A biological function for cadmium in marine diatoms

Todd W. Lane* and François M. M. Morel

Department of Geosciences, Princeton University, Princeton, NJ 08544-1003

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The oceanic distribution of cadmium follows closely that of major algal nutrients such as phosphate. The reasons for this ''nutrient-like'' distribution are unclear, however, because cadmium is not generally believed to have a biological function. Herein, we provide evidence of a biological role for Cd in the marine diatom *Thalassiosira weissflogii* under conditions of low zinc, typical of the marine environment. Addition of Cd to Zn-limited cultures enhances the growth rate of *T. weissflogii*, particularly at low pCO_2. This increase in growth rate is reflected in increased levels of cellular carbonic anhydrase (CA) activity, although the levels of TWCA1, the major intracellular Zn-requiring isoform of CA in *T. weissflogii*, remain low. ^{109}Cd label comigrates with a protein band that shows CA activity and is distinct from TWCA1 on native PAGE of radiolabeled *T. weissflogii* cell lysates. The levels of the Cd protein are modulated by CO_2 in a manner that is consistent with a role for this enzyme in carbon acquisition. Purification of the CA-active fraction leads to the isolation of a Cd-containing protein of 43 kDa. It is now clear that *T. weissflogii* expresses a Cd-specific CA, which, particularly under conditions of Zn limitation, can replace the Zn enzyme TWCA1 in its carbon-concentrating mechanism.

Marine microorganisms are largely responsible for the cycling, and therefore the distribution, of many nutrients in the sea, and it is the availability of these nutrients that, in turn, controls oceanic primary production. This relationship is as true of essential trace elements as it is of major nutrients. In oceanic systems, the water column distribution of many biologically important trace metals is thus similar to that of the major nutrients: phosphate, nitrate, and silicate. At the surface, where photosynthetic activity depletes algal nutrients, such metals are present at extremely low concentrations, presumably as the result of biological uptake. Like those of major nutrients, these metal concentrations increase at depth because of decomposition of organic matter and remineralization.

Zinc, which has many known biological functions (1), is a good example of a metal that follows this type of nutrient-like profile. Cadmium, however, which is generally thought to be universally deleterious to living organisms, also has a nutrient-like profile (2); its concentration is so well correlated to that of phosphate that the accumulation of Cd in the fossilized tests of marine invertebrates is used as a measure of past nutrient concentrations in the sea (3). In the absence of a demonstrated biological function for Cd, it has been widely thought that abiotic processes, such as adsorption onto organic particles, modulate the distribution of this metal. Recently, it has been proposed that the depletion of Cd from surface seawater is caused by its use by marine phytoplankton (4). Under conditions of Zn limitation, Cd enhances the growth rate of some marine phytoplankton in culture (4–6), and it thus seems that Cd may replace Zn in some essential biochemical function.

In *Thalassiosira weissflogii*, the enzyme carbonic anhydrase (CA) constitutes a major use of cellular Zn (7), and the expression of TWCA1, the major intracellular CA, depends directly on the Zn nutrition of the organism (7, 8). TWCA1 concentrations are also modulated by the pCO_2 in the medium, and it is clear that the enzyme plays a role in inorganic carbon acquisition. Although the total dissolved inorganic carbon in surface seawater is 2 mM, only about 1% of it is in the form of CO_2, the species of carbon required by ribulose-1,5-biphosphate carboxylase/oxygenase in the first step of the Calvin cycle (9). Several species of microalgae are known to compensate for low CO_2 availability by operating a carbon-concentrating mechanism. This mechanism is generally thought to have two components, a pump for the active transport of HCO_3^- and the enzyme CA that catalyzes the dehydration of HCO_3^- to form CO_2.

In view of the zinc requirement in CA for carbon acquisition, it is not surprising that marine phytoplankton have evolved mechanisms for dealing with the very low zinc concentration in surface seawater: inorganic Zn (Zn′) = 2–50 pM (10). There is good evidence from laboratory experiments that, in *T. weissflogii*, Co can substitute for Zn at the active site of TWCA1 *in vivo* and thus partially overcome Zn/CO_2 limitation (4, 7, 8, 11). There are three obvious mechanisms by which Cd could alleviate Zn/CO_2 limitation in *T. weissflogii*. (*i*) Cd could substitute for Zn in TWCA1 in much the same manner as Co does. (*ii*) Cd could serve as the metal center in a Cd-specific form of CA. (*iii*) Cd could displace Zn from other metalloproteins, liberating it to function in TWCA1. Because Cd and Zn are isoelectronic, the replacement of Cd for Zn in some metalloproteins is at least plausible. CAs with Cd substituted for Zn *in vitro* typically show significantly reduced activity (12).

