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93. We thank H. Erdjument-Bromage of the Sloan-Kettering Microchemistry Facility for $NH_2$-terminal sequence and mass spectroscopic analyses, K.A. McAllister and R. Wiseman for the rat Brca2 cDNA clone, M. Wold for the RPA expression plasmid, the staff of the Cornell High Energy Synchrotron Source MacChess, of the National Synchrotron Light Source X9A and X4A beamlines, and of the Argonne National Laboratory Advanced Photon Source ID19 and ComCAT beamlines for help with data collection. This work was supported by the NIH, the Howard Hughes Medical Institute, the Dewitt Wallace Foundation, the Samuel and May Rudin Foundation, and the Arthur and Rochelle Belfer Foundation. Coordinates have been deposited in the RCSB Protein Data Bank (accession codes 1MIU, 1IYJ, and 1MJE).

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Figs. S1 to S3

17 July 2002; accepted 14 August 2002.

REPORTS

# A Proton Buffering Role for Silica in Diatoms

**Allen J. Milligan and François M. M. Morel***

**For 40 million years, diatoms have dominated the reverse weathering of silica on Earth. These photosynthetic protists take up dissolved silicic acid from the water and precipitate opaline silica to form their cell wall. We show that the biosilica of diatoms is an effective pH buffer, enabling the enzymatic conversion of bicarbonate to $CO_2$, an important step in inorganic carbon acquisition by these organisms. Because diatoms are responsible for one-quarter of global primary production and for a large fraction of the carbon exported to the deep sea, the global cycles of Si and C may be linked mechanistically.**

Aquatic protists of the class Bacillariophyceae (diatoms) have an absolute requirement for the element Si from which the cell wall, known as a frustule, is produced (*1*). Silicic acid is taken up by a specific transporter (*2*) and polymerized intracellularly within a specialized vesicle onto an organic matrix consisting of cationic polypeptides (*3*). After polymerization, the amorphous hydrated silica with a general formula of $Si_nO_{2n-x}(OH)_{2x}$ (where n and x are whole numbers) is moved to the exterior of the cell (*1*). Diatoms often dominate the phytoplankton assemblage in regions of high productivity where algal nutrients, including Si, are available. These nutrients are brought to surface waters by rivers, strong vertical mixing, or upwelling and allow diatoms to "bloom" because of their high intrinsic growth rates. Thus, it has been hypothesized that the precipitation of silica must somehow provide an ecological advantage to diatoms. Suggested functions for the siliceous cell wall include serving as an ultraviolet (UV) filter (*4*), as armor to protect against grazing by zooplankton (*5*), and as ballast to control water column position (*6*). It has also been suggested that it is energetically cheaper to construct a cell wall with silica rather than with organic carbon (*7*). But to date there has been no experimental demonstration of an actual physiological function for silica in diatoms or of its supposed advantage in cell wall formation.

Whereas dissolved nutrients in upwelled water are at concentrations that do not limit growth rates, the concentration of $CO_2$ in these waters is not necessarily saturating to photosynthesis (*8*). This is due to the poor affinity and specificity of the main carboxylating enzyme, ribulose-1,5-bisphosphate carboxylase-oxygenase (RubisCO), which, at the $CO_2$ and $O_2$ concentrations of seawater, is much undersaturated with $CO_2$ and catalyzes the oxygenation as well as the carboxylation of ribulose-1,5-bisphosphate (Rubp) (*9*). Many photosynthetic protists have overcome this limitation by using a carbon concentrating mechanism (CCM) that augments the $CO_2$ concentration in the vicinity of RubisCO (*10*). As part of their CCM, diatoms, like several other microalgae, possess an extracellular carbonic anhydrase (CA) that catalyzes the slow reaction (uncatalyzed half-life ~30 s) between bicarbonate ($HCO_3^-$) and $CO_2$ at their surface (*11*). The expression of this CA is regulated, increasing at low ambient $CO_2$ concentrations (*12*). Although the mechanism of the CCM of diatoms is not completely understood, there is general agreement that the activity of the external CA is an important part of it (*13*).

