Regulation of Carbonic Anhydrase Expression by Zinc, Cobalt, and Carbon Dioxide in the Marine Diatom *Thalassiosira weissflogii*[1]

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TWCA1 is the major Zn-requiring isoform of carbonic anhydrase (CA) in the marine diatom *Thalassiosira weissflogii.* We have examined the roles that trace metals and CO_2 play in the regulation of TWCA1 expression over ranges of concentrations that bracket those encountered in the marine environment. Both steady-state levels of TWCA1 and the kinetics of induction were measured by western analysis. TWCA1 levels correlated well with cellular CA activity levels. TWCA1 was induced at a low CO_2 concentration but the level of induction, as determined by western analysis, was dependent on the availability of Zn. Co effectively substituted for Zn in regulating TWCA1 expression and promoting TWCA1 activity. Upon shift from low to high CO_2, the concentration of TWCA1 decreased. The expression of TWCA1 is diel cycle regulated, and cellular TWCA1 decreased during the dark phase. These results provide the basis for studying the expression of CA in field populations and, taken together with previous radiolabeling studies, provide strong evidence of in vivo metal substitution of Co for Zn in a CA. Our data also support the conclusion that TWCA1 plays a central role in carbon acquisition in *T. weissflogii*.

In marine environments, the predominant form of dissolved inorganic carbon is bicarbonate, whereas CO_2 accounts for less than 1% of the total dissolved inorganic carbon (Millero, 1996). The carboxylating enzyme Rubisco, however, requires CO_2 as a substrate (Cooper et al., 1969). To maintain efficient photosynthesis in spite of low CO_2 availability, many phytoplankton species possess a carbon-concentrating mechanism (CCM) that functions both to increase the CO_2 concentration in the vicinity of Rubisco (for review, see Badger et al., 1998; Kaplan and Reinhold, 1999) and to exploit the large pool of dissolved inorganic carbon that is in the form of HCO_3^-. The CCM is generally thought to have two key components: a mechanism for directly or indirectly taking up HCO_3^- and at least one carbonic anhydrase (CA), normally a Zn-requiring enzyme, that catalyzes the inter-conversion of HCO_3^- and CO_2 (Badger and Price, 1994).

In marine diatoms (which are dominant primary producers in marine ecosystems), there is growing evidence from both the laboratory and the field of external HCO_3^- utilization, either by direct transport of the ion or by conversion of HCO_3^- to CO_2 via an external CA. Recently, Nimer et al. (1997) demonstrated the direct uptake of HCO_3^- by the marine diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana.* Korb et al. (1997) demonstrated that at least three species of diatoms utilize external HCO_3^-, most likely by a direct uptake mechanism, although they were not able to completely rule out an external CA. Tortell et al. (1997) showed that natural assemblages of bloom-forming diatoms were able to take up HCO_3^- in an ethoxyzolamide (a CA inhibitor)-insensitive manner, and, furthermore, were able to generate internal inorganic carbon pools that were significantly more concentrated than the external medium.

The coastal diatom *Thalassiosira weissflogii* has been shown to have at least one CA, designated TWCA1. Its cDNA was recently cloned and sequenced (Roberts et al., 1997). The *twca1* cDNA encodes a protein of 34 kD, which is processed by cleavage of the amino terminus to a truncated form with a predicted molecular mass of 26 kD. Antiserum raised against the cleaved form of TWCA1 recognized two bands on an SDS-PAGE gel of 27 and 26 kD (Roberts et al., 1997). The amino termini of both of these proteins had the same sequence, and it is possible that they may represent closely related isoforms, further processing, or partial degradation of the TWCA1 peptide.

The relationship of TWCA1 to other forms of CA is not completely clear. As *twca1* is the only cDNA encoding a diatom CA that has been sequenced, information on the conservation of specific residues in the peptide sequence is not available. A direct comparison of the derived peptide sequence of TWCA1 to that of a large number other known CAs from a diversity of taxa indicates that the conserved residues defining the α-, β-, and γ-forms of CA are not found in the same relative positions in TWCA1 (Roberts et al., 1997). However, a protein database search (PROPSEARCH) based on protein properties (Hobohm and Sander, 1995) and using the full 34-kD derived peptide se-

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quence of TWCA1 indicated that it may represent a distant homolog of α-CAs, CAH1 of *Chlamydomonas reinhardtii* being the most closely related protein. The same result is not obtained when we use the sequence of the 27-kD truncated form of TWCA1 that is found in vivo and known to be active (Roberts et al., 1997). A comparison of fragments of the TWCA1 sequence to that of CAH1 shows that the amino-terminal fragment of TWCA1 that is cleaved off in vivo contains a short stretch of amino acids that includes two His residues and resembles a portion of the active site of an α-CA. Because there is no evidence that this fragment reassociates with the larger fragment, final conclusions as to the relationship of these diatom CAs to other known forms should await sequence data from more than one example.