Previous work with *T. weissflogii* strongly suggests that Cd may indeed be playing a direct role in CA. Addition of Cd to Zn-limited cultures not only enhances their growth rate but results in the increased expression of a CA (6) that is clearly distinct from the major Zn requiring isoform TWCA1. In ^{109}Cd-labeling experiments, the ^{109}Cd label seems to migrate with this band of CA activity in nondenaturing PAGE (6, 7), providing support for the existence of a Cd-dependent CA. Herein, we expand on these previous data and show that the effect of Cd on the growth rate of Zn-limited cells is mediated through an increase in cellular CA activity and that this increase in activity is due to the expression of a Cd-specific CA.

Materials and Methods

Strains and Culture Conditions. Axenic *T. weissflogii* (clone Actin) cultures were grown at 20°C and 1,000 μmol·photons·m^{-2}·s^{-1} in modified Aquil growth medium without Co (ref. 13; 2.2 mM dissolved inorganic carbon/300 μM NO_3^-, pH 8.2) with the Zn and Cd levels as indicated in each individual experiment. Inorganic trace metal concentrations were calculated from total metal concentrations with the computer program MINEQL (14). Zn-limited stock cultures were grown under Zn-limited conditions for six generations before the inoculation of experimental cultures. Experimental cultures were grown under the appropriate conditions for five or more generations.

Gas mixtures, used to control CO_2 levels in cultures, were prepared in a standard air (N_2/O_2) background with the desig-

Abbreviations: CA, carbonic anhydrase; Zn′, inorganic Zn concentration; Cd′, inorganic Cd concentration.

*To whom reprint requests should be addressed. E-mail: tlane@princeton.edu.

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nated concentration of CO_2. Cell concentrations were followed by using a Coulter Multisizer II.

CA Assays. CA was detected in native polyacrylamide gels either by using a modification of the assay developed by Patterson *et al.* (15) or by using bromthymol blue. Equal amounts of protein were run on a 10% or 12% nondenaturing gel (16). For the bromthymol blue assay, the gel was soaked in native gel running buffer in which 0.1% bromthymol blue was dissolved until the gel was dark blue in color. When saturated CO_2 was blown over the gel, green or yellow bands appeared against a blue background. The gel was photographed with transmitted white light with Polaroid 669 color film.

CA activity was measured in cell lysates by the method of Sültemeyer (17) in 25 mM veronal buffer.

Western Analysis. Each phytoplankton strain was grown at both 100 and 750 ppm CO_2 then harvested and subjected to Western analysis. Samples were harvested by filtration then pelleted by centrifugation, resuspended in SDS/PAGE sample buffer, and stored at −7°C until use. SDS/PAGE and Western analyses were carried out by standard methods (16) with high titer polyclonal antiserum from rabbits directed against TWCA1 (18) and ^{125}I-labeled protein A. Relative amounts of radioactivity were measured by phosphorimaging.

Labeling Experiments. Two 400-ml cultures of *T. weissflogii* were grown at 100 ppm CO_2, the first containing 3 pM Zn′, 45 pM inorganic Cd (Cd′), and 20 μCi/liter ^{109}Cd and the second containing 15 pM Zn′ and 20 μCi/liter ^{65}Zn. When a cell density of 2.5×10^4 to 5×10^4 cells per ml was achieved, both cultures were harvested by filtration, resuspended in lysis buffer (50 mM Tris·HCl, pH 7.0/1 mM EDTA/50 mM KCl/0.5 mM DTT/0.1 nM PMSF), and lysed by sonication on ice. The progress of cell lysis was monitored by phase contrast microscopy. Samples representing equal cell numbers were analyzed by nondenaturing PAGE and subsequent in-gel CA detection assay. The gels were then dried and phosphorimaged.

