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Relations Between D-Ribulose-1,5-Bisphosphate Carboxylase, Carboxysomes and CO_2 Fixing Capacity in the Obligate Chemolithotroph *Thiobacillus neapolitanus* Grown Under Different Limitations in the Chemostat

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Abstract. An adaptation of the D-ribulose-1,5-bisphosphate carboxylase (RuBPCase) activity to changing CO_2 concentrations in the growth medium in the chemostat was observed in the obligate chemolithotroph *Thiobacillus neapolitanus.* RuBPCase activity has been separated in a soluble and particulate fraction. The activity of the particulate fraction appeared to be associated with the carboxysomes.

The total activity of RuBPCase of CO_2 limited cultures was about 5-fold higher than the activity of thiosulphate limited cultures grown in the presence of 5% CO_2 whilst the particulate activity and the soluble activity were about 8- and 1.5-fold higher, respectively. The fluctuation of the total and particulate RuBPCase activity correlated with the changes in volume density of carboxysomes in the cell.

An inverse correlation between maximal CO_2 fixing capacity by whole cells and the volume density of carboxysomes was observed. The change in ratio of soluble RuBPCase activity to particulate RuBPCase activity paralleled the change in maximal CO_2 fixation by whole cells during the different growth conditions.

Key words: Carboxysomes – D-Ribulose-1,5-bisphosphate carboxylase – CO_2 fixing capacity – *Thiobacillus neapolitanus* – Chemolithotroph – Autotroph.

In common with the other specialist chemolithotrophs, methylotrophs and photolithotrophs, *Thiobacillus neapolitanus* has been shown to be metabolically rigid (Smith and Hoare, 1977). In contrast to the facultative chemolithotrophs (mixotrophs) the obligate chemolithotrophs are known to be unable to repress the enzymes of the Calvin cycle in the presence of organic compounds (Taylor and Hoare, 1969; Kuenen and Veldkamp, 1973) though organic compounds are incorporated to some extent into high molecular weight cell material. To date only very few examples have been reported showing that the obligate chemolithotrophs are able to adapt their metabolism to changing growth conditions. For example in *T. neapolitanus* the RNA-content of the cells doubled when the growth rate increased 4–6 fold (Kuenen, in press). In the same organism the activity of D-ribulose-1,5-bisphosphate carboxylase (RuBPCase) increased threefold during CO_2 limitation (Kuenen and Veldkamp, 1973).

The RuBPCase has been shown to be present in the polyhedral bodies (carboxysomes) of *T. neapolitanus* (Shively et al., 1973) and also in the polyhedral bodies of *Anabaena cylindrica* (Codd and Stewart, 1976) and *Nitrobacter agilis* (Shively et al., 1977). Thus far no other enzyme activities have been detected (i.e., Ribulose-5-phosphate kinase) in the carboxysomes (Fellman, 1977).

The presence of RuBPCase activity in both the carboxysomes and the cytosol (Shively et al., 1973; Codd and Stewart, 1976) suggested a role of carboxysomes in the regulation of RuBPCase activity. The aim of the present investigation was to examine a possible correlation between number of carboxysomes, RuBPCase activity and maximal CO_2 fixation capacity by whole cells under energy and under CO_2 limitation in the chemostat.

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Materials and Methods

Organism and Growth Conditions

Thiobacillus neapolitanus strain X was grown in the chemostat under thiosulphate- and CO_2-limitation as has been described by Kuenen and Veldkamp (1973). Carbon dioxide-limited cultures were aerated with air which had been stripped from CO_2 with sodiumasbestos

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(Merck). CO_2 was administered to the culture as a 0.05 M Na_2CO_3 solution. 1 M NaOH was used for titration. During the other growth conditions 1 M Na_2CO_3 was used for titration.