CAs are generally known to require Zn at the active site (Coleman, 1998), but evidence of in vivo utilization of Co and Cd in CA has been published (Price and Morel, 1990; Morel et al., 1994; Lee and Morel, 1995; Yee and Morel, 1996). CA constitutes a major use of Zn in at least some marine diatoms. At the concentrations of trace metals found in the surface waters of the open ocean, inorganic carbon utilization by *T. weissflogii*, a neritic species, has been shown to be impaired (Morel et al., 1994). For example, inorganic Zn concentrations (Zn′) in the surface waters of the central North Pacific are as low as 2 pM (Bruland, 1989). As a result, in some areas of the open ocean, sufficient trace metals may not be present for the efficient utilization of HCO_3^- by marine phytoplankton.

Because of the spectroscopic qualities of Co, the in vitro substitution of Co for Zn in α-CAs is well documented. The Co-containing form of the enzyme generally shows a marked decrease in activity compared with the native Zn form (Tu and Silverman, 1985). The demonstration that Zn can be extracted from a protein and replaced with Co in vitro is not evidence that such metal substitution can or does take place in vivo. The only evidence for in vivo metal substitution in a CA was provided by ^{65}Zn and ^{57}Co labeling studies in *T. weissflogii* (Morel et al., 1994; Yee and Morel, 1996), in which ^{65}Zn and ^{57}Co bands were found to co-migrate with a single band of CA activity on a native gel of diatom proteins.

Although the regulation of CA expression is well studied in the chlorophytic microalgae, there is a paucity of data for *Bacillariophyceae* and over the range of CO_2 concentrations that are environmentally relevant. These studies are then of limited utility for marine systems. Furthermore, there is no information on the role of trace metals in the regulation of CA in microalgae, and only two studies of in vivo Zn/Co substitution. We examined the regulation of CA in the model marine diatom *T. weissflogii* over a range of CO_2 and trace metal concentrations that are known to occur in the oceanic environment. In doing so, we provide further evidence that Co is substituting for Zn in CA in vivo. We also examined the regulation of TWCA1 protein levels by a diel cycle.

RESULTS

Zn/Co/CO_2 Colimitation of Growth

Figure 1 illustrates the Zn/CO_2 limitation of growth typical of *T. weissflogii*. At 3 pM Zn′, *T. weissflogii* showed a significant decrease in growth rate at 100 μatm CO_2 compared with cultures grown at 15 pM Zn′. As previously observed (Morel et al., 1994), this deficit is partially relieved by growth at 750 μatm CO_2, indicating that Zn limitation of growth is at least partly caused by an inadequate supply of inorganic carbon to the organism. The Zn/CO_2 colimitation of growth was completely alleviated by the addition of 21 pM inorganic Co (Co′) to the culture, as essentially the same growth rate was observed as in Zn-sufficient cultures.

Modulation of TWCA1 Protein Levels by CO_2, Zn, and Co

In order to characterize the regulation of TWCA1, we grew cultures of *T. weissflogii* under various concentrations of CO_2, Zn′, and Co′, and then measured the relative amounts of TWCA1 by western analysis and phosphor imaging. As shown in Figure 2, A and B, the steady-state levels of TWCA1 in 100 μatm CO_2-adapted, Zn-sufficient (15 pM Zn′) cultures were 10-fold higher than those seen in 750 μatm CO_2-adapted cells under the same trace metal conditions. In Zn-limited cultures (3 pM Zn′), the steady-state levels of TWCA1 protein were markedly reduced compared with Zn-sufficient cells, about 10-fold at 100 μatm CO_2. Adding Co to Zn-limited cultures

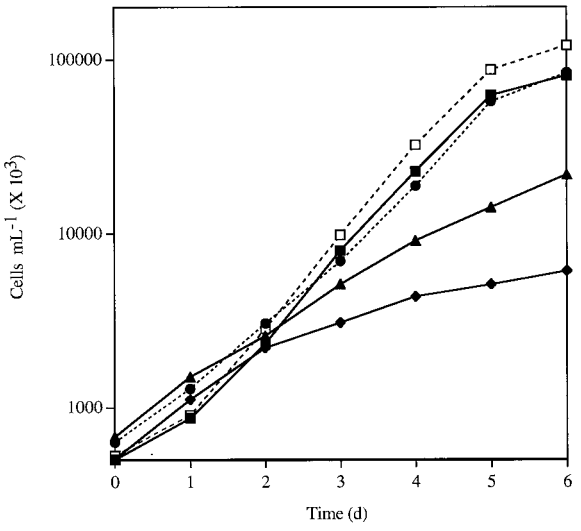

Figure 1. Typical growth curves of *T. weissflogii* grown under different conditions of trace metals and CO_2. ■, 15 pM Zn′, 100 μatm CO_2; ◆, 3 pM Zn′, 100 μatm CO_2; ●, 21 pM Co′, 100 μatm CO_2; ▲, 3 pM Zn′, 750 μatm CO_2; □, 15 pM Zn′, 750 μatm CO_2.

