Energetic aspects of CO₂ uptake in *Thiobacillus neapolitanus*

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Abstract. Uptake of inorganic carbon (C_i) in the form of CO₂ and/or HCO₃⁻ was studied in the chemolithoautotroph *Thiobacillus neapolitanus* under energy (thiosulphate) or carbon (CO₂) limitation. Uptake of C_i was found to be a metabolic energy dependent process since in the presence of uncouplers no uptake was observed. The accumulation level of C_i was higher in the CO₂-limited cells (1000- to 1500-fold) in comparison to the thiosulphate-limited cells (500- to 800-fold). The process of uptake could be influenced by addition of ionophores. Inhibition of uptake and accumulation of C_i was found after addition of valinomycin which completely dissipated the electrical potential ($\Delta\psi$). After addition of nigericin an increase in the uptake and accumulation of C_i was observed with a concomitant increase of the $\Delta\psi$. These results suggest that the $\Delta\psi$ is the main driving force for uptake of C_i. However, uptake of C_i could never be found in the absence of electron transfer, or in cells in which the electron transfer chain was blocked by potassium cyanide. Electron transfer therefore appears to be an additional requirement for C_i uptake. Kinetic experiments on the uptake of inorganic carbon at different pH values suggest that CO₂ is the carbon species taken up by *T. neapolitanus*.

Key words: Electrochemical proton gradient – $\Delta\psi$ – Electron transfer – CO₂ uptake – *Thiobacillus neapolitanus*

Transport of CO₂ or HCO₃⁻ across the cytoplasmic membrane or plasmalemma of many green algae, cyanobacteria and green plants has been reported (Badger and Andrews 1982; Kaplan et al. 1980; Miller and Colman 1980; Lucas 1983; Spalding and Ogren 1983). These organisms can use CO₂ as the only carbon source for growth and fix CO₂ with the first enzyme of the Calvin cycle, ribulose-1,5-bisphosphate carboxylase (Cooper et al. 1969).

Extensive studies have been performed on the mechanism of uptake of inorganic carbon by the cells. Many aquatic plants and algae are capable of taking up both CO₂ and HCO₃⁻ (Lucas 1983; Prins et al. 1982) while in some algae (Beardall and Raven 1981; Lucas et al. 1983) and cyanobacteria (Miller and Colman 1980; Badger and

Andrews 1982; Kaplan et al. 1984) only one of these carbon species can cross the membrane. The mechanism(s) of transport of both CO₂ and HCO₃⁻ in cyanobacteria and green algae is not entirely understood. Badger et al. (1980) and Beardall and Raven (1981) reported a "CO₂-concentrating mechanism" in *Chlamydomonas reinhardtii* and *Chlorella emersonii*, respectively. For the uptake of HCO₃⁻ a carrier-mediated facilitated diffusion process is considered (Miller and Colman 1980; Kaplan et al. 1984; Volokita et al. 1984). Two mechanisms of this energy requiring process have been proposed: a primary electrogenic pump mechanism (Kaplan et al. 1984) and a secondary transport system driven by one or both components of the proton motive force (Miller and Colman 1980).

In this paper we report on the mechanism of uptake of inorganic carbon by the bacterium *Thiobacillus neapolitanus*. This obligate chemolithotrophic organism responds to changes in the external concentration of CO₂ by altering the concentration of the first enzyme of the Calvin cycle for autotrophic CO₂ fixation, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Under CO₂-limiting conditions the level of RuBisCO increased 3- to 5-fold, and most of this enzyme is packed in tiny bodies, the carboxysomes. At excess supply of CO₂ relatively little particulate bound, i. e. carboxysomal, ribulose-1,5-bisphosphate carboxylase is found (Beudeker et al. 1980). The data presented in this paper indicate that transport of CO₂ across the cytoplasmic membrane is mediated by a saturable transport system which is driven by the proton motive force and requires electron transfer in the respiratory system.