Cultivation in the presence of 5% (v/v) CO_2 in the gas mixture was realized by mixing air with CO_2 from a gas cylinder. Under these conditions consumption of CO_2 did not significantly alter the % CO_2 in the gas mixture. The basal medium for cultivation of *T. neapolitanus* contained (% w/v): NH_4Cl, 0.04%; $MgSO_4 \cdot 7\ H_2O$, 0.08; KH_2PO_4, 0.05; K_2HPO_4, 0.05; $Na_2S_2O_3 \cdot 5\ H_2O$, 1.0.Two milliliter of the trace element mixture (Vishniac and Santer, 1957) were added to a liter of medium.

All experiments described were carried out on samples taken from steady state cultures.

D-Ribulose-1,5-Bisphosphate Carboxylase (E.C. 4.1.1.39)

Steady state cultures were harvested by centrifugation for 40 min at 20,000 × *g* (4°C) and washed in the assay buffer [100 mM tris (hydroxymethyl)methylamine (TRIS) containing 5 mM dithiothreitol (DTT), 20 mM $MgCl_2 \cdot 6\ H_2O$ and 5 mM $NaHCO_3$ that had been adjusted to pH 8.2 at 20° C with HCl (Lorimer et al., 1977].

Cell-free extracts were made by sonification for 5 × 30 s in the presence of 1 : 1 (w/w) ballotini beads (0.11 mm diameter). Debris were removed by centrifuging for 10 min at 40,000 × *g* (4°C). To 100 µl of the resulting supernatant 170 µl assay buffer was added and this mixture was activated by incubating for 10 min at 30° C. To this activated mixture 10 µl of 50 µCi $NaH^{14}CO_3$/50 µmol · ml was added 15 s prior to the beginning of the reaction. The reaction was started by addition of 20 µl 0.010 M ribulose bisphosphate (RuBP) and was allowed to proceed for 2.5 min. Samples containing 50 µl were taken every 0.5 min and transferred into a scintillation vial at 60° C, containing 100% acetic acid, to remove unfixed CO_2. Traces of acetic acid that remained after 20 min of incubation at 60° C did not quench the countings in the scintillation counter (Nuclear Chicago, Mark 1). Radioactive counts were converted to desintegrations and subsequently to moles of CO_2 using conventional standard procedures. In all cases the activities were expressed in terms of RuBP-dependent CO_2 fixation.

Determination of the Ratio of Particulate and Soluble RuBPCase Activity

In order to obtain a quantitative estimate of the distribution between particle bound RuBPCase (i.e. in the carboxysomes) and soluble enzyme the following procedure was developed. When cells of *T. neapolitanus* were sonified in 0.1 M TRIS buffer in the presence or absence of ballotini beads (see above) all the activity of RuBPCase became soluble and remained in the supernatant fraction after centrifuging for 60 min at 48,000 × *g*. Electron microscopical investigations of the particulate fraction showed that during sonification under these conditions the carboxysomes were rapidly converted into empty shells, which subsequently desintegrated. The individual RuBPCase molucules, that are easily identifiable owing to the characteristic "doughnut" shape, could be seen next to the empty shells. However, when cells of *T. neapolitanus* were sonified (in the absence of ballotini beads) in ten times less concentrated TRIS buffer (0.01 M) the activity of RuBPCase could be separated in a soluble and particulate fraction by centrifuging for 60 min at 48,000 × *g*. The particulate (P) fraction appeared to contain intact carboxysomes only and virtually no ghosts or individual RuBPCase molecules. The obtained supernatant fraction (S) did not contain either ghosts or particles. Resuspending the intact carboxysomes in 0.1 M TRIS buffer immediately resulted in desintegration of the particles and formation of empty shells. A time course experiment showed that the S/P ratio increased during the first three to five consecutive half minute intervals of sonification. This is obviously due to the desintegration of debris whereby trapped enzyme is released. After this period the ratio remained constant for at least 5 more half minute intervals of sonification. This was confirmed further by electron microscopical observations.

The reproducibility of the method was also checked by using different amplitudes settings on the sonification apparatus. In the two different laboratories virtually identical S/P ratios were found using different ultrasonic equipment.