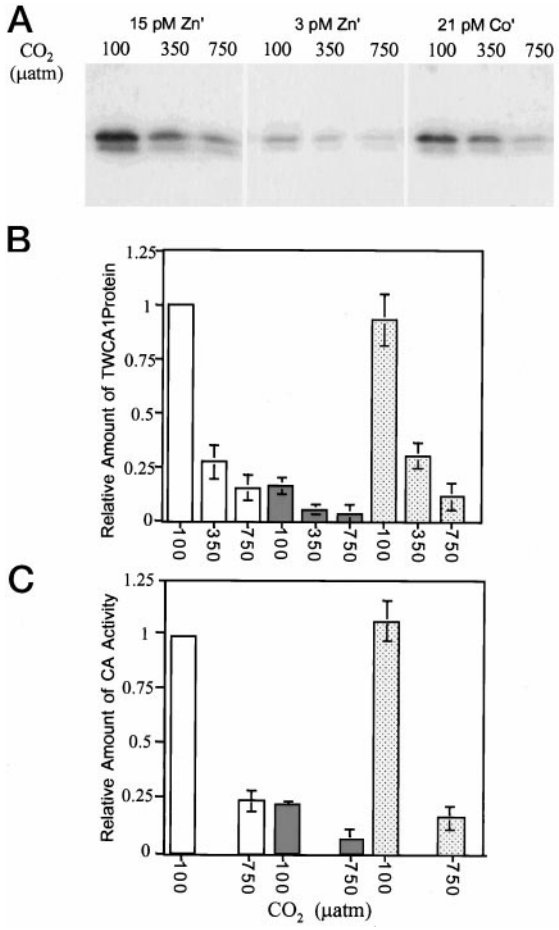

Figure 2. A, Typical phosphor image of western blot of whole-cell lysates of *T. weissflogii* grown under different conditions of CO_2 and trace metals. B, Relative TWCA1 levels per cell determined by western analysis and phosphor imaging in cells grown under different concentrations of CO_2 and trace metals. 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 μatm CO_2, 15 pM Zn culture. C, Relative amounts of CA activity per cell in lysates of cells grown under different concentrations of CO_2 and trace metals. The graph represents the average of three measurements from each of two independent sets of cultures. White bars, 15 pM Zn'; shaded bars, 3 pM Zn'; stippled bars, 21 pM Co'.

restored essentially the same levels of TWCA1 protein as observed in the Zn-sufficient cultures.

Modulation of CA Activity Levels

The relative amounts of CA activity, normalized per cell, were determined for lysates of cells grown under 100 and 750 μatm CO_2 (Fig. 2C). The amount of CA activity resembled the TWCA1 protein levels measured by western analysis (compared in Fig. 2, B and C). The levels increased markedly at low pCO_2, decreased under Zn limitation, and were restored by Co addition to Zn-limited cultures. The relative levels of activity did not vary in exact quantitative accord with the TWCA1 protein level, however. Western analysis may underrepresent the relative CA levels in high-CO_2 cells. This may represent the inherent imprecision of each analysis or it may be due to the presence of other isoforms of CA not recognized by anti-TWCA1 serum.

Time Course of TWCA1 Induction or Degradation by CO_2 Shift

The time required for high-CO_2-adapted cells to respond to a rapid decrease in CO_2 was determined in a series of CO_2-shift experiments. At t = 0, the experimental culture was shifted to 100 μatm CO_2 while the control remained at 750 μatm. The relative amount of TWCA1 present in the cells was determined at regular intervals for 24 h (Fig. 3A). A parallel series of experiments was carried out on cultures in which Zn had been replaced with Co (Fig. 3B). TWCA1 protein levels began to increase approximately 5 h after the CO_2 shift, and steady-state levels of TWCA1 were attained approximately 18 h after the