Materials and methods

Organism and growth conditions

Thiobacillus neapolitanus (strain X) as grown in continuous culture at 28°C at pH 6.8 as described by Kuenen and Veldkamp (1973). The medium contained in demineralized water 2.8 mM K₂HPO₄, 3.7 mM KH₂PO₄, 7.5 mM NH₄Cl, 3.2 mM MgSO₄ · 7 H₂O, 40.3 mM Na₂S₂O₃ · 5 H₂O and 2 ml per litre of a trace element mixture (Vishniac and Santer 1975). Air was continuously led through the culture. The oxygen concentration was monitored with an oxygen electrode and controlled automatically at 50% air saturation by varying the stirring rate. The pH of the culture was controlled by a pH electrode and maintained at pH 6.8 by the addition of 1 M Na₂CO₃. CO₂ limiting conditions were obtained by bubbling the inflowing air through soda lime and supplying the desired quantities of CO₂ (as a 40 mM

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Abbreviations. RuBisCO, ribulose-1,5-bisphosphate carboxylase; DCCD, N,N'-dicyclohexylcarbodiimide; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; FCCP, carbonyl cyanide p-trifluoromethoxyphenyl hydrazone; EDTA, sodium ethylene diamine tetraacetate

NaHCO₃ solution) via a separate pump. A solution of 1 M NaOH was used to reset the pH. The culture liquid removed from the chemostat was stored up to 3 days at 4°C.

Pretreatment of the cells

Cells were harvested by centrifugation (10,000 × *g* for 10 min at 4°C), washed once in 25 mM potassium phosphate (pH 7.0) and resuspended in this buffer to a final protein concentration of 5–10 mg protein/ml. If required, N,N'-dicyclohexylcarbodiimide (DCCD), nigericin or valinomycin were added before the cells were stored at 4°C.

In Gram-negative bacteria sodium-ethylene diamine tetraacetate (EDTA) is commonly used to make the outer membrane permeable for the ionophores. However, EDTA itself appeared to decrease the uptake of inorganic carbon (C_i) drastically in *T. neapolitanus* and was therefore omitted.

Assay of CO₂ uptake

Uptake studies of CO₂/HCO₃⁻ were carried out at 28°C in glass test tubes with the filtration technique as described by Konings (1979). The continuously stirred reaction mixture (100 µl) contained 25 mM potassium phosphate (air saturated), buffered at the required pH. One minute prior to the start of the experiment 1 µl cell suspension (5–10 mg protein/ml) was added to the reaction mixture. After 30 s 1 µl of the electron donor Na₂S₂O₃ · 5 H₂O (2M) was added. The uptake experiment was started by the addition of radioactively labeled substrate (2 µl NaH¹⁴CO₃, 3.7 MBq/mmol) and non-labeled substrate (1 µl 30 mM NaHCO₃) simultaneously. The reaction was stopped by addition of 2 ml of a solution containing 0.1 M LiCl and 0.1 M NaOH. The suspension was filtrated immediately over prewetted (with 0.1 M LiCl plus 0.1 M NaOH) cellulose acetate filters (Schleicher and Schüll, Dassel, FRG, pore size 0.46 nm); the glass tube and filter were washed once with 2 ml 0.1 M LiCl – 0.1 M NaOH solution. Filters were placed in counting vials containing a thoroughly stirred mixture of 4 ml hydroluma (Baker Chemicals, Deventer, The Netherlands) and 200 µl carbosorb (Packard, Downers Grove, IL, USA). The whole filtration procedure was performed in 5 to 7 s.

To determine the concentration of inorganic ¹⁴C present in the incubation mixture during the experiment the radioactivity was followed in parallel series of uptake experiments. Carbo-sorb (200 µl) was added to parallel series of tubes at the same experimental time intervals as in the uptake experiments. The mixture was removed and transferred to a counting vial. Each glass tube was washed twice with 2 ml hydroluma and this solution was also added to the counting vial. Quenching of the radioactive label due to the addition of carbosorb was not observed. Uptake of inorganic carbon was also determined in cells in which a potassium diffusion potential was induced. This artificial electrical potential was generated by diluting cells, suspended at 60 mg protein/ml in 100 mM potassium phosphate, pH 7.0 (plus 40 µM valinomycin), hundredfold into 100 mM sodium phosphate pH 7.0 plus HCO₃⁻/H¹⁴CO₃⁻ (same concentration as above). A control sample was diluted with 100 mM potassium phosphate pH 7.0 instead of sodium phosphate with the same concentrations HCO₃⁻/H¹⁴CO₃.