Chromatography. A 2-liter culture was grown at 3 pM Zn′, 45 pM Cd′, 20 μCi/liter ^{109}Cd, and 100 ppm CO_2 until a cell density of 1×10^5 cells per ml was achieved. Cells were harvested by filtration and frozen at −70°C until use. Frozen cell pellets were thawed in ice-cold lysis buffer and lysed by sonication on ice as described above. Frustules and other debris were removed by centrifugation at 10,000 × *g* for 20 min at 4°C. Membranes were removed by centrifugation at 100,000 × *g* for 2 h at 4°C. The supernatant was subjected to sequential ammonium sulfate fractionation of 30 and 70% with the pellet from the 70% fraction being retained and redissolved in 10 mM phosphate buffer (pH 6.8). The residual salt was removed by extensive dialysis into the dissolution buffer. The 30–70% ammonium sulfate fraction was dialyzed into loading buffer (10 mM phosphate, pH 6.8) and loaded onto a 10-ml bed volume DEAE-Sephacel column. The column was then washed with 50 ml of loading buffer, and proteins were eluted by a linear 25-ml 0–500 mM NaCl gradient in loading buffer.

Fractions (0.5 ml) were collected and analyzed by native page and CA Assay. The Cd-CA eluted very early in the gradient. The Cd-CA-containing fractions were pooled and run on a preparative native polyacrylamide gel. After a CA assay, the band of CA activity was excised and electroeluted. The semipurified CA was then subjected to SDS/PAGE in the absence of β-mercaptoethanol and phosphorimaging.

A silver stain of the SDS gel revealed two bands at 43 and 60 kDa. The ^{109}Cd label comigrates with the 43-kDa protein.

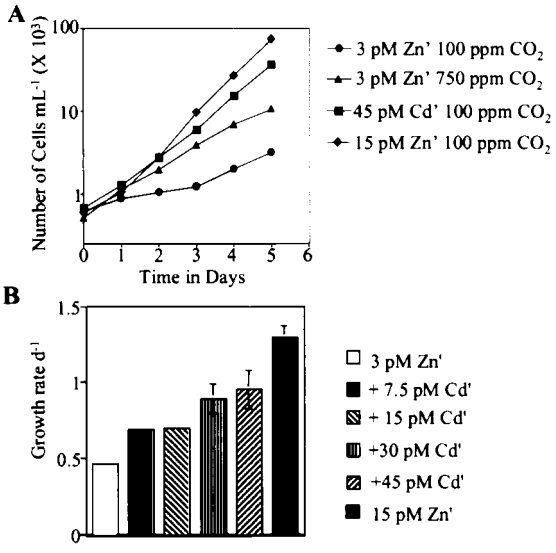

Fig. 1. (*A*) Typical growth curves of *T. weissflogii* grown under different conditions of trace metals and pCO_2. (*B*) Growth rates at 350 ppm CO_2 of *T. weissflogii* cultures grown under different conditions of trace metals. The graph is the summary of two separate sets of cultures. Error bars represent the range of values observed (if not shown, error bars are too small to be resolved).

Results

Growth Rate. Cultures of *T. weissflogii* grown at various Zn′ and Cd′ and pCO_2s provide confirmation that Cd is beneficial to the growth of the organism under Zn- and CO_2-limiting conditions (Fig. 1). At 3 pM Zn′ in the absence of Cd, the growth rate is greatly reduced compared with growth in normal Aquil medium (15 pM Zn′) at 350 ppm CO_2 (0.4 vs. 1.3 day^{-1}), consistent with previous reports (7). Normal growth rate can be restored completely by increasing the Zn to Zn′ = 15 pM and partially by increasing the CO_2 to pCO_2 = 750 ppm (Fig. 1*A*), thus illustrating the role of Zn in carbon acquisition (8). Adding Cd (Cd′ = 45 pM) to the low Zn, low CO_2 cultures is almost as effective as adding Zn. At pCO_2 = 350 ppm, supplementation of the Zn-limited cultures with Cd at concentrations in the range of Cd′ = 7.5–45 pM alleviates Zn limitation, growth rate increasing with Cd′ over the whole range tested (Fig. 1*B*). Even at the highest Cd′ (45 pM), however, the growth rate is somewhat lower than at high Zn′ (average: 0.95 vs. 1.3 day^{-1}).