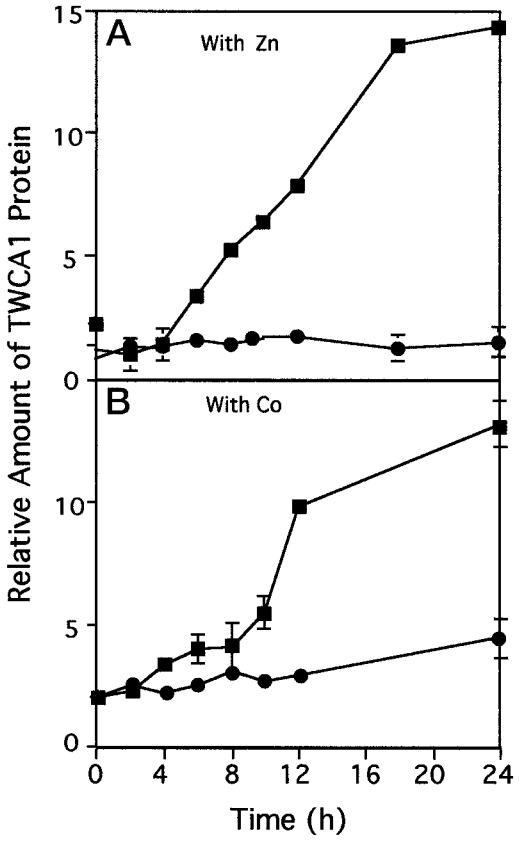

Figure 3. A, Time course of TWCA1 induction by a shift in CO_2 concentration in Zn-containing cultures. B, Time course of TWCA1 induction by shift in CO_2 in Co-containing cultures. Cultures were preadapted to 750 μatm CO_2. At t = 0, experimental cultures were shifted to 100 μatm CO_2 and samples were withdrawn at the times indicated. Relative amounts of TWCA1 per cell were determined by western analysis and phosphor imaging. The graphs each represent the average of two separate experiments. When not shown, error bars are within the symbol. ■, 100 μatm CO_2; ●, 750 μatm CO_2.

shift. The results from Zn- and Co-containing cultures did not differ significantly in terms of the time required for the initiation of induction (Fig. 3).

Adaptation to increased levels of CO_2 was tested in a shift experiment in which Zn-sufficient cultures were preadapted to 100 μatm CO_2 and then shifted to 750 μatm (Fig. 4). To compensate for dilution by cell division, protein from equal volumes of culture (rather than equal cell numbers) was loaded on the gel. Upon the shift to 750 μatm CO_2, there was an immediate and rapid decline in the amount of TWCA1 present in the cells. A 10-fold decrease in the amount of TWCA1 protein was achieved in 24 h. This ratio between the levels of TWCA1 at the start and at the end of the experiment (Fig. 4) was the same as between cultures grown continuously at 100 versus 750 μatm CO_2 (Fig. 2B).

Time Course of TWCA1 Induction by Zn or Co Addition

Experiments to determine the time response to an increase in trace metal availability required large concentrations of slow-growing, Zn-limited cells at low CO_2. Cultures were thus initially grown at 350 μatm CO_2 to a density of 20,000 cells mL^{-1}, then adapted to 100 μatm CO_2 for at least 24 h prior to the start of the experiment. At t = 0, EDTA-buffered Zn or Co was added to a final concentration of 15 pM Zn′ or 21 pM Co′. Protein from an equal number of cells was loaded on the gel for each time point. Ten hours after the shift, a rapid increase in the amount of TWCA1 protein was observed, and steady-state levels of protein were present within 24 h (Fig. 5)—somewhat longer than the time required to respond to a CO_2 shift. The responses to both metals were essentially identical.

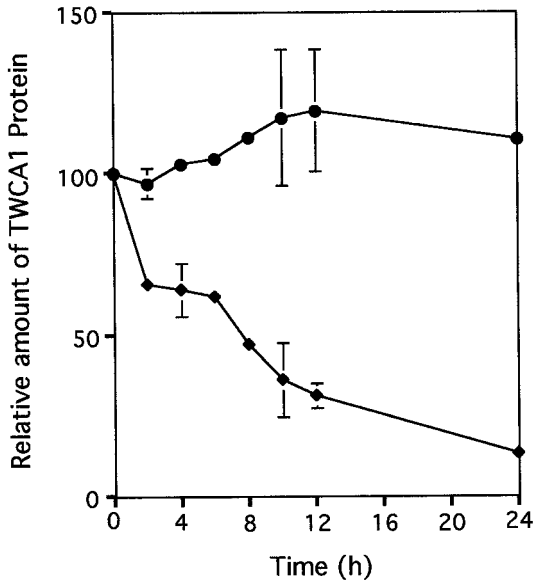

Figure 4. Time course of TWCA1 decrease in response to increased CO_2 concentration. Cultures were preadapted to 100 μatm CO_2. At t = 0, experimental cultures were shifted to 750 μatm CO_2 and samples were withdrawn at the times indicated. Relative amounts of TWCA1 were determined by western analysis and phosphorimaging. All values were normalized to that of the initial timepoint (t = 0). The graph represents the average of two separate experiments. ●, 100 μatm CO_2; ♦, 750 μatm CO_2.