Assay of CO₂ fixation

The amount of inorganic carbon (C_i) fixed into organic material by the cells was determined from the non-volatile

products of C_i fixation. The filtration technique described above was used, after which the filters were placed in counting vials containing 200 µl 98% acetic acid. The vials were placed for 20 min in a thermostatically controlled waterbath at 60°C. After cooling to room temperature 4 ml hydroluma and 200 µl carbosorb were added.

Measurements of the electrical potential (ΔΨ)

The ΔΨ was measured with an ion-selective tetraphenylphosphonium (TPP⁺)-electrode as described by Elferink et al. (1983) inserted in a screwcapped 3 ml vessel in which a cell suspension (0.09 mg protein/ml) in 25 mM potassium phosphate (pH 7.0) was stirred. Simultaneously oxygen consumption could be measured with a Clark-type oxygen electrode which was also inserted in the vessel. Before the start of the experiment the buffer was air-saturated. Subsequently the vessel was closed with the screw-cap to prevent entrance of oxygen by diffusion.

Measurements of the pH gradient (ΔpH)

ΔpH was measured using the automated flow-dialysis technique as described by Hellingwerf and Konings (1980), with the silicon oil centrifugation technique with benzoic acid as probe as described by ten Brink and Konings (1982) or with an ion-selective salicylate electrode as described by Hellingwerf and van Hoorn (1985). In order to detect a possible ΔpH, inside acid, the filtration technique as described by Konings (1979) with (¹⁴C)-methylamine as distribution probe was used.

ATP synthesis

ATP synthesis of a 1 ml cell suspension (0.1–0.15 mg protein/ml) in 25 mM potassium phosphate (pH 7.0) was followed at 30°C. The well-stirred cell suspension was incubated for 2 min prior to the addition of Na-thiosulphate (20 mM). The reaction was stopped after 2 min by adding 1 ml 14% (w/w) HClO₄ plus 9 mM EDTA. The solution was neutralized with 1 N KOH/KHCO₃ and centrifuged. The supernatant was kept at 4°C and the ATP contents were measured the same day by the luciferine-luciferase assay as described by Melandri et al. (1980).

Oxygen consumption

The oxygen consumption was measured polarographically in 3 ml reaction mixtures at 28°C as described by Matin and Konings (1973).

Protein

Protein was determined according to the method of Lowry et al. (1951).

Chemicals

NaH¹⁴CO₃ (58.5 Ci/mol, 5 mCi/ml) and (¹⁴C)-methylamine HCl (40–60 Ci/mol) were obtained from the Radiochemical Centre (Amersham, Buckinghamshire, UK). All other chemicals were of analytical grade.

Fig. 1. Inorganic carbon uptake in washed, whole cell suspensions of *T. neapolitanus*, pregrown in the chemostat under thiosulphate limitation, in the presence and absence of KCN. Addition of cells at $t = -1$ min, of thiosulphate at $t = -30$ s and $\text{H}^{14}\text{CO}_3^-$ at $t = 0$. In the presence of thiosulphate: (●) total carbon taken up; (○) inorganic total carbon minus fixed carbon; (▼) fixed carbon; (□) total carbon taken up in the presence of $1 \mu\text{M}$ KCN. (*) Uptake of carbon in the absence of thiosulphate

Fig. 2. Uptake and fixation of inorganic carbon in washed cell suspensions of *T. neapolitanus*, pregrown under thiosulphate (●, ▼) and CO_2 (○, ▽) limitation. (●, ○) Total inorganic carbon taken up, (▼, ▽) fixed carbon

Results

Energy-dependent uptake of inorganic carbon

^{14}C labeled bicarbonate was added to washed suspensions of *Thiobacillus neapolitanus* precultivated in continuous culture under thiosulphate limitation. Radioactive label was rapidly taken up and accumulated by the cells (Fig. 1). This uptake of inorganic carbon (C_i) occurred only in energized cells. In the absence of the electron donor thiosulphate no label was found in the cells. In the energized cells high accumulation ratios of inorganic carbon (between 500- and 800-fold) were found. This accumulation was not due to high fixation levels, as is shown in Fig. 1. After 2 min only 12% of the accumulated C_i was incorporated in cell material. In CO_2 -limited cells a much higher fraction (approx. 45%) of the