TWCA1 Levels. A major fraction of the cellular Zn in Zn-sufficient *T. weissflogii* is in TWCA1, its principal intracellular CA isoform. We thus tested whether the beneficial effect of Cd on growth was reflected in increased levels of TWCA1 in Zn-limited cultures supplemented with Cd. The TWCA1 levels measured by Western analysis in cells from cultures grown under various conditions of pCO_2, Zn′, and Cd′ are shown in Fig. 2 *A* and *B*. As expected, the relative amount of TWCA1 protein is reduced about 10-fold in cells from the Zn-limited cultures as compared with those from the Zn-replete cultures and is greatly enhanced at low pCO_2. However, when the Zn-limited cultures are supplemented with Cd, significantly less, rather than more, TWCA1 is detected compared with the unsupplemented Zn-limited cultures. These Western analyses were performed on denaturing gels, and the lack of reactivity of the polyclonal antiserum (raised against the apoenzyme) is a clear indication that Cd is neither substituting into TWCA1 nor releasing Zn from other metalloenzymes for use in TWCA1.

CA Activity. Because supplementation of Zn-limited cultures with Cd results in increased growth rate but decreased amounts of

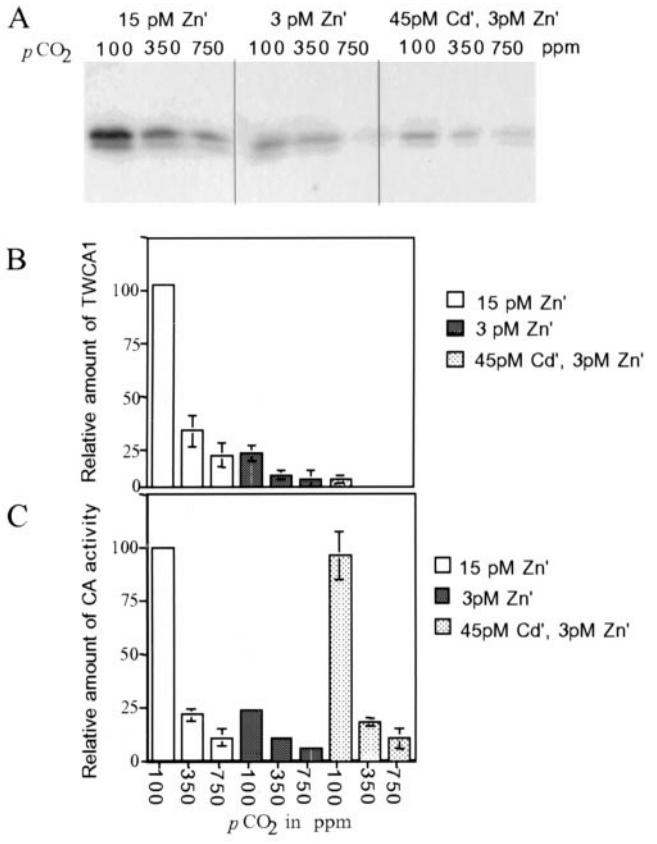

Fig. 2. (*A*) Typical phosphorimage of a Western blot of whole-cell lysates of *T. weissflogii* grown under different conditions of pCO_2 and trace metals. (*B*) Relative TWCA1 levels determined by Western analysis and phosphorimaging in cells grown under different conditions of pCO_2 and Zn′. The graph represents the average of measurements from three independent sets of cultures. All measurements from the same blot were normalized to the value from the 100-ppm, 15-pM Zn culture. (*C*) Relative amounts of CA activity in lysates of cells grown under different conditions of pCO_2 and trace metal concentrations. The graph represents the average of three measurements from each of two independent sets of cultures. Error bars represent the ranges of values measured (if not shown, error bars are too small to be resolved).

TWCA1, it seems most likely that the addition of Cd alleviates Zn limitation through the synthesis of a Cd-specific CA distinct from TWCA1. If this mechanism were true, the addition of Cd to a low Zn culture should result in the restoration of CA activity in these cells despite the low levels of TWCA1. The CA activity present in cell lysates determined by *in vitro* CA assays (Fig. 2*C*) indicates that the addition of Cd to Zn-limited cultures does indeed result in an increase in CA activity. Zn-limited cells have a CA activity that is only one-fifth of that of Zn-sufficient cells—roughly in accord with the relative concentrations of TWCA1 (compare Fig. 2 *B* and *C*). Addition of 45 pM Cd′ in the culture restores full CA activity in the Zn-limited cells.