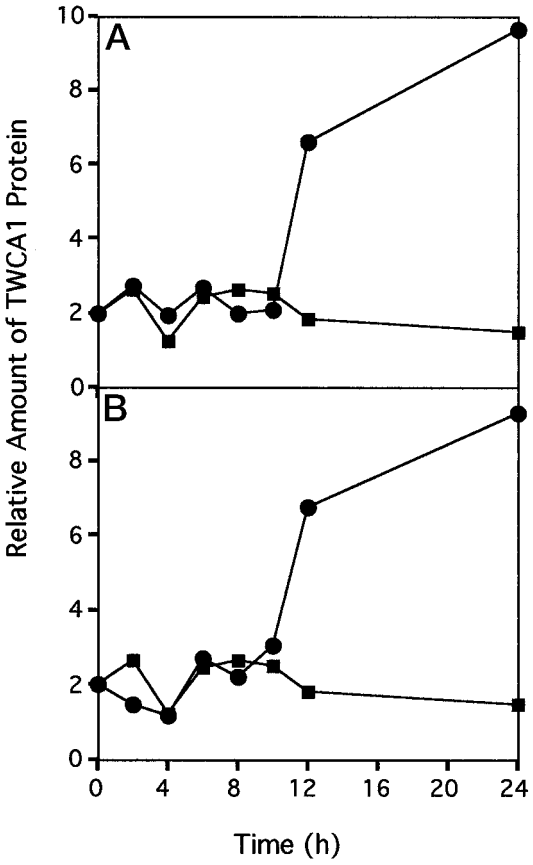

Figure 5. Time course of TWCA1 induction in response to addition of Zn (A) or Co (B). Cultures were preadapted to 100 μatm CO_2 and 3 pM Zn′. At t = 0, experimental Zn-EDTA was added to a final Zn′ of 15 pM and samples were withdrawn at the times indicated. Relative amounts of TWCA1 were determined by western analysis and phosphor imaging. In A: ●, Zn added, and ■, Zn limited; in B: ●, Co added, and ■, Zn limited.

Diel Cycle Regulation

Cells were grown at 100 μatm CO_2 on a 12-h light/dark cycle for six generations prior to the start of the experiments. Cell division was initiated during the last third of the light phase and continued for the first third of the dark phase. The amount of TWCA1 protein per unit culture volume steadily increased during the light phase until cell division was initiated (Fig. 6A). During the portion of the light phase in which cell division was occurring, the amount of TWCA1 protein per unit of culture volume remained constant and the amount of TWCA1 per cell decreased accordingly (Fig. 6B). The mean TWCA1 content per culture volume of the samples taken during

the dark phase (excluding the low point marked with a "?") was 15% lower than the final samples taken in the light phase (significant at the 95% confidence level). TWCA1 protein synthesis decreased or the rate of degradation increased early in the dark phase, and the levels of TWCA1 were stable for the remaining 8 h of the dark phase. This reduction in the amount of TWCA1 was much less dramatic than that brought about by an increase in CO_2 (Fig. 4), with which the amount of TWCA1 protein decreased by 75% over a 12-h time interval.

Northern Analysis

To determine if the regulation of induction of TWCA1 by CO_2 availability is at the level of mRNA abundance, we examined by northern analysis the effect of a CO_2 shift on the amount of *twca1* transcript. Parallel Zn-sufficient cultures were grown at 750 μatm CO_2. A t = 0, one culture was shifted to 100 μatm CO_2 and incubation was continued for 3 h (Fig. 7). Comparing the relative amounts of radiolabel (as quantified by a phosphor imager) in uninduced (Fig. 7, lane A) versus induced cultures (Fig 7, lane B), the increase in *twca1* mRNA levels in the culture shifted to low CO_2 was approximately 5-fold. Replicate gel samples that were stained with ethidium bromide (Fig. 7, lanes C and D) clearly indicated that the amount of material loaded was roughly equivalent and could not account for the difference observed in mRNA levels.

