestnoy et al. (22) exhibited an emission maximum near 490 nm. The redox potential of CdS crystallites is greatly enhanced in small quantum clusters, so reduction reactions that cannot occur in bulk material can occur in quantum particles (30). Methyl viologen (reduction potential, −0.55 V) has been employed as the electron acceptor in most semiconductor studies (29–31). Methyl viologen also serves effectively as an acceptor in the anaerobic irradiation of GSH-CdS particles. Photoexcitation of the particles aerobically leads to decomposition of the complex accompanied by the diminution of titratable sulfide ions. Deposition of synthetic clusters has been attributed to localization of positive holes to form S⁺ radical anions followed by oxidation of S⁻ by oxygen (31, 32). This aerobic reaction is in competition with the radiative recombination of electron and positive holes. It appears that particle decomposition and luminescence have a common intermediate that is scavenged by methyl viologen at the surface of the CdS particle (31).

The properties of the excited electronic states and the correlation of optical properties with particle size suggest that the glutathione-coated CdS complex is indeed a semiconductor-type cluster analogous to the γEC-coated CdS crystallites.

The glutathione/γ-glutamylcysteine coating of the clusters does not stabilize the CdS crystallite as effectively as the γEC peptides. The GSH particles are unstable at neutral pH as accretion (referred to as Ostwald ripening) occurs with prolonged incubation (data not shown). The γEC peptide-con-
taining particles show cluster accretion only slowly at neutral pH but more facile at pH 3.5 (22). Instability of GSH particles may explain the greater heterogeneity in size of GSH-coated clusters compared with γEC peptide-coated particles in C. glabrata. Each CdS cluster is dynamic at neutral pH as sulfide ions readily mediate accretion to generate larger particles at the expense of smaller clusters. Sulfide ions will mediate accretion of GSH particles to sizes with band gap transitions in the visible region, whereas CdS particles coated with γEC peptides of n3 and n4 are terminally red-shifted near 320 nm (20).

The nomenclature of the γEC peptides is vague. Multiple terms including cadystin and phytochelatin have been used for this family of chelating peptides. None of these terms are satisfactory. It is now clear that the metal complexes can form with a continuum of coating peptides from the γ-GluCys dipeptide to polymerized dipeptide units. The polymerization reaction is mediated by metal ions in an undefined process. The regulation of this polymerization and the nutrient regulation of the coating material are significant issues to be resolved.

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