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Inorganic carbon uptake by an Antarctic sea-ice diatom, *Nitzschia frigida*

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Abstract There have been no studies to date on the mechanisms of inorganic carbon acquisition by Antarctic microalgae. Consequently, we have examined inorganic carbon (DIC) use in *Nitzschia frigida*, a diatom typical of the Antarctic bottom-ice community. The $K_{0.5}(CO_2)$ of photosynthesis in this organism was estimated to be 1.09 μM at pH 7.5. The internal concentration of DIC was approximately 4050 μM at an external [DIC] of 45 μM. At air-equilibration levels of inorganic carbon this would be sufficient for a ten-fold accumulation ratio of CO_2. Cells of *N. frigida* are capable of carbon-dependent photosynthesis at rates that exceed that expected from uncatalysed CO_2 supply to the cell. About 25% of the total carbonic anhydrase activity appears to be associated with the cell surface in *N. frigida*. These results support the hypothesis that *N. frigida*, like many microalgae from temperate waters, has an active carbon-concentrating mechanism, associated with the ability to utilize external HCO_3^- for photosynthesis.

Introduction

Algae colonising sea-ice habitats are thought to be responsible for a significant proportion of the carbon fixed in the ice-covered regions of the Southern Ocean (Garrison et al. 1986, Grossi et al. 1987). Despite their importance to ecological processes – at least in the in-shore areas of the Southern Ocean – the physiological aspects of carbon acquisition for photosynthesis by ice algae have received relatively little attention.

A range of microalgae and cyanobacteria have been shown to be able to make efficient use of low levels of dissolved inorganic carbon (DIC) when they are grown at air-equilibrated levels of CO_2 (Berry et al. 1976; Badger and Andrews 1982; Burns and Beardall 1987; Beardall 1989; Beardall and Raven 1990). This phenomenon is most frequently explained by the existence of an inducible carbon-concentrating mechanism (CCM) (Badger et al. 1980; Kaplan et al. 1980), which acts to maintain internal CO_2 concentrations higher than can be accounted for merely by diffusion-mediated entry (Raven 1985). The elevated CO_2 concentrations occur at the active site of ribulose 1,5-biphosphate carboxylase oxygenase (RUBISCO), thus increasing the $CO_2:O_2$ ratio and ensuring that the oxygenase activity of that enzyme is reduced or suppressed (Kerby and Raven 1985; Beardall 1989).

Most of the studies that have been conducted on the carbon-concentrating mechanism of algae have been restricted to temperate species. However, microalgae colonising Antarctic sea ice experience concentrations of O_2 considerably higher than do marine organisms from temperate or tropical latitudes. For example, the dissolved oxygen present in air-saturated seawater at -1.0°C may be 1.6 times greater than in the same seawater at 20°C. Furthermore, the uncatalysed rate of CO_2 supply from HCO_3^- and the diffusion of CO_2 in seawater are considerably less (by factors of nine-fold and two-fold respectively, Johnson 1982; Table 5.1 in Raven 1984) at 1.0°C than at 25°C. On the other hand, dissolved CO_2 in equilibrium with air is also higher at low temperatures and the air–equilibrium ratio of CO_2:O_2 is about 15% higher at 1°C than at 20°C (Skirrow 1975). These differences are likely to have an impact upon the maintenance of adequate carbon for photosynthesis. In the ecological context, the ability to use

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HCO_3^- and to accumulate inorganic carbon may be relatively more important in ice algae than in other microalgae from marine habitats. This study, therefore, examined carbon uptake in *Nitzschia frigida*, an Antarctic marine diatom characteristic of the Antarctic sub-ice assemblage.

Materials and methods

Algal culture

Nitzschia frigida was isolated from Davis Bay, Australian Antarctic Territory (68°35′S, 77°58′E) and was maintained in clonal culture on f/2-enriched seawater media (Guillard and Ryther 1962; McLachlan 1973) at a salinity of 35‰. Batch cultures (125 ml) were grown in 250-ml Ehrlenmeyer flasks and harvested in early- to mid-log phase. The low cell densities and relatively high surface area: volume meant that it was unlikely that the cultures deviated significantly from air-equilibrium. Cultures were grown under a continuous photoperiod with a photon flux of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a temperature of -1.5 ± 0.5°C.