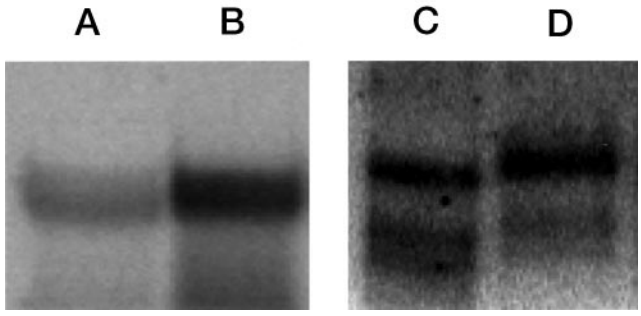

Figure 7. A and B, Phosphor image of a northern blot of total RNA probed with ^{32}P-labeled antisense *twca1* mRNA. Cultures were initially grown at 750 μatm CO_2. At t = 0, the induced culture was shifted to 100 μatm CO_2. After 3 h, cells were harvested. For both the uninduced (A) and induced (B) cultures, equal amounts of total RNA (30 μg) was loaded on the gel. There is a 5-fold increase in *twca1* mRNA levels (as determined by phosphor imaging) in the 100 versus the 750 μatm CO_2 sample. C and D, Negative image of ethidium bromide staining of duplicate samples loaded on the same gel. C, Uninduced; D, induced.

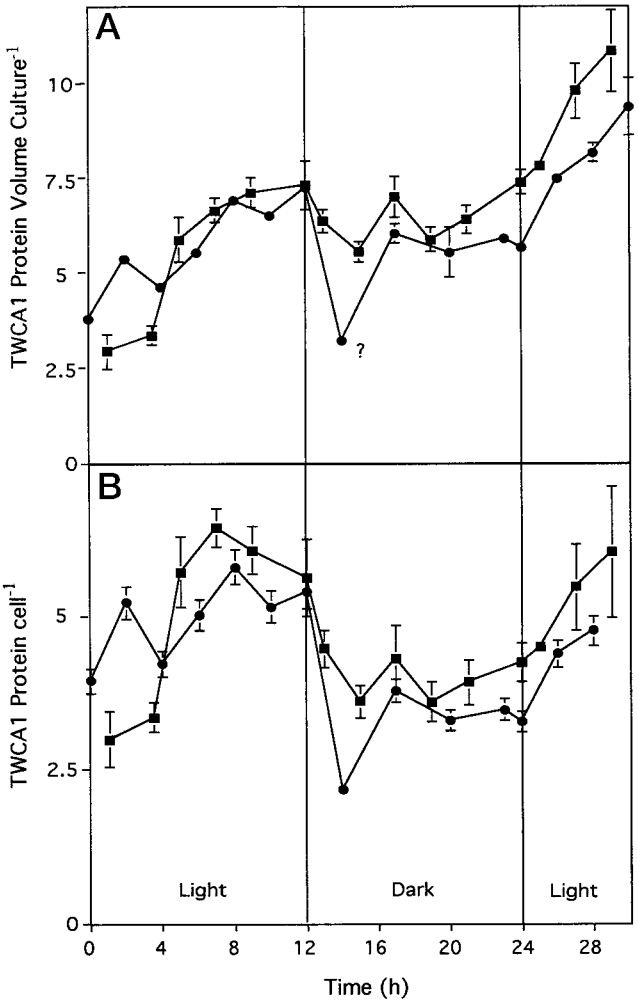

Figure 6. Time course of TWCA1 regulation by the diel cycle. Relative amounts of TWCA1 were determined by western analysis and phosphorimaging. A, Relative amounts of TWCA1 per unit culture volume B, Relative amounts of TWCA1 per cell. Each line represents data from an independent culture. ●, Experiment 1; ■, experiment 2.

DISCUSSION

The range of CO_2 concentrations used in this study is consistent with the roughly 100 to 700 μatm range reported for CO_2 in coastal waters (Kemp and Pegler, 1991; Frankignoulle et al., 1996; Boehme et al., 1998). Over this range, there is not a significant change in total inorganic carbon in seawater, indicating that TWCA1 is indeed modulated by CO_2 and not by total C. The Zn' concentrations we used are based on values reported by Bruland (1989) for the North Pacific Ocean.

We previously reported (Roberts et al., 1997) that TWCA1 protein is induced by low CO_2. We have now quantified this effect by western analysis and shown that the steady-state levels of TWCA1 protein are 10-fold greater in cultures grown at 100 than at 750 μatm CO_2. This is approximately the same magnitude of induction as seen when *Chlamydomonas reinhardtii* is shifted from 50,000 to 350 μatm CO_2 (Sültemeyer et al., 1990). In *T. weissflogii*, however, this difference is seen over a much narrower range of CO_2

concentrations. Induction of an internal CA at low CO_2 is consistent with active uptake of HCO_3^-.

