Euglena gracilis Cadmium-binding Protein-II Contains Sulfide Ion*

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Sulfide ions are a constituent of the cadmium-binding protein-II in the alga Euglena gracilis. Their presence was demonstrated by the methylene blue assay, by acid labilization induced reductions in the Cd-S charge transfer band at 254 nm and by reactions with the thiol reagent, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). Direct reduction of DTNB by sulfide and precipitation of CdS yield a complex stoichiometry for the DTNB reaction. The S2-/Cd2+ ratios determined, 1.25 ± 0.10 (methylene blue) and 1.37 ± 0.16 (DTNB), are in good agreement.

Euglena gracilis is an established model for studying cellular metal metabolism (1). Recently we have shown that this alga metabolizes zinc and cadmium differently than mammalian cells (2–4). The inducible cadmium-binding proteins (Cd-BP-I and II)† of E. gracilis have unique physiochemical properties which distinguish them from metallothionein: greater negative charge, higher half-titration pH for metal displacement, more variations in amino acid composition between BP-I and II, and faster reactions with EDTA (2, 3). Such differences are undoubtedly related to the unusual biology of the Cd-BPs: inducibility only at high levels of Cd2+, lack of induction by Zn2+, and lack of cross-reactivity with antibodies to rat liver metallothionein (2, 3).

With one exception, cofactors other than metals have not been observed in mammalian metallothioneins or related metal-binding proteins of lower species (5). Sulfide ions in the inducible cadmium-binding peptide-1 of the fission yeast Schizosaccharomyces pombe constitute, to our knowledge, the only exception (6). Sulfide ions also occur in all phyta as components of iron-sulfur clusters, (Fe,S, (SCys),)−x; x, y, and z = 2, 2, and 4 or 4, 4, and 4, etc. (7). During pH titrations of E. gracilis Cd-BP-II, we noticed an irreversible change in A254 as the pH was lowered and detected the odor of HPS, suggesting the presence of sulfide ions in the protein. Our investigations of this hypothesis are presented here.

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§ The abbreviations used are: Cd-BP-I and -II, cadmium-binding protein-I and -II; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid.

EXPERIMENTAL PROCEDURES

Materials—From Aldrich was obtained N,N-dimethyl-1,4-phenylenediamine monohydrochloride; from Sigma, DTNB and Trizma (Tris base); and from EM Science (Cherry Hill, NJ), NaS.

Preparation of BP-II—Crude Euglena gracilis Cd-BP was prepared using Cd2+ to displace Zn2+ as previously described (2). BP-II was purified on a Waters Model 480 HPLC using a Protein-Pak DEAE-5PW column: 0–800 mM KCl gradient, 50 mM Tris-HCl, pH 6.8. The cadmium applied was recovered as BP-I (10–20%) and BP-II (75–85%). Each was concentrated using an Amicon Diaflo YM-2 ultrafiltration membrane and then stored under liquid nitrogen.

Spectrophotometric pH Titrations—BP-II (3.0 ml, 10 μM Cd2+) was titrated stepwise to pH 2 with 1 N HCl. After each addition, the spectrum (240–300 nm) was recorded using a Hewlett-Packard 8451A Spectrophotometer. The reversibility was determined by incrementally increasing the pH to 12 with 1 N NaOH. The reference for each spectrum was 50 mM Tris-HCl, adjusted to the sample pH ± 0.2 A254 was plotted versus pH after normalizing the absorbance values from 0.0 to 1.0.

DTNB Measurement of Thiol and Sulfide—BP-II (10–20 μM Cd2+) in 0.2 ml of 50 mM Tris-HCl, pH 7.5, was added to DTNB solution (0.8 ml in 100 mM phosphate buffer, pH 7.5) (8). The final DTNB concentration was 8.0 mM. The reaction was monitored at 412 nm until complete (30–45 min) and then was analyzed as previously described (9). The TNB/Cd2+ ratios were calculated using A4 and the Cd2+ content was determined by atomic absorption spectroscopy.