Chlorophyll determinations

Pigments were extracted in cold 100% acetone. In order to ensure complete extraction, the cells were left in the dark at -15°C for at least 1 h, blended for 1 min at high speed in a Turrix blender and sonicated for 3 min. The pigment extract was adjusted to 90% acetone and total chlorophyll was calculated according to the equations of Jeffrey and Humphrey (1975).

Measurement of $K_{0.5}$ (CO_2)

Inorganic carbon-dependent production of O_2 was measured using a method modified from that described by Berry et al. (1976) and Badger et al. (1977). Culture material was centrifuged ($\times 1600\ g$ for 5 min) and resuspended in 2 ml CO_2-free media (see below), to a known concentration of approximately $1.0–2.0 \times 10^6 \cdot ml^{-1}$ (usually equivalent to 10–15 $\mu g\ chl \cdot ml^{-1}$).

This cell suspension was placed in the chamber of a Hansatech DW1 oxygen electrode and purged with N_2 until oxygen evolution ceased. Known amounts of $NaHCO_3$ were then added to the chamber and ensuing rates of DIC-dependent photosynthetic O_2 evolution determined. The experimental temperature was 4°C, and a photon flux of 120 $\mu mol\ quanta \cdot m^{-2} \cdot s^{-1}$ was supplied to the surface of the electrode chamber from a 200-W quartz halogen projector lamp. Although this photon flux could be considered high for ice algae, there was no evidence of photoinhibition.

Kinetic parameters of DIC-dependent O_2 evolution were estimated by fitting the data to the Michaelis-Menton equation using the non-linear regression module of SYSTAT.

The CO_2-free medium was prepared by autoclaving 100 ml f/2 media containing 50 mM HEPES (2-hydroxyethylpiperazine-2-ethanesulphonic acid). After autoclaving, the medium was titrated against fresh NaOH to achieve a pH of 7.5, purged with N_2 gas and kept in a sealed vessel until use. The pH used for these experiments was chosen to minimise the time taken to deplete the cells and medium of DIC. Aliquots of medium for experimental purposes were further purged with N_2 immediately prior to use.

Measurement of the internal inorganic carbon pool

Intracellular pools of inorganic carbon were determined by a silicone-oil centrifugation method based on that described by Werden et al. (1972) and Badger et al. (1980). Cells were harvested by centrifugation ($\times 1100\ g$ for 3 min) and resuspended in 3 ml of media. They were transferred to a 1.5-ml Eppendorf tube and centrifuged for 10 s in a Beckman Microfuge before being resuspended into CO_2-free media. DIC was added to 45 μM, approximately 0.1 of the air-equilibrated levels available in seawater at a pH of 7.5 and 4°C. The Eppendorf tubes and contents were chilled prior to use; the experiment was conducted on ice and the experimental temperature was estimated to be 4°C. Incubations were run over a time course up to 150 s.

The intracellular space and extracellular water taken through the silicone-oil layer were estimated by using [^3H_2O] and [^{3}H-dextran] respectively (Badger et al. 1977; Badger et al. 1980).

Internal pH was measured using the 5,5-dimethyl-[2-^{14}C] oxazolidine dione (DMO) method (Heldt et al. 1973; DeMichelis et al. 1979; Badger et al. 1980). The pKa for DMO was estimated to be 6.5 at 4°C and at the molarity of seawater used (Boron and Roos 1976). Calculated internal pH values were used to determine the internal concentration of free CO_2. The $pKa_{(out)}$ for carbonic acid was taken to be 6.116 (Skirrow 1975) and the $pKa_{(in)}$ of 6.276 was assumed by accepting arguments that Na^+ and other inorganic ions remain at a concentration less than 100 mM at all times within the cell and that organic solutes play a significant role in the osmoregulation of algae (Katz and Avron 1985; Raven 1976, Paul 1979; Brown and Hellebust 1980).