Electrophoretic Analysis. A CA other than TWCA1 seems to be responsible for the increase in CA activity in Zn-limited cultures supplemented with Cd. This Cd-induced CA can be detected by native PAGE of radiolabeled cell lysates from Cd-supplemented cultures followed by an in-gel colorimetric pH shift assay (Fig. 3). The higher mobility band is the Zn-dependent CA, TWCA1, and the lower mobility band is the putative Cd-specific CA, Cd-CA. Extensive labeling experiments have shown that Cd is never detected at the position of TWCA1 and that, conversely, Zn is never detected at the position of the Cd-CA.

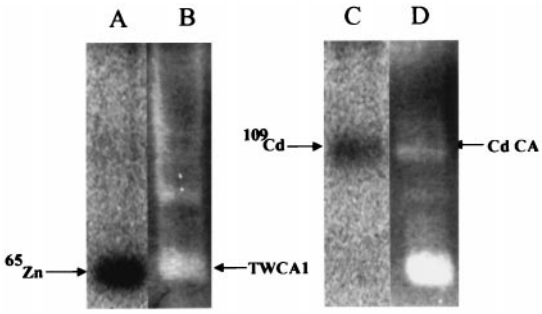

Fig. 3. Native PAGE and CA assay of cell lysates of cells grown at 100 ppm CO_2 under different trace metal concentrations. (*A*) Phosphorimage of a native gel of a lysate of cells grown under 15 pM Zn′ labeled with 65 Zn. (*B*) CA assay of the same lane shown in *A*. (*C*) Phosphorimage of a native gel of a lysate of cells grown under 3 pM Zn′ and 45 pM Cd′ labeled with ^{109}Cd. (*D*) CA assay of the same lane shown in *C*.

Purification. We attempted to purify the Cd-CA by affinity chromatography with p-aminomethylbenzene sulfonamide resin, a standard and specific method of purifying CAs that was used with success with TWCA1 (18). Unlike TWCA1, the Cd-CA did not bind to the column, however (data not shown), another indication that this enzyme is distinct from TWCA1. We then proceeded to purify the Cd-CA by standard chromatographic and electrophoretic techniques.

Ion exchange chromatography of ammonium sulfate fractions with DEAE-Sephacel showed that the ^{109}Cd label and the Cd-CA activity coelute very early in the salt gradient, much earlier than TWCA1. Pooled Cd-CA-containing fractions from the ion exchange column were subjected to native PAGE, and the band of activity was excised, eluted, and run on SDS/PAGE. Two distinct protein bands with molecular masses of 43 and 60 kDa were found associated with the band of CA activity (Fig. 4*A*). The positions of these bands were not altered by the addition of β-mercaptoethanol to the SDS sample buffer. Neither band was recognized by anti-TWCA1 serum. Subsequent phosphorimaging of an SDS gel run with labeled protein (after purification) indicated that the ^{109}Cd label was associated with the 43-kDa protein (Fig. 4*B*).

CO_2 and Trace Metal Modulation of Cd-CA Levels. To verify that the level of expression of the Cd-CA is consistent with our mea-

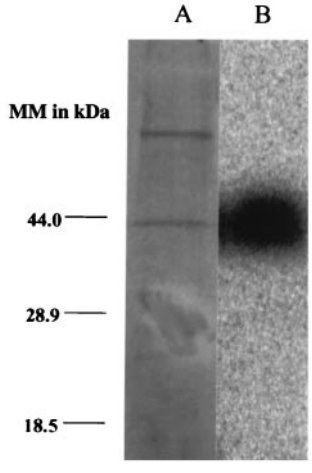

Fig. 4. SDS/PAGE of semipurified Cd-CA. (*A*) Silver-stained SDS gel of purified Cd-CA. (*B*) Phosphorimage of SDS gel of ^{109}Cd-labeled, purified Cd-CA. MM, molecular mass.