Forms of CA that have high catalytic rates ($k_{cat} > 10^4\ s^{-1}$) require a pH buffer to either provide or receive the proton involved in the reaction between $HCO_3^-$ and $CO_2$ because proton exchange with water is relatively slow (*14*, *15*). The proton transfer reaction between the pH buffer and the active site has been shown to be the rate-limiting step in catalysis (*16*). In seawater the principal natural buffers are, in order of importance, bicarbonate, borate, and silicate. The bicarbonate-carbonate system has been shown to be an effective buffer for some

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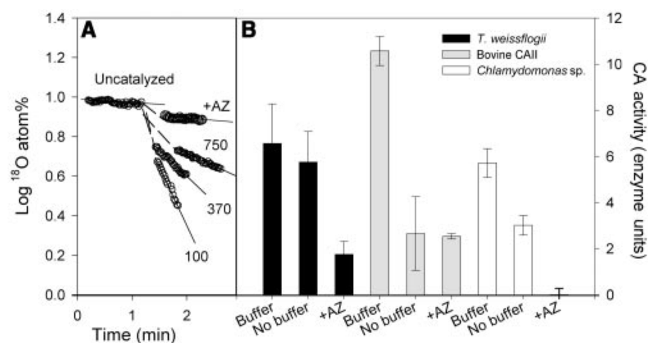


**Fig 1.** (**A**) Time course of $^{18}O$ loss from $^{13}C^{18}O_2$ (*19*). Uncatalyzed rate refers to the rate in the absence of cells. *T. weissflogii* cells grown under 100, 370, and 750 parts per million (ppm) $CO_2$ pressure ($pCO_2$) were added at 1.2 min (25 mM Hepes buffer, pH = 7.5). The initial slope (broken line) is the acceleration of the $^{18}O$ exchange rate from a combination of internal and external CA (data points not shown). The secondary slope (data points shown) represents acceleration from external CA alone (*19*). CA inhibited with 40 μM AZ (+AZ). (**B**) Buffer dependence of CA activity in whole cells and bovine CA II. Whole-cell activities represent external CA activity in *T. weissflogii* ($9.2 \times 10^5$ cells) and *Chlamydomonas* sp. ($4.7 \times 10^6$ cells) with and without 25 mM Hepes buffer (pH = 7.5) in 500 mM sorbitol. Bovine CA II (10 units) with and without 25 mM Hepes buffer in 510 mM NaCl. CA inhibited with 40 μM AZ (+AZ) in the presence of buffer is included for comparison. Enzyme units [U ± 95% confidence interval (CI); $n = 3$] represent the increase in the reaction rate compared with the uncatalyzed rate.

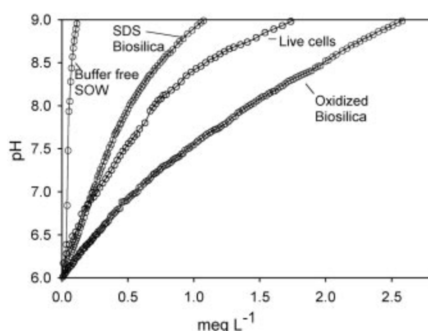


**Fig. 2.** Titration curves with 0.1 N NaOH of live cells and cleaned biosilica from *T. weissflogii*. Biosilica from $5.7 \times 10^7$ cells representing 45 mM Si in buffer-free synthetic ocean water (SOW) (15 ml). The titration of SOW is included for comparison.

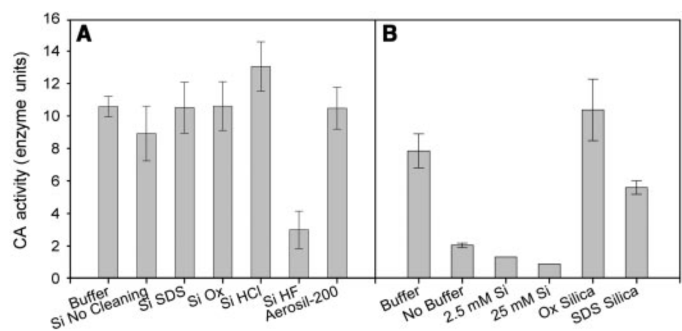


**Fig. 3.** (**A**) Activity assays for bovine CAII with the use of phosphate or variously treated silica as the buffer system: phosphate buffer (50 mM in 510 mM NaCl), biosilica rinsed with 510 mM NaCl (Si no cleaning), biosilica cleaned with 10% SDS (Si SDS), biosilica cleaned with 10% SDS and oxidized with perchloric acid (Si Ox), biosilica digested with hydrochloric acid (Si HCl), biosilica digested with hydrofluoric acid (Si HF), and synthetic amorphous silica (Aerosil-200). All Si treatments contained 25 mmol Si $l^{-1}$ in 510 mM NaCl, pH = 8.1. (**B**) Activity assays for internal CA purified from *T. weissflogii* with and without phosphate (50 mM), silicic acid (2.5 and 25 mM Si), SDS cleaned biosilica (Si = 10 to 13 mM), or oxidized biosilica (Si = 17 to 27 mM) as buffers (pH = 8.1) in 510 mM NaCl. Errors are 95% CI ($n = 3$, except for 2.5 mM and 25 mM silicic acid where $n = 1$).