Species of carbon utilised by *Nitzschia frigida*

The carbon species utilised by *N. frigida* was determined by calculating the uncatalysed rate of CO_2 supply under the experimental conditions and comparing the availability of CO_2 with the rate of carbon-dependent oxygen evolution (Beardall 1985; Miller 1985). Rates of uncatalysed supply of CO_2 were calculated from the equations of Johnson (1982).

Carbonic anhydrase activity

Carbonic anhydrase (CA) activity was assayed as described by Miyachi et al. (1983) and modified from the method of Wilbur and Anderson (1948). Prior to the assay, cells were harvested by centrifugation ($\times 1500\ g$ for 5 min) and resuspended in 18 mM Veronal buffer, pH 8.3.

Intact cells were assayed for the presence of an external CA whilst total activity was determined from assays using cell homogenates (sonication at 20 W for 30 s using a Branson Sonifier-microscopic observation showed that cell breakage was 90% or greater). The reaction mixture was 3 ml Veronal buffer to which 0.4 ml cell suspension or cell homogenate and 2.0 ml CO_2-saturated water was added. Where necessary, 35 $g \cdot l^{-1}$ NaCl was used as an osmoticum.

The activity, in Wilbur Anderson (WA) units, of the suspension/sonicate was estimated using the equation

$$WA = 10[(t_0/t_x) - 1]$$

where t_0 and t_x represent the time taken for the pH of the control and test suspensions respectively to drop from 8.3 to 7.3 (Kimpel et a1. 1983; Shiraiwa and Miyachi 1983). The experiment was conducted on ice at 2°C.

The control consisted of the assay buffer to which 0.4 ml distilled water was added instead of the cell suspension.

Results

Kinetics of DIC-dependent photosynthetic O_2 evolution

The response of photosynthesis by *N. frigida* to DIC concentration is shown by the pooled data from three experiments in Fig. 1. The $K_{0.5}$(DIC), calculated from fitting the data to the Michaelis Menton equation by non-linear regression, was estimated to be 27.51 ± 3.88 µM. The $K_{0.5}(CO_2)$ was therefore calculated to be 1.09 ± 0.04 µM. The maxmimum photosynthetic rate was estimated to be 53.27 ± 2.18 µmol $O_2 \cdot (\text{mg Chl})^{-1} \cdot h^{-1}$.

Intracellular volume

Measurements of ^{3}H-dextran and ^3H_2O uptake resulted in estimates for the extracellular water and cell volume of 0.88 and 0.9787 $\mu l \cdot \mu g\ Chl^{-1}$ respectively. The high value for extracellular volume presumably reflects the large amount of extracellular mucilage produced by *N. frigida*.

Internal concentration of DIC

Cells of *N. frigida* showed accumulation of DIC. Internal DIC appeared to be at equilibrium within the first 10–20 s of incubation (data not shown). The estimated internal pH was 7.580 ± 0.003 (SE., $n = 4$). The average accumulation ratio for DIC ($[DIC]_{in}$: $[DIC]_{out}$) for this experiment was 90 ± 21 (SE., $n = 6$) and the concentration of total internal inorganic carbon was 4050 ± 945 µM. Table 1 also gives the estimates of internal CO_2 and accumulation ratios for CO_2 at the external [DIC] used, and, assuming the same internal [DIC] and pH, in air-equilibrated seawater.

Supply of dissolved inorganic carbon

Figure 2 shows the uncatalysed rate of CO_2 supply (*dotted line*) compared to rates of photosynthesis by concentrated cell suspensions (*symbols*, *solid line*). Clearly, the DIC-dependent rate of photosynthetic oxygen evolution by *N. frigida* is greater in this experiment than can be accounted for simply by the uncatalysed supply of CO_2 from HCO_3^- in the bulk medium. This implies that this organism is most likely to be able to utilise HCO_3^- from the bulk medium in addition to CO_2. This HCO_3^- use could be due either