Maximal CO_2-Fixation by Whole Cells

Cells from steady state cultures were collected by centrifugation at 23,000 × *g* (28° C) and resuspended in growth medium without thiosulphate. The reaction was carried out in a series of 5 well-aerated small glass tubes, each containing 100 µl of cells. To each glass tube 10 µl 50 mM thiosulphate was added 1 min prior to the addition of labelled bicarbonate. The reaction was started by adding 10 µl $NaH^{14}CO_3$ (25 µCi/50 µmol · ml) to each glass tube. Samples without thiosulphate served as a blank for the nonenzymatic CO_2 absorption.

A time-course of 5 min was taken by passing the content of one glass tube every minute over a 0.45 µm membrane filter, with the aid of a vacuum pump. The filters were washed twice with 2 ml of a 0.05% potassium phosphate buffer pH 6.8 containing 50 mM $NaHCO_3$. After drying for three minutes at 105°C the filters were counted in a gasflow counter with an efficiency of 17%. Incubation of the filters in HCl fumes did not effect the countings. Rates were linear for at least 5 min.

This method has been checked carefully for effects of different concentrations of the components in the assay mixture. HCO_3^-, O_2 and thiosulphate were all present at saturating concentrations and uptake rates were linear with the cell protein added.

Electron Microscopy

Pellets of cells were fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2. Postfixation occurred in a solution of 1% OsO_4 and 2.5% $K_2Cr_2O_7$ in 0.1 M cacodylate buffer pH 7.2. After dehydration in a graded alcohol series the cells were imbedded in Epon and sectioned with a diamond knife on a LKB microtome and examined in a Philips EM 300.

Volume densities of carboxysomes were determined with the test point technique according to Weibel and Bolender (1976).

Student's *t*-test was used for statistical analysis at a confidence limit of 0.005%.

Protein Determination

Protein of cell-free extracts was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard with appropriate corrections for TRIS and DTT in the buffer.

Protein of whole cells was determined with a modification of the microbiuret method of Goa (1953) as has been described by Kuenen and Veldkamp (1972).

Contamination by other Bacteria

The cultures were frequently checked for contaminants as has been described by Kuenen and Veldkamp (1973).

Inorganic Carbon

The contents of inorganic carbon in the supernatant were determined in the inorganic channel of a Total Organic Carbon Analyzer (Beckman).

Thiosulphate

Thiosulphate concentrations in the culture were determined by the method of Sörbo (1957).

Table 1. Dry weight, protein, thiosulphate and inorganic carbon in chemostat-grown cultures of *Thiobacillus neapolithanus* at different growth limitations on a mineral medium with 40 mM thiosulphate. $D = 0.07\,h^{-1}$; $pO_2 = 50\%$ air saturation

Growth limiting substrate	Dry weight mg/l	Protein mg/l	Inorganic carbon mg/l	Thiosulphate mmol/l
CO_2	65	33	<1	10
Thiosulphate	160	80	6	< 0.05
Thiosulphate (+5% CO_2)	170	81	30	< 0.05

Chemicals

$NaH^{14}CO_3$ was purchased from the Radiochemical Centre Amersham (England). RuBP was from Sigma Chemicals Co (St. Louis, Missouri, USA).

All other chemicals were of analytical grade.

Results

To study the effect of different growth conditions on the activity of the D-ribulose-1,5-bisphosphate carboxylase (RuBPCase) *Thiobacillus neapolitanus* was grown under different limitations in the chemostat. Dry weight, protein per volume of culture, thiosulphate concentration in the culture and inorganic carbon in the supernatant were determined. As expected the CO_2 was not detectable under CO_2 limitation whereas under thiosulphate limitation CO_2 could easily be measured. Addition of 5% CO_2 in the air supply caused a 5-fold increase of the steady state CO_2 concentration (Table 1).

The protein was 48–50% of the dry weight under the different growth conditions (not shown).

Under CO_2 limitation about 10 mM (of the original 40 mM) thiosulphate was left in the culture whereas under thiosulphate limitation, as expected from the chemostat theory, the concentration was below the detection level (0.05 mM). The cell yield per mol consumed thiosulphate is considerably lower under CO_2 limitation. Apparently the cells exhibit energy-spilling reactions under these conditions.