CAs, but concentrations in the range of 150 to 200 mM are required for full activity (*17*), much higher than the 2 mM found in seawater. Boric and silicic acids with $pK_a$'s (where $K_a$ is the acid constant) in excess of 9.0 (compared with a seawater pH ~ 8.1) and concentrations less than 0.5 mM are not likely to be effective buffers for external CA either. However, polymerized silica is more acidic than silicic acid (*18*) and is present at a high concentration at the diatom surface. Therefore, we hypothesized that biosilica might serve as a buffer for the activity of the extracellular CA in diatoms.

To assay CA activity in the presence and absence of various buffers, we used membrane inlet mass spectrometry in which gases in solution phase pass through a permeable membrane and are measured in real time. This method allows one to follow the loss of oxygen from $CO_2$ and $HCO_3^-$ using triply labeled $^{13}C^{18}O_2$ (mass/charge ratio = 49) and equilibrating with unlabeled water (*19*). Figure 1A shows that before the addition of the marine diatom *Thalassiosira weissflogii* to a Hepes buffer solution (25 mM; pH = 7.5), there is slow exchange of $^{18}O$ with water. Addition of whole cells accelerates the reaction markedly in a manner that is dependent on the cells before exposure to partial pressures of $CO_2$. As expected for the activity of an extracellular CA, this catalysis is inhibited by the addition of the specific CA-inhibitor acetazolamide (AZ), which does not penetrate into cells.

If the silica cell wall serves as an effective buffer for the activity of the external CA of *T. weissflogii*, one should be able to observe catalysis of the reaction between $HCO_3^-$ and $CO_2$ in live cells suspended in unbuffered medium (*19*). As seen in Fig. 1B, removal of the Hepes buffer from the medium in suspensions of rinsed cells had no effect on the activity of the enzyme. The same experiment performed with either bovine CAII or live cells of the marine green alga *Chlamydomonas* sp., which has a glycoprotein wall and a well-characterized external CA, resulted in a depression of CA activity that illustrates the dependence of CA activity on a pH buffer (Fig. 1B). In all cases, AZ addition resulted in little to no activity. Thus, the cell wall of *T. weissflogii* maintains a completely efficient buffer for external CA activity, whereas the polysaccharides and other organic compounds in the cell wall of *Chlamydomonas* are insufficient to maintain full activity.

To assess the surface buffering capacity of biosilica, we obtained frustules from *T. weissflogii*, cleaned them of associated membranes using SDS detergent and, in some samples, oxidized away the organic coating using perchlorate and heat treatment (*19*). The resulting materials titrated with strong base exhibit buffering capacity over a range of pH relevant to aquatic systems (Fig. 2). Similar results were obtained for live cells titrated in the same manner. A difference in the buffer capacity between the two cleaning methods is observable and likely reflects the occupation by organic material of sites that are protonated in the oxidized sample. But in all cases, the proton exchange capacity of the biosilica is quite high: 0.2 equivalents (eq) per mol Si between the pH values of 8 and 9 in live cells.

We then tested diatom frustules for their ability to provide buffering to bovine CAII. Cells were broken up with a sonicator and were rinsed of cytoplasmic material with NaCl solution. Addition of bovine CA to this material resulted in full activity, demonstrating that a component of the diatom cell wall can indeed serve as a proton donor to the enzyme (Fig. 3A). To assess whether the responsible cell wall component is silica, we cleaned the cell wall material of organic matter. The process of removing bound membranes by detergent treatment and removing tightly bound organic material (mostly glycoproteins) (*20*) by oxidation did not hinder the ability of the frustules to provide buffering to CA. When we removed the silica from diatom cell walls with hydrofluoric acid (1 N) and retained the organic material with the use of a 3000 molecular weight (MW) cutoff filter,

the cell wall material no longer supported high CA activity. The loss of activity is not a result of acidification because material digested by 1 N HCl still supported full CA activity (Fig. 3A). The same experiment conducted with a synthetic amorphous silica (Aerosil-200), which has surface properties similar to biogenic silica (*21*), provided good buffering for CA as well. We conclude that it is the silica of diatom cell walls that is responsible for buffering rather than an organic component of the cell wall.