Aliquots of Cd-BP-II were also adjusted to pH 2, exhaustively evacuated to remove H2S, returned to pH 7.5, and then analyzed.

Methylene Blue Determination of Sulfide—The procedure of King and Morris (10) was followed. NaS stock solutions were standardized iodometrically (11).

RESULTS

Cd-BP-II was titrated with HCl to displace the cadmium by proton competition for the bound thiolates according to Equation 1.

\[ \text{BP(CysS,Cd)} + z\text{H}^+ \rightarrow \text{apo-BP(CysSH)} + y\text{Cd}^{2+} \]  

(1)

As shown in Figs. 1 and 2, the absorbance at 254 nm previously assigned to a cadmium-thiolate charge transfer band (2, 3) is lost as the pH is lowered to 1.5, consistent with Equation 1. The inflection point is pH 5.6 ± 0.2 (Fig. 1). Thus, under acidic conditions, Cd-BP-II is less stable than metallothionein, which has a half-titration point of about pH 3 (12, 13).

However, when the pH is restored to 9, A254 is only partially recovered (Fig. 2). About 70% of the absorbance is lost after the acidification and neutralization cycle, suggesting that an irreversible change may have occurred. Metallothionein, in contrast, can be taken through several cycles of acidification/neutralization with nearly complete recovery of the 254 nm absorbance. Thus, acid dissociation of the cadmium-thiolate clusters of metallothionein is reversible. Among possible explanations for the irreversible loss of absorbance, the presence of acid-labile sulfide was considered most likely. Further tests of the hypothesis were undertaken.

Samples of BP-II were analyzed for the presence of sulfide ion by the methylene blue procedure of King and Morris (10). Fresh NaS, well protected from oxidation, was used for standardization. The results were compared to the number of moles of cadmium in the preparations (Table I). The average value of S2-/Cd2+ for five determinations on three independent preparations of Cd-BP-II was 1.25 ± 0.10, providing strong
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The stoichiometry differs from that of the thiol reaction (Equation 2b) because two TNBs are released for every sulfide ion present. Thus, the presence of sulfide ions in a binding protein could give an apparent value for the moles of thiols which is even greater than the sum of the thiols and sulfide ions: \( n_{TNB} = 2n_{S^2-} + n_{RSH} \).

We attempted to determine the extent of the DTNB reaction due to the sulfide ions in the Cd-BP-II by exploiting their acid lability. Solutions of the BP were divided into 2 aliquots. One was analyzed as prepared, while the second was adjusted to pH 2, exhaustively evacuated, and then restored to pH 7 and analyzed (Fig. 3). When the difference in TNB generated with and without acidification (Table II) was attributed to sulfide, according to Equation 2a, the resulting value, 0.74 S^2-/Cd^2+, was lower than that determined using methylene blue. The moles of DTNB determined after acidification and neutralization, 0.56 (per original cadmium), also seemed low since the preliminary amino acid analysis (4) demonstrates a high cysteine content.

A possible complication is the precipitation of CdS, which is extremely insoluble, \( K_{sp} = 3.6 \times 10^{-29} \). To determine whether the cadmium released during the titration would inhibit the reaction of sulfide ion with DTNB, we monitored the reactions of DTNB (8 mM) with \( S^2- \) solutions (70 \( \mu \)M) to evidence for the presence of sulfide.

Cd-BP-I and Cd-BP-II react with the thiol reagent 5,5'-dithio-2-nitrobenzoic acid (DTNB = \( \phi SS\phi \)), which was used to measure the thiol content of the protein (2, 3). However, the presence of the sulfide required a reexamination of this reaction, since sulfide ions might also react with DTNB, releasing 5-thio-2-nitrobenzoic acid (TNB = \( \phi S^- \)). When freshly prepared Na_2S is added to DTNB under anaerobic conditions, the absorbance change at 412 nm indicates that it reacts according to Equation 2a.