In order to follow a possible change in number of carboxysomes with changing RuBPCase activity the enzyme activities under the different growth conditions were compared with results from measurements on electronmicroscopical photographs of thin sections. In order to obtain a quantitative measure of the number of carboxysomes both the volume density per cell of the carboxysomes and the number of carboxysomes per at random diameter were determined.

Table 2 shows the apparent correlation between the volume density of carboxysomes and the RuBPCase activity under the different growth conditions. The

Table 2. Volume densities of carboxysomes in thin sections, number of carboxysomes per at random diameter in thin sections as observed under the electron microscope, and D-ribulose-1,5-bisphosphate carboxylase (RuBPCase) activities of *T. neapolitanus* grown under different limitations in the chemostat. $D = 0.07\,h^{-1}$; $pO_2 = 50\%$ air saturation. Statistical analysis of the data listed in this table showed that the differences were significant (see "*Materials and Methods*")

Growth limiting substrate	Volume density of carboxysomes % cell volume	Number of carboxysomes per at random diameter	RuBPCase spec. act.[a]
CO_2	8.7	1.4	240
Thiosulphate	4.4	0.5	72
Thiosulphate (+ 5% CO_2)	1.5	0.09	45

[a] nmol/min · mg protein

Table 3. Activities of RuBPCase in the particulate fraction (P) and in the soluble fraction (S) of chemostat-grown cultures of *T. neapolitanus* at different limitations. $D = 0.07\,h^{-1}$; $pO_2 = 50\%$ air saturation

Growth limiting substrate	Particulate nmol/min · mg	Soluble total cell protein	S/P
CO_2	80	27	0.34
Thiosulphate	18	21	1.10
Thiosulphate (+ 5% CO_2)	11	18	1.67

RuBPCase activity did not only increase during CO_2 limitation it also appeared to decrease by addition of extra CO_2 to the culture, indicating that the observed changes are due to an effect of CO_2 rather than to different physiological states of the cells (i.e. energy or carbon limitation). The results obtained by electron microscopical investigations of thin sections (Table 2) were confirmed by measurements of the RuBPCase activity in the soluble and particulate fraction of cell free extracts. The particulate fraction activity was shown to be associated with the carboxysomes. The activity in the supernatant was due to the activity of the RuBPCase in the cytosol (see materials and methods). In order to be able to compare the activities of the RuBPCase in the particulate and soluble fraction the activities of the fractions were calculated per mg total cell protein. Thus the distribution of the RuBPCase over the carboxysomes and cytosol can be estimated. Table 3 shows that during CO_2 limitation the activity of RuBPCase in the particulate fraction (P) increased about 7–8-fold whereas the activity in the soluble fraction (S) changed by a factor less than 2. This

Table 4. Relations between CO_2-fixing capacity by whole cells, RuBPCase activities and S/P ratios of chemostat-grown cultures of *T. neapolitanus* at different limitations. $D = 0.07\ h^{-1}$; $pO_2 = 50\%$ air saturation

Growth limiting substrate	RuBPCase	Maximal CO_2-fixing capacity by whole cells	S/P
	nmol/min · mg protein		
CO_2	240	115	0.34
Thiosulphate	72	271	1.10
Thiosulphate (+ 5% CO_2)	45	450	1.67

increase in activity of the particulate fraction resulting in a lower S/P ratio, correlates with the observed higher volume density of carboxysomes during CO_2 limitation. In the presence of 5% CO_2 the opposite effect can be observed. The finding that RuBPCase in the particulate fraction showed activity under our assay conditions does not necessarily mean that the enzyme in the carboxysomes is active "in vivo". For it may well be that during the preparation of cell-free extracts the RuBPCase in the carboxysomes was activated.

To examine whether carboxysomes may be active "in vivo" we compared the maximal CO_2 fixation capacity by whole cells with the RuBPCase activity. The maximal CO_2 fixation capacity was tested in the presence of excess thiosulphate and CO_2.