Fig. 3. Uptake of inorganic carbon by *T. neapolitanus* energized by thiosulphate (pH 7.0) in the presence of valinomycin ($15 \mu\text{M}$), nigericin ($5 \mu\text{M}$), CCCP ($10 \mu\text{M}$; or $10 \mu\text{M}$ FCCP, $10 \mu\text{M}$ S_{13} or $10 \mu\text{M}$ SF_{6847}). Total inorganic uptake with (■) nigericin, (▲) valinomycin, (□) CCCP, (●) no addition, and (▼) fixed carbon

internal C_i was present as fixed material (Fig. 2). In these cells the accumulation ratios of C_i were also higher (1000- to 1500-fold). Cells grown under thiosulphate-limited conditions were further used in this study to reduce fixation of C_i to a minimum and to be able to measure the accumulated free C_i accurately.

To investigate whether the uptake of C_i by *T. neapolitanus* was indeed a metabolic-energy requiring process the effects of uncouplers on the uptake of C_i were determined. Uptake of C_i by cells in the presence of thiosulphate was fully inhibited by the uncouplers carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP), S_{13} or SF_{6847} (Fig. 3). These results show that $\text{CO}_2/\text{HCO}_3^-$ enters these cells by a metabolic-energy dependent transport process.

Energetics of C_i uptake

We have considered two metabolic energy intermediates in the uptake of C_i : ATP, as has been found in several transport systems of metal ions and other solutes (Heefner and Harold 1980; Harold 1977; Rhoads and Epstein 1977), and the proton motive force (Δp), as was originally suggested by Mitchell (1961) to be involved in secondary transport processes (Konings et al. 1981).

ATP levels in cells before and after the addition of thiosulphate were determined. Non-energized cells contained 0.15 mM ATP, but no uptake of labeled C_i was observed (Fig. 1). After energization the cells contained up to 0.93 mM ATP. In these cells uptake of C_i occurred. If ATP would be the energy source for the transport system, uptake and accumulation of C_i should decrease when ATP synthesis is blocked. Therefore DCCD, a H^+ ATPase inhibitor which is commonly used to block the synthesis of ATP, was added to the cells. This caused a slight decrease of the level of ATP to 0.83 mM , while an increase of the uptake of C_i was found (data not shown). The presence of ATP in cells that do not take up C_i and the (stimulatory) effect of DCCD suggest that ATP is not an energy source for the uptake of C_i .

To test whether Δp or its components, $\Delta\Psi$ (the electrical potential) and ΔpH (the pH-gradient), are involved in the uptake of C_i experiments were performed in the presence of the ionophores valinomycin and nigericin. Valinomycin is an electrogenic potassium carrier which causes, in the presence of K^+ , a dissipation of the electrical potential ($\Delta\Psi$). Nigericin acts as an electroneutral potassium/proton antiporter and dissipates the pH-gradient (ΔpH).

Valinomycin (15 μM) reduced the uptake of C_i by cells in the presence of thiosulphate and only a slight accumulation (up to 20-fold) in the first minute was found (Fig. 3). After 8 min radioactive label in these cells was only present as fixed carbon. The electrical potential as measured with the TPP^+ electrode was -67 mV (inside negative) in non-energized and energized cells grown under thiosulphate-limiting conditions and -80 mV in non-energized and energized cells grown under CO_2 -limiting conditions. This $\Delta\Psi$ was completely dissipated by 15 μM valinomycin.

When thiosulphate-limited cells were treated with 5 μM nigericin both the initial rate of uptake and the accumulation ratio (at most 2000-fold) of inorganic carbon increased (Fig. 3). This increase in the uptake of C_i could be the result of an interconversion of the ΔpH into a $\Delta\Psi$ which leads to an increase of the $\Delta\Psi$. After addition of nigericin indeed an increase of the $\Delta\Psi$ to -125 mV was recorded. However, a ΔpH was not measurable in non-energized nor in energized cells neither by flow-dialysis nor by silicone oil centrifugation nor with the salicylate electrode. The increase of the $\Delta\Psi$ after addition of nigericin, however, strongly indicates that a ΔpH exists at pH 7.0 which cannot be recorded with the methods applied. The role of the ΔpH as driving force for C_i uptake is therefore not clear but the results clearly indicate that the electrical potential ($\Delta\Psi$) is a driving force for C_i uptake.