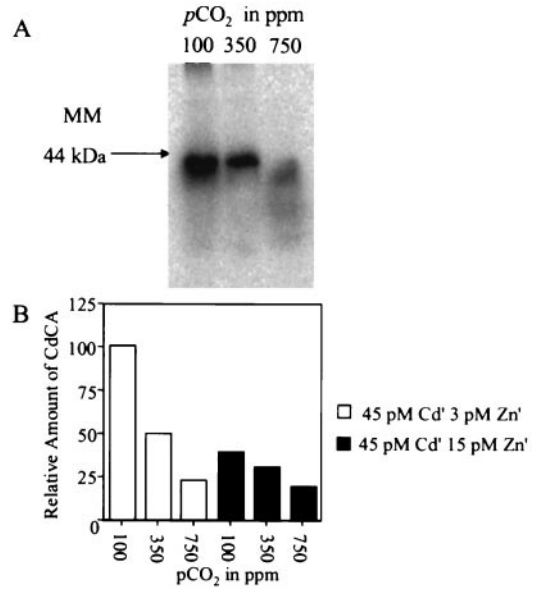

Fig. 5. Modulation of Cd-CA levels by trace metals and pCO_2. (*A*) Phosphorimage of SDS/10% PAGE of total cellular protein of ^{109}Cd-labeled cultures grown at 45 pM Cd′ and 3 pM Zn′ and the indicated pCO_2. MM, molecular mass. (*B*) Graphic representation of results of a typical labeling experiment. ^{109}Cd-labeled cultures were grown at 45 pM Cd′ at either 3 pM or 15 pM Zn′ at the indicated pCO_2. Relative amounts of Cd-CA were determined by SDS/PAGE and phosphorimaging.

surements of CA activity (Fig. 2*C*), cultures labeled with ^{109}Cd were grown at Cd′ = 45 pM at various concentrations of Zn′ and pCO_2. The levels of the Cd-CA were then quantified by the amount of ^{109}Cd label incorporated in the 43-kDa band on SDS/PAGE. (Extensive testing indicated that boiling in SDS sample buffer—without β-mercaptoethanol—had no discernible effect on the amount of radiolabel retained by the protein.) Fig. 5*A* shows a typical phosphorimage from these experiments, and Fig. 5*B* is a quantitative representation of these results. The amount of ^{109}Cd label present in the 43-kDa protein is well correlated with the amount of CA activity present in cultures grown under the same conditions of trace metals and CO_2. Further, the modulation of the levels of the ^{109}Cd label, showing an increase in the expression of the Cd protein at low pCO_2 and low Zn′, is consistent with its role as a CA involved in carbon acquisition.

Discussion

The data we have presented clearly show that the beneficial effect of Cd on the growth of Zn-limited *T. weissflogii* is due to the synthesis of a Cd-CA. (*i*) Cd addition enhances CA activity in the cells but not the concentration of TWCA1, its main intracellular Zn-CA isoform. (*ii*) Cd and CA activity comigrate in nondenaturing protein gels. (*iii*) Purification of the CA fraction leads to the isolation of a Cd-containing protein of 43 kDa. (*iv*) Expression of this Cd protein with pCO_2 is well correlated with variations in CA activity (under low Zn conditions) and is modulated as expected for a CA involved in inorganic carbon acquisition.

That Cd-CA is distinct from TWCA1 and not simply a Cd-substituted form of the same enzyme is shown principally by two results. First, the Cd-CA is not recognized by a polyclonal antiserum raised against the apo-TWCA1, which recognizes the nondenatured Zn and Co forms of the enzyme as well as the denatured form after boiling in SDS and β-mercaptoethanol. Second, and more simply, on a denaturing gel, the Cd-CA has a molecular mass of 43 kDa: 16 kDa larger than the 27-kDa molecular mass of TWCA1 (18).

To our knowledge, the Cd-CA of *T. weissflogii* is the first cadmium enzyme ever reported. Other Cd-containing biological macromolecules are known of course. For example, metal storage or detoxification molecules such as metallothioneins and phytochelatins usually contain Cd *in vivo*. The enzyme phytochelatin synthase, which catalyzes the polymerization of glutathione into phytochelatin, requires binding to a cation such as Cd^{2+} to be active. But Cd-CA is, to our knowledge, the first reported Cd-specific enzyme, one in which, by analogy to Zn-CA, Cd^{2+} is almost certainly playing the role of the catalytic metal center. Further characterization of this enzyme, including its derived amino acid sequence, its activity, and the nature of its metal center will follow the cloning and sequencing of its cDNA and the purification of sufficient quantities of the protein—as a native enzyme or as a fusion protein.