The time required for *T. weissflogii* to adapt to decreased CO_2 is significantly longer than that reported for chlorophytes. An increase in the amount of TWCA1 protein present in the cells was not detected before 5 h, and steady-state levels of the protein were achieved only after 18 h. In *C. reinhardtii,* induction kinetics have been characterized for CAH1, a periplasmic CA. The CAH1 polypeptide can be detected 2 h after induction (Yang et al., 1985; Dionisio-Sese et al., 1990), and steady-state levels of CA are reached 4 to 6 h after induction. Similar rapid kinetics of CA induction in response to a CO_2 shift have been reported for *Chlorella* and *Dunaliella* spp. (Sültemeyer, 1997).

T. weissflogii responded rapidly to an increase in CO_2. A significant decrease in TWCA1 protein was detected within 2 h of an increase in CO_2, and a 75% reduction in TWCA1 levels was attained within 12 h. In *C. reinhardtii*, the periplasmic CAH1 enzyme remained stable after an increase in CO_2, but the mitochondrial form of the enzyme, MCA1, was rapidly degraded under similar conditions (Eriksson et al., 1998). TWCA1 was isolated from the soluble fraction of cell lysates. It seems likely that at high CO_2 concentrations, inappropriate internal CA activity would be more deleterious to the cell, and thus more likely to be targeted by regulated degradation than an external CA activity.

Co appears to function in essentially the same manner as Zn in TWCA1 regulation and activity. Co-containing cultures are virtually indistinguishable from Zn-sufficient cultures in both their production of TWCA1 protein and their growth rates. Co-containing cultures also displayed the same level of CA activity in cell lysates as those containing Zn. In contrast, when Co is substituted in vitro for Zn in α-CA, a significant decrease in activity is observed (Tu and Silverman, 1985). Our results are in agreement with previous results (Morel et al., 1994) showing that in ^{57}Co-labeled cells, the radioactivity co-migrated with a band of CA activity in a native gel. This band of activity was shown by Roberts et al. (1997) to be TWCA1. Furthermore, Yee and Morel (1996), using a standard affinity chromatographic method for the purification of CAs, demonstrated that both CA activity and ^{57}Co label co-purified. There was thus little doubt that a Co-CA could be found in *T. weissflogii*, and it seemed highly probable that this protein was indeed TWCA1. Our western analysis strongly affirms this conclusion, because antiserum raised against TWCA1 recognizes an identical set of bands on blots of total cellular protein from cultures grown with either Zn or Co. TWCA1 has little similarity to any of the previously described forms of CA (Roberts et al., 1997), and may represent a form of the enzyme that evolved around the ability to accept either Zn or Co at its active site. Western analysis of several diatom, chlorophyte, and haptophyte strains indicates that TWCA1 may represent a form of CA common in and limited to diatoms (T.W. Lane and F.M.M. Morel, unpublished data).

The responses of Zn/Co/CO_2 co-limited cultures to Zn or Co addition were essentially identical to each other and significantly delayed compared with the response to a shift in CO_2 concentration. Rapid increase in the amount of TWCA1 protein was not seen until 12 h after metal addition, whereas it was seen 5 h after a drop in CO_2. The growth rate of the Zn-limited *T. weissflogii* cultures at low CO_2 was about 5-fold lower than that of Zn-sufficient cultures. Therefore, the delay in TWCA1 increase in response to metal addition likely reflects the generally slower metabolism in metal-limited cells.

In *T. weissflogii* cultures grown on a light-dark cycle, there was an initial 15% decrease in the amount of TWCA1 during the dark phase, which was not due to dilution by cell division. This decrease is much lower than the 75% decrease observed 12 h after a shift from 100 to 750 μatm CO_2, and brings up the question of the mechanism of regulation of TWCA1. If this regulation was normally affected in response to CO_2 demand, one would expect complete degradation of TWCA1 during the dark phase. It may be that the major regulator of TWCA1 responds to internal CO_2 concentration (or some closely related parameter), and that, during the dark phase, respiration results in elevated internal CO_2 levels and a slight decrease in TWCA1 protein levels. The fate of the Zn associated with degraded CA is of interest because TWCA1 accounts for a large fraction of the intracellular Zn concentration in *T. weissflogii,* which is known to be regulated (Ahner and Morel, 1995). During the dark phase, the liberated Zn may be used by another metalloenzyme or it may be stored in some unknown peptide.