The role of electron flow in C_i -uptake

The electrical potential of non-energized cells was the same (-67 mV) as in cells energized by thiosulphate. If a reversed ΔpH (inside acid) was present with the same magnitude as the existing $\Delta\Psi$ the total proton motive force would be zero. This could explain the absence of uptake of C_i under non-energized conditions. However, a reversed ΔpH was not found using methylamine as distribution probe (see Materials and methods).

We have checked if the recorded $\Delta\Psi$ in the non-energized cells was indeed a true $\Delta\Psi$ and not an artifact of the measurement, or a Donnan potential, caused by impermeable charged molecules in the cytoplasm. The uncoupler CCCP and the ionophore valinomycin dissipated completely the $\Delta\Psi$ and treatment with nigericin resulted in an increase of the $\Delta\Psi$ to -100 mV . Moreover, the $\Delta\Psi$ in CO_2 -limited cells was higher (-80 mV) both under energized and non-energized conditions than cells grown under thiosulphate limitation. These results indicate that indeed a $\Delta\Psi$ of -67 mV was present in non-energized cells. However, these cells did not take up inorganic carbon, not even to equilibration levels. Also in the presence of nigericin, when the $\Delta\Psi$ increased to -100 mV , or in cells in which a potassium diffusion potential was imposed, no uptake of C_i was observed in the absence of the electron donor thiosulphate.

Only energized cells would take up and accumulate C_i (Fig. 1). If potassium cyanide, a potent inhibitor of the terminal oxidase, was added to the cells, oxygen consumption

Fig. 4A, B. Eadie-Hofstee plot of the initial rate of uptake of inorganic carbon at different pH values. A Initial rate of uptake of CO_2 and B initial rate of uptake of HCO_3^- . (●) pH 6.0, (★) pH 6.5, (▲) pH 7.0, (■) 7.5

was completely stopped in the presence of thiosulphate. Cyanide had no effect on the electrical potential, but the uptake of C_i was blocked completely (Fig. 1).

The importance of thiosulphate oxidation in the C_i -transport process was also demonstrated by several other experiments. In standard uptake experiments the dissolved oxygen in the medium buffer was consumed by the cells within 2 min, due to the high rate of oxygen consumption ($1540\text{ nmol/min}^{-1}\text{ mg}^{-1}\text{ protein}$). During these 2 min the uptake of C_i occurred at a high rate (Fig. 1). After this period the uptake rate decreased to a level which was dependent on the rate of oxygen diffusion in the suspension. If the stirring rate in the uptake-tubes was low, a relatively low accumulation of C_i in the cells occurred. High stirring rates resulted in high uptake rates. However, at too high stirring rate and oxygen flow over the samples in the tubes, the ^{14}C -labeled bicarbonate was blown away as $^{14}\text{CO}_2$. This problem made experiments at pH 6.0 difficult and impossible at pH 5.0.

Transported substrate: CO_2 or HCO_3^- ?

To obtain information which of the carbon species, CO_2 or HCO_3^- , are transported by whole cells, the kinetics of uptake of C_i was determined. The K_m for transport was estimated assuming that either CO_2 (Fig. 4A) or HCO_3^- (Fig. 4B) was the transported species. The estimations were done in the pH range 6 to 7.5, since below pH 6.0 and above pH 7.5 the cells oxidized thiosulphate at a low rate. In the pH range 6.0 to 7.5 the percentage of the total C_i present in the medium as CO_2 decreased from 69.2 at pH 6.0, 41.6 at pH 6.5, 18.4 at pH 7.0 to 6.6 at pH 7.5 (according to Kern 1960). The initial rates of C_i uptake were plotted as functions of the concentrations of both carbon species (Fig. 4).

If CO_2 would be the accumulated species (Fig. 4A) the K_m for uptake would vary between 87 and 326 μM . If HCO_3^- would be the accumulated species (Fig. 4B) as 350-fold increase of the K_m would be found between pH 6.0 (K_m 32 μM) and pH 7.0 and 7.5 (K_m 2.7 and 11.3 mM, respectively). Since it seems reasonable to assume that the affinity of the transport protein for its substrate does not change drastically in the pH range studied the most likely accumulated species appears to be CO_2 .