Cd-CA may not be the only Cd enzyme, and marine phytoplankton may not be the only organism that synthesizes Cd-CA. In *T. weissflogii*, it is clear that the Cd-CA is the major Cd enzyme if not the only one: the only protein bands visible on gels of cell lysate labeled with ^{109}Cd are Cd-CA and phytochelatins (which run close to the front). As seen in the growth data, Cd does not completely alleviate Zn limitation. This residual growth deficiency may be because Cd-CA is not as effective an enzyme as Zn-CA. However, it would seem that synthesis of additional enzyme, at sufficiently high Cd′, should make up for any difference in activity, and our whole-cell activity data do not support this interpretation. More likely, at very low Zn, Zn-dependent functions besides CA become limiting, and these cannot be alleviated by Cd. There have been scattered reports of beneficial effects of Cd in various organisms (19, 20); these may be spurious or represent the function of some Cd enzymes, CA or others yet to be discovered.

The Cd-CA of *T. weissflogii* is probably the first enzyme whose discovery was based on the geochemical behavior of a trace element in nature. Indeed, it is the nutrient-like behavior of Cd in the oceans that led to the initial Cd addition experiments in low Zn cultures (4) and the subsequent search for its beneficial role (5, 6). Coming full circle, the discovery of the Cd-CA, which is based on the oceanic distribution of Cd, should help us understand the geochemical behavior of Cd in the oceans. The use of Cd in Cd-CA probably accounts for the nutrient-like profile of this metal: Cd-CAs may be widespread in natural populations, and Cd use may be a general capability of diatoms or of marine phytoplankton in general. Lee *et al.* (6) found that, in three of seven marine species that were limited at Zn′ = 3 pM, addition of Cd to Cd′ = 5 pM increased growth rate significantly. Two of these species were diatoms of the genus *Thalassiosira*, and one was the green alga *Tetraselmis maculata*. Following the idea that Cd may play a role in inorganic carbon acquisition, Cullen *et al.* (21) recently measured Cd uptake in natural phytoplankton assemblages from waters off the California coast and found that Zn and CO_2 modulated Cd uptake in a manner consistent with Cd use in Cd-CA; Cd in the phytoplankton increased with decreasing surface water pCO_2 and was inversely related to the Zn concentration in the biomass. Incubation experiments with natural assemblages confirmed the causal relationship between Cd uptake, CO_2, and Zn′. In view of the correlation that is seen between Cd depletion and pCO_2 in surface waters of some regions of the oceans, these results may be generalizable and further data are likely to show that surface Cd depletion is the result of Cd-CA use for inorganic carbon acquisition. Besides providing an explanation for the oceanic geochemical cycle of Cd, such a result would also point to the critical importance and difficulty of inorganic carbon acquisition by marine autotrophs.

The effective *in vivo* substitution of Co for Zn in TWCA1 (8, 11) and the replacement of TWCA1 by a Cd-specific enzyme at low Zn bear witness to the extreme low metal concentrations

under which marine phytoplankton have evolved. It is indeed a mystery at this point how some species are able to grow rapidly with cellular concentrations of metals that are a fraction of what is absolutely necessary in other species. For example the oceanic diatom *Thalassiosira oceanica grows* at a rate of 1.0 day^{-1} with a cellular iron quota of only 2.0 μmol:mol C (22), only a fraction of the quota that supports the growth of other diatoms and of the amount estimated necessary for biochemical functions (23). It is very likely that substitution of metals in active metalloproteins and replacement of metalloenzymes by others containing other metals or none—as observed for CA—are the mechanisms by which oceanic microorganisms have adapted to an environment of extremely low metal concentrations. It is thus not surprising that we should find common enzymes such as CA with uncommon metal requirements. What we are observing is the unique biogeochemistry of the oceanic environment reflected in the unique biochemistry of its flora.

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