Largely due to the lack of a system for the genetic manipulation of diatoms, there is as yet no direct evidence for the role of TWCA1 in the CCM of diatoms. However, the data presented here provide strong circumstantial evidence for such a role. Zn limitation results in both low growth rates and low cellular levels of TWCA1. This limitation by Zn could be reversed by the addition of Co, a metal with few known functions in eukaryotes other than vitamin B12, and which we have shown can substitute for Zn in TWCA1. Thus, it is clear that the low growth rate observed under Zn limitation is at least partly the result of low cellular concentrations of TWCA1. Since the effect of Zn limitation can also be alleviated by increasing CO_2, it would appear that the major function of TWCA1 is in the acquisition of carbon for photosynthesis. We note further that CA inhibitors have been shown to be effective in reducing very short-term (<10 s) carbon fixation in *T. weissflogii* (Tortell et al., 1997).

Northern analysis clearly demonstrates modulation of *twca1* mRNA abundance by CO_2, and this may indicate the level at which TWCA1 expression is regulated by CO_2. The kinetics of TWCA1 accumulation upon induction by a decrease in CO_2 is in good agreement with regulation at the level of transcript abundance rather than at the level of translation. Because the measurements of TWCA1 levels by western analysis do not distinguish between the apoenzyme and the holoenzyme, the variations in protein levels with varying Zn or Co concentrations and the kinetics of response to metal addition also argue that the regulation by trace metal availability is at the level of transcript abundance.

TWCA1, likely a central element of the CCM, represents a diatom-specific form of CA. It is the largest fraction of soluble Zn in *T. weissflogii*, and, as such, it constitutes a major use of the cell's potentially limited metal resources (Morel et al., 1994). The expression of TWCA1 appears to be tightly regulated over the range of CO_2 and trace metal concentrations that the organism is likely to encounter in the natural environment. The ability of Co to substitute effectively for Zn at the active site of the enzyme is reflected in the regulation of its expression. Elucidating the regulation of CA activity in diatoms in response to environmental conditions should help us understand how these organisms acquire inorganic carbon and effect a major fraction of oceanic primary production.

MATERIALS AND METHODS

Culture Conditions

Thalassiosira weissflogii (clone Actin) cultures were grown at 20°C and 1 mmol photons m^{-2} s^{-1} in modified Aquil growth medium (Price et al., 1988/1989): 2.2 mM dissolved inorganic carbon and 300 μM NO_3^-, pH 8.2, with the Co and Zn levels as indicated in each individual experiment. Inorganic trace metal concentrations (M′) were calculated from total metal concentrations using the computer program MINEQL (Westall et al., 1976). Zn-Limited stock cultures were grown at 3 pM Zn′ for six generations prior to the inoculation of experimental cultures. Experimental cultures were grown under the appropriate conditions for five or more generations and harvested in the early exponential phase (approximately 25,000 cells mL^{-1}). Gas mixtures were prepared in air with the designated concentrations of CO_2. Cell concentrations were followed using a particle counter (Multisizer II, Coulter, Hialeah, FL).

CA Assays

CA assays were carried out by the method of Sületemeyer (1997) in 25 mM veronal buffer.

Western Analysis

SDS-PAGE and western analysis were carried out by standard methods (Sambrook et al., 1989) using high-titer polyclonal antiserum from rabbits directed against TWCA1 (Roberts et al., 1997) and [^{125}I]protein A (NEN Life Sciences, Boston). Relative amounts of radioactivity were determined with a phosphor imager (Molecular Dynamics, Sunnyvale, CA). In time course experiments, phosphor imager values were normalized to the endpoint sample with the highest value: the final sample in induction experiments and the first sample in degradation experiments. Samples were harvested by filtration, pelleted by centrifugation, resuspended in SDS-PAGE sample buffer, and stored at −70°C.

Northern Analysis

Parallel cultures were grown with 15 pM Zn′ at 750 μatm CO_2. When cell densities reached 50,000 cells mL^{-1}, one culture was shifted to 100 μatm CO_2. Incubation was continued for an additional 3 h, during which time cells were harvested by filtration, resuspended in TriReagent (Sigma, St. Louis), and stored at −70°C. Total cellular RNA was isolated by the method of Chomcyznski and Sacchi (1987) using TriReagent. RNA was electrophoresed in a formaldehyde-containing denaturing agarose gel (Sambrook et al., 1989) and transferred to a nylon membrane using a vacuum blotter (Stratagene, La Jolla, CA) and following the instructions of the manufacturer. The prehybridization, hybridization, and washing steps were carried out at 65°C using a reagent kit (Northern Max, Ambion, Austin, TX). The blot was probed with a single-stranded antisense RNA probe of the complete *twca1* cDNA sequence labeled with ^{32}P (NEN Life Sciences) using an in vitro transcription kit (MAXIscript, Ambion). The blot was washed at 65°C, and relative amounts of mRNA were determined by phosphor imaging.

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