\[
S^{2-} + \phi SS\phi \rightarrow S^- + 2\phi S^- \tag{2a}
\]

\[
RSH + \phi SS\phi \rightarrow RSS\phi + \phi S^- \tag{2b}
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\]
which increasing concentrations of \( Cd^{2+} \), up to a 1/1 ratio, were added. The results demonstrated that cadmium effectively inhibited the reaction of 1 (or slightly more than 1) eq of sulfide ion with DTNB due to precipitate formation.

\[
Cd^{2+} + S^{2-} \rightleftharpoons CdS_{1}\tag{3}
\]

The data for the DTNB reaction was reanalyzed, assuming that the cadmium ion present stoichiometrically precipitated the sulfide ion.

\[
CdS_{1}(SCys), + HSSH \rightarrow zCdS_{1} + zCysSSH + (z + 2y - 2x)SH + (y - x)S^{2-}\tag{4}
\]

Each BP cysteine \((z)\) generates one TNB molecule, but only the sulfides \((y)\) in excess of cadmium \((z)\) would generate TNB. Thus, the stoichiometry of TNB released should be \(z + 2(y - x)\). The cadmium content determined by atomic absorption spectroscopy, the sulfide content determined as methylene blue, and the moles of TNB generated from methylene blue, respectively, which allow the ratios \(S^{2-}/Cd^{2+}\) and \(SH/Cd^{2+}\) to be calculated (Table I). The value of \(S^{2-}/Cd^{2+}\) obtained, 1.38 \pm 0.08, is in excellent agreement with the methylene blue value first determined. The \(SH/Cd^{2+}\) value calculated in this manner, 1.56, is larger than the value measured after acidification, 0.56. The latter may reflect oxidation during the acidification/neutralization cycle.

The kinetics, as well as the extent, of the DTNB reaction were altered by the acid treatment (Fig. 3 and Table II). The reaction of untreated Cd-BP-II is biphasic and the two rates differ by an order of magnitude. The acid-treated protein reacted in a single phase, with a rate equal to the faster rate for the native protein. The loss of one reaction phase suggests that Cd-BP-II, reconstituted without the sulfides, lacks the native structure and has a different metal-binding geometry. This is in agreement with the 69% loss of the A_{294} after the acidification/neutralization cycle (Fig. 2).

**DISCUSSION**

The discovery of sulfide ions in the inducible cadmium-binding protein-II of *E. gracilis* was unexpected. Since this is the second organism in which sulfide is present in a cadmium-binding peptide/protein, the first being the yeast *Schizosaccharomyces pombe* (6), the phenomenon may be widespread among lower organisms. The inefficient induction of the *Euglena* Cd-BPs by low levels of \( Cd^{2+} \) in the media, the kinetic lability of the \( Cd^{2+} \), and the thermodynamically weaker strength of cadmium binding suggest that the structure and the induction mechanisms of this sulfide-containing BP are not as highly evolved as those of the mammalian metallothioneins.

The greater lability and lower stability of Cd-BP-II compared to metallothioneins may result in part from the presence of sulfide ions used in lieu of cysteine thiolates. The ratio of sulfur to cadmium is close to the values of 2.75 and 3.0 for the \( \alpha \) and \( \beta \) clusters of metallothionein, but the use of sulfide ions will reduce the shielding of the clusters by protein backbone and other residues and also decrease stability since the chelate effect of the protein is lost for the sulfides. If the sulfide ions bridge two or more cadmium ions while the cysteines are terminal ligands, as in Fe-S proteins, the 69% loss of A_{294} is reasonable since each sulfide can generate two or three times as many Cd-S bonds as does each cysteine.

The analogies between the Cd-BP and iron-sulfur proteins are intriguing. Both involve metals which form stable tetrahedral coordination environments with soft ligands, such as the sulfide and thiolate anions. Both proteins use cysteine and sulfide as ligands. The absolute stoichiometries of Cd-BP-II are not yet available because of uncertainties in the molecular weight, but the estimated ratio of cysteine:sulfide:cadmium is 1.5:1.4:1. This falls close to the values of 2.75 and 3.0 for the 2Fe-2S and 4Fe-4S proteins, 2:1:1 and 1:1:1, respectively.

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