As can be seen from Table 4 there exists an inverse correlation between the RuBPCase activity and the CO_2-fixation capacity by whole cells and consequently between the volume density of carboxysomes and the maximal CO_2-fixation capacity of whole cells. In contrast, the CO_2-fixing capacity parallels the change in S/P ratio.

It should be noted that the actual specific rate of CO_2 fixation in the growing culture in the chemostat at a dilution rate of $0.07\ h^{-1}$, of course, must be virtually the same for all growth conditions since the specific growth rates are identical and the protein content of the cells shows little variation. Given a constant carbon content of 50% of the dry weight (unpublished results) it can be calculated, using the data from Table 1, that the required specific rate of fixation is about 70 µg C/h · mg protein

$$\left(D \times \frac{\text{mg C/l}}{\text{mg protein/l}}\right)$$

which is equivalent to 1.2 µg C/min · mg protein. This is equivalent to 100 nmol CO_2/min · mg protein, which is only slightly less than the observed maximal rate of CO_2-fixation in CO_2-limited cells (Table 4).

Discussion

The data presented show that *Thiobacillus neapolitanus* is able to adapt its RuBPCase activity to changing CO_2 concentrations in the medium. The fluctuation in RuBPCase activity correlated with the fluctuation of volume density of carboxysomes. This is in good agreement with the finding of Shively et al. (1973) that these particles contain RuBPCase. The role of carboxysomes in the fixing of CO_2 "in vivo" remains unclear. The measurements of the maximal capacity to fix CO_2 by intact cells in the presence of excess thiosulphate and CO_2 show a change of this activity which parallels the change in S/P ratio of the RuBPCase activity. The higher the activity of the soluble RuBPCase relative to the particulate RuBPCase the higher the CO_2 fixing capacity by whole cells.

In contrast, the absolute activity of RuBPCase as measured "in vitro" showed an inverse relationship with the maximal capacity to fix CO_2, demonstrating that the activity of this enzyme, both soluble and particulate, is not rate-limiting for the maximal CO_2 fixation. The rate-limiting step during CO_2 fixation by whole cells may be another factor e.g. another Calvin cycle enzyme or the energy generation.

Somewhat surprisingly the maximal CO_2 fixation rate by whole cells from CO_2-limited cultures is just high enough to account for the growth rate in the chemostat. Apparently these cells do not react immediately to addition of excess CO_2 by an increase in CO_2 fixation. Carbon dioxide-limited cells contain a different thiosulphate-oxidizing system compared to that of thiosulphate-limited cells; this is expressed in a different sensitivity to inhibitors of the respiratory chain and a different affinity for thiosulphate (Beudeker and Kuenen, unpublished results). The observed low cell yield per mol consumed thiosulphate indicates the operation of a regulatory mechanism in order to prevent an excessive ATP production. In the presence of excess CO_2 and thiosulphate of our assay system this limited ATP production may cause the observed low maximal CO_2 fixing capacity by intact cells. Because of this different thiosulphate oxidizing system in fact CO_2-limited cells cannot be compared with energy limited cells. However, thiosulphate-limited cells with and without additional supply of CO_2, which should be comparable, show the same correlations.

The parallel change of S/P ratio and CO_2-fixing capacity does not support an active function in CO_2 fixation for the carboxysomes as has been suggested for the carboxysomes of the facultatively chemolithotroph *Thiobacillus intermedius* (Purohit et al., 1976). At first sight the observed parallel change of S/P ratio and CO_2 fixing capacity can be explained considering an enzyme

storage function of the carboxysomes. Stewart and Codd (1975) suggested a storage function for the carboxysomes of the symbiotic Nostoc of *Blasia pusilli*, which is unable to fix CO_2. But since the varying soluble RuBPCase activities do not correlate with the CO_2 fixing capacity by whole cells other regulatory mechanisms should operate to control the activity of the soluble RuBPCase activity "in vivo". It seems possible that the carboxysome itself plays an important role in this regulation. In this context it may be significant that Westphal and Bock (1974) have shown that the polyhedral particles of *Nitrobacter* contain DNA.

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