Discussion

The data presented indicate that the electrical potential is a main driving force for the uptake of inorganic carbon. A stimulation of the uptake and an increase in the accumulation were found whenever the $\Delta\Psi$ increased. The involvement of the $\Delta\Psi$ in the uptake of CO_2 indicates that cations like K^+ , Na^+ or H^+ are transported in symport with CO_2 . Uptake of inorganic carbon together with Na^+ was reported previously by Miller and Calvin (1985) and Kaplan et al. (1984). The role of the ΔpH in the uptake process is still not clear. This is mainly due to the inability to record a pH with the methods applied. Uptake of C_i was only observed in the presence of respiration and never in the absence of electron transfer whatever the magnitude of the $\Delta\Psi$. These results could possibly be explained by a regulation of the C_i transport system by the electron transfer system. Such an interaction between the electron transfer chain and solute transport was previously observed by Elferink et al. (1984, 1985) in *Rhodospseudomonas sphaeroides* and *Escherichia coli*. The mechanism of this interaction is unknown. For *Thiobacillus neapolitanus* it would be a useful mechanism to block the uptake of C_i in the absence of an electron donor to survive long periods of energy starvation (Beudeker 1981). The existence of this interaction and the possible role of the ΔpH could be elucidated by using membrane vesicles in the C_i uptake experiments. The isolation of these vesicles from *T. neapolitanus* has been described but the activity of these preparations (as could be seen from the transport of amino acids) was very low (Matin et al. 1974). We have also attempted to isolate active membrane vesicles but were unsuccessful.

CO_2 represents the unprotonated form of inorganic carbon which apparently binds to a carrier protein as it does to RuBisCO. In our system CO_2 appears to be transported across the cytoplasmic membrane with the electrical potential as a main driving force. Volokita et al. (1984) proposed a model for CO_2 uptake by cells of the cyanobacterium *Anabaena variabilis* in which CO_2 binds to a carrier. Due to conversion of CO_2 to HCO_3^- by the carbon anhydrase-like

activity of this carrier HCO_3^- was transferred and released at the inside of the cytoplasmic membrane. According to this model HCO_3^- would be the transported species. This transport of HCO_3^- would occur against the electrochemical gradient which is at variance with our results.

Our results demonstrate that uptake of C_i as well as the level of fixation is significantly higher in CO_2 -limited cells than in thiosulphate-limited cells. Since CO_2 -limited cells show a RuBisCO activity which is five-fold higher than in thiosulphate-limited cells (Beudeker et al. 1980) it should be expected that the fixation is higher under CO_2 limitation. However, Beudeker et al. (1980) found in the CO_2 -limited cells of *T. neapolitanus* an inverse relationship with the amount of RuBisCO and the rate of fixation. The discrepancy with our results could be a consequence of the moment of harvesting and the physiological condition of the cells. Beudeker used *T. neapolitanus* cells straight from the CO_2 -limited continuous culture. In the CO_2 -limited cultures 50% more thiosulphate is respired than would be required for the equivalent cell yield under thiosulphate limitation. This implies that in CO_2 -limited cells oxidation and energy generation are uncoupled (Kuenen and Veldkamp 1973). In addition cell free extracts of these cells contain high ATPase activity (J. G. Kuenen, unpublished works). In our experiments, however, the harvested cells were starved for 2–3 d at 4°C. These cells took up immediately C_i and showed high fixation rates (Fig. 2). This indicates that the starvation period abolishes the uncoupling which ultimately leads to a fixation rate which is in accordance with the amount of RuBisCO present.

The rates of fixation in Figs. 1 and 2 show that CO_2 fixation in thiosulphate-limited cells starts 30 s later than in CO_2 -limited cells. In thiosulphate-limited cells 8.5 mM C_i is present after energization. Due to the high internal pH the inorganic carbon will be present as HCO_3^- and only a small fraction of C_i (at pH 7.5 6.6%) will be in the form of CO_2 . At pH 7.5 only 0.56 mM CO_2 is present in the cells. Since the K_m (CO_2) for RuBisCO isolated from *T. neapolitanus* has been estimated to be 0.85 mM (Snead and Shively 1978) this internal concentration of CO_2 would not be saturating. When CO_2 -limited cells were used for the uptake experiment 27.5 mM C_i and 1.8 mM CO_2 are present after 20 s. The higher intracellular CO_2 concentration in CO_2 limited cells could explain why fixation starts immediately after addition of thiosulphate.

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