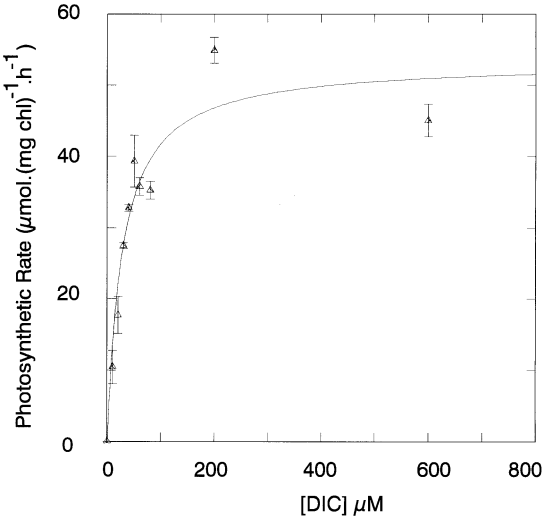

Fig. 1 Photosynthetic rate of *N. frigida* as a function of [DIC], mean of 3 experiments (*bars* = SE); external pH was 7.5, temperature was 4°C

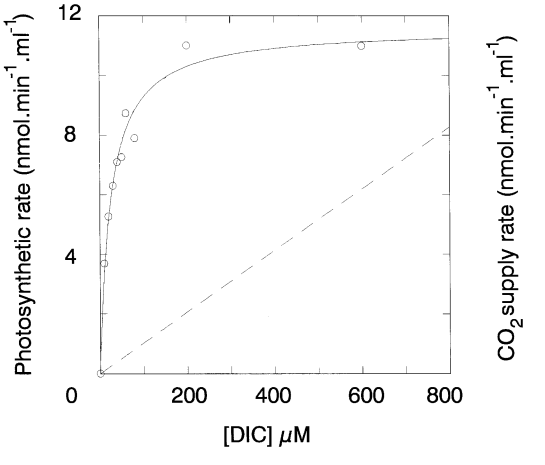

Fig. 2 Rates of oxygen evolution by a concentrated suspension of *N. frigida* (*circles*, *solid line*) and calculated rates of uncatalysed conversion of HCO_3^- to CO_2 (*dotted line*) as a function of [DIC]. External pH was 7.5, temperature was 4°C

Table 1 Internal and external pool sizes for DIC and CO_2 in *Nitzschia frigida*. Internal pH was 7.580 ± 0.003 and external pH was 7.5. Values are means ± standard errors ($n = 6$). Assay temperature was 4 °C

$[DIC]_{ext}$ (µM)	$[CO_2]_{ext}$ (µM)	$[DIC]_{int}$ (µM)	$[CO_2]_{int}$ (µM)	Accumulation ratio for DIC ($[DIC_{in}/[DIC]_{ext}$)	Accumulation ratio for CO_2 ($[CO_2]_{in}/[CO_2]_{ext}$)
45	1.79	4050 ± 945	192 ± 45	90 ± 21	104.7 ± 36.9
489	19.45	4050 ± 945[a]	192 ± 45[a]	8.3 ± 1.9[a]	9.9 ± 2.3[a]

[a] Calculations based on the assumption that under air equilibration the internal DIC pool also saturates at 4050 µM

Table 2 Carbonic anhydrase determinations for *Nitzschia frigida*. The errors quoted are standard errors of the mean ($n = 5$). There were 6.5×10^6 cells in the sample. Assay temperature was 4°C

Fraction	Carbonic anhydrase activity		
	WA units per 10^6 cells	WA units per μg chl	%Total activity
External	0.76 ± 0.11	0.123 ± 0.01	25.5
Internal	2.22 ± 0.03	0.36 ± 0.006	74.5

to HCO_3^- transport or to enhanced supply of CO_2 following the action of external carbonic anhydrase on bulk HCO_3^-.

Carbonic anhydrase

Table 2 shows the results of measurements of internal and external carbonic anhydrase activity in *N. frigida*. Cells contained nearly 3 WA units/10^6 cells. Assuming that the CA activity in the sonicate measured the total CA activity associated with the cells and that the "whole cell" data represent external CA activity, then approximately 25% of the CA in *N. frigida* was externally bound, or available at the surface of the cell.