There is currently no protocol to purify the external CA of diatoms, so we purified the major internal CA (*22*) of *T. weissflogii* to determine the ability of biosilica to serve as a proton donor for this enzyme, which has no homology to other known CAs (*23*). The activity of this CA was high when either phosphate or biosilica was used as a buffer (Fig. 3B). Removing the buffer or using dissolved silicic acid as a buffer resulted in low catalytic rates. The lower rates observed in the SDS-cleaned frustules were a result of a lower biosilica concentration (about half the biosilica) added to the sample. The biosilica concentration was found to correlate well with CA activity (enzyme units = 0.37x mmol $l^{-1}$ Si + 1.8; $P < 0.05$, $r^2 = 0.87$, $n$ = 5 CA assays). The predicted CA activity in the absence of biosilica (1.8 enzyme units) agrees well with the observed value for incubations with no buffer (2.0 enzyme units). These results show that polymerized biosilica, unlike silicic acid, is an efficient buffer for diatom CA at the pH of seawater.

Absent a thorough understanding of the carbon acquisition systems of microalgae, it is difficult to assess the advantage that the buffering capacity of the siliceous cell wall might impart to diatoms. We note, however, that the large buffering capacity of polymerized silica would add to the energetic economy that may come from making the cell wall out of inorganic rather than organic material. This extracellular buffering may be useful for enzymatic functions other than that of CA. The necessity or usefulness of silicon in biological systems has been debated for a long time (*24*). Besides its obvious structural role, biogenic silica may well serve as a buffer in a variety of biochemical processes. It is conceivable, for example, that the documented but unexplained importance of silica in bone formation (*25*) may be linked to its role as a buffer.

In marine systems, diatoms play a major role in the export of organic carbon to the deep sea (*26*) and potentially influence the concentrations of $CO_2$ in the atmosphere (*27*). The last stages of the radiation of diatoms in the oceans and their dominance of the oceanic silica cycle occurred during the Eocene, about 40 million years ago (*28*). There is evidence that this is also the time when atmospheric $CO_2$ reached its low modern value (*29*) and organic carbon in sediments started to become enriched in $^{13}C$ (*30*). It is conceivable that all these phenomena are linked together mechanistically, not only because diatoms are efficient at exporting fixed $CO_2$ to the deep oceans but perhaps also because their siliceous cell wall makes them particularly efficient at acquiring inorganic carbon from solution.

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31. We thank T. Lane for helpful discussions regarding the experimental design and M. Bender and B. Barnett for access to and assistance with mass spectrometer measurements. K. Keller, P. N. Froelich, and two anonymous reviewers provided comments which improved the manuscript. Supported by the Center for Environmental Bioinorganic Chemistry funded by NSF and DOE.


**Supporting Online Material**

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Materials and Methods

11 June 2002; accepted 6 August 2002

# Water Diffusion and Clustering on Pd(111)

**T. Mitsui,[1] M. K. Rose,[2] E. Fomin,[2] D. F. Ogletree,[1] M. Salmeron[1]***

**The adsorption, diffusion, and clustering of water molecules on a Pd(111) surface were studied by scanning tunneling microscopy. At 40 kelvin, low-coverage water adsorbs in the form of isolated molecules, which diffuse by hopping to nearest neighbor sites. Upon collision, they form first dimers, then trimers, tetramers, and so on. The mobility of these species increased by several orders of magnitude when dimers, trimers, and tetramers formed, and decreased again when the cluster contained five or more molecules. Cyclic hexamers were found to be particularly stable. They grow with further exposure to form a commensurate hexagonal honeycomb structure relative to the Pd(111) substrate. These observations illustrate the change in relative strength between intermolecular hydrogen bonds and molecule-substrate bonds as a function of water cluster size, the key property that determines the wetting properties of materials.**

Intermolecular hydrogen bonds between water molecules can be comparable to or stronger than those formed between the molecule and many substrates (*1*, *2*). The relative strength of these two bonds determines the wetting properties of water. Fundamental questions regarding the adsorption of water on surfaces, from isolated molecules to clusters, complete layers, and beyond, are still unanswered. Cluster formation, including dimers and trimers at submonolayer coverage, has been analyzed by several groups using vibrational spectroscopies such as high-resolution electron energy loss spectroscopy (*3*–*7*), infrared absorption spectroscopy (*8*–*11*), and helium atom scattering (*12*). However, it is no

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