Discussion

The data presented here are clearly consistent with the operation of a CCM in *N. frigida*. The value for $K_{0.5}(CO_2)$ falls within the range of 0.25 to 1.5 μM reported by Burns and Beardall (1987) for microalgae from temperate environments. Direct measurements of internal DIC concentrations at, admittedly, a low external concentration, confirm that *N. frigida* is capable of accumulating DIC to internal levels approximating 4 mM. This value is unlikely to be lower at higher external DIC (Beardall 1989, but see Raven 1991). Even at air-equilibration values of external CO_2 this would be sufficient to allow a nine-fold accumulation of CO_2 at the active site of RUBISCO with a consequently marked effect on the oxygenase activity of this enzyme and on photorespiration. The possession of a CCM may not be ubiquitous among microalgae or sufficiently active to result in DIC saturation of growth (Munoz and Merrett 1989; Riebesell et al. 1993). Given that the mechanism is energetically costly (Raven 1985) and repressible, there must be some ecological advantage for ice algae such as *N. frigida* to maintain a CCM under the low energy supply (low light) conditions in their natural environment.

Raven (1991) has argued that, provided air-equilibration of seawater can be maintained, the need for a CCM is decreased at low temperatures. Thus, the increased solubility of CO_2, increased $CO_2 : O_2$ ratio and increased pKa tend to increase the availability of CO_2 under such conditions. Furthermore, in some species the kinetic behaviour of RUBISCO changes in such a way that the $K_{0.5}(CO_2)$ decreases at low temperatures (Davison 1987; Descolas-Gros and DeBilly 1987), again decreasing the necessity for active transport of DIC to maintain the supply of CO_2 to the RUBISCO-active site. A lowered CCM activity is also consistent with observed differences in carbon isotope discrimination between temperate and polar phytoplankton (Raven 1990; Raven 1991; Rau et al. 1989).

However, polar waters frequently have dissolved CO_2 concentrations less than air equilibration (Takahashi 1989; Rau et al. 1989). This phenomenon could be more marked in the under-ice communities in which *N. frigida* is found, where the presence of ice cover would prevent equilibration of seawater with the atmosphere. This would be exacerbated by the decrease in the CO_2 diffusion coefficient at low temperatures (Raven 1991) and the copious mucilage production by *N. frigida*. The possession of mucilage would also possibly lead to increases in dissolved oxygen concentration surrounding the cells and a higher $O_2 : CO_2$ ratio. Oxygen concentrations under ice have been shown, in some situations, to increase to levels up to 3.7 times greater than air equilibrium values (Parker et al. 1982). Furthermore, although the affinity of RUBISCO for CO_2 (and for RuP_2) does generally increase with lower temperatures, it has been reported that in the Antarctic microalga *Nitzchia turgiduloides*, $K_{0.5}(RuP_2)$ was actually higher (affinity lower) at 1°C (close to the normal environmental temperature for that species) than at 3°C and 30°C (Descolas-Gros and DeBilly 1987).

If CO_2 were, then, to be potentially limiting to *N. frigida*, the ability to make use of the high HCO_3^- pools present in seawater would confer an ecological advantage. Given the lower rate of uncatalysed conversion of HCO_3^- to CO_2 and low rates of diffusion of CO_2 at low temperatures, the presence of an external carbonic anhydrase to improve CO_2 supply to the cell surface could be important. CA activities in this study were high compared to those of a number of temperate microalgae (Burns and Beardall 1987). It is evident that *N. frigida* can make use of HCO_3^- from the bulk medium and it is suggested that this is achieved by the presence of the relatively high levels of externally accessible carbonic anhydrase found in this organism. Such a mechanism for DIC acquisition appears to be common in temperate microalgae (Beardall 1989; Booth and Beardall 1991).

Whether the presence of an active CCM is a feature of polar microalgae as a whole or whether it is restricted, for the reasons described above, to organisms characteristic of the under-ice communities, remains to be determined.

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