Ammonia Assimilation by *Thiocapsa roseopersicina*
Grown on Various Nitrogen and Carbon/Electron Sources

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Batch cultures of the phototrophic bacterium, *Thiocapsa roseopersicina*, were grown anaerobically in the light either on sulfide with various ammonia concentrations, \( \text{N}_2 \), or amino acids as nitrogen sources, or on several simple organic substrates in the absence of reduced sulfur compounds using 6 mm \( \text{NH}_4\text{Cl} \) as source of nitrogen. At high ammonia concentrations high activities of (NADPH-linked) glutamate dehydrogenase (GDH), but low transferase and no biosynthetic activity of glutamine synthetase (GS) were obtained, while under conditions of ammonia deficiency (growth with \( \text{N}_2 \) or glutamate) GDH activity was very low and both GS activities were strongly increased. Glutamate synthase (GOGAT) activity (NADH-dependent) showed little variation. These data indicate that at high NH\(_4^+\) concentrations ammonia is assimilated via GDH, under \( \text{NH}_2\text{H} \) limitation, however, via the GS/GOGAT system. Glutamine as nitrogen source may be utilized via GOGAT as well as via an active glutaminase plus GDH. Ammonia, but not glutamine, seems to cause repression and inactivation of GS. Alanine and asparagine inactivate the enzyme inhibiting the biosynthetic, but not the transferase activity. These amino acids in part also influence the activities of GDH, GOGAT, malate dehydrogenase (MDH) and isocitrate dehydrogenase (ICDH).

Cultures grown on acetate or pyruvate instead of sulfide showed increased GDH activities and high GS transferase activities possibly reflecting an increase of intracellular \( \alpha \)-ketoglutarate concentration. On malate or fructose also increased GS transferase activities, but rather low GDH activities were observed. High biosynthetic GS activities and elevated GOGAT activities were found only in fructose-grown cells. On the organic substrates the ICDH activities always were somewhat higher than after lithoautotrophic growth. With the exception of acetate, the MDH activities were considerably elevated, especially on pyruvate. The different pathways of \( \alpha \)-ketoglutarate formation and their influence on the enzymes of ammonia assimilation are discussed.

All species of the purple sulfur bacteria (Chromatiaceae) grow readily on ammonia as source of nitrogen [1]. Many strains also can fix molecular nitrogen (summarized by Stewart [2], Dalton [3], Bast [4]), while other nitrogenous compounds including amino acids are less commonly utilized [4, 5]. As in other bacteria the assimilation of ammonia by Chromatiaceae may proceed via two alternative pathways involving either glutamate dehydrogenase (GDH) (E. C. 1.4.1.2/1.4.1.4), or glutamine synthetase (GS) (E. C. 6.3.1.2) and glutamine synthase (GOGAT) (E. C. 2.6.1.53) [4, 6, 7]. The findings hitherto reported indicate that the ammonia concentration and the kind of nitrogen source in the growth medium as well as possibly the carbon source may affect synthesis and activity of the enzymes of ammonia assimilation.

Since we found an active GS, NADH-linked GOGAT and NADPH-linked GDH in *Thiocapsa roseopersicina* strain 6311 [4], we chose this organism to look for variations in the activities of the ammonia-assimilating enzymes after growth on different nitrogen or carbon/electron sources. In order to detect possible differences in the catabolism of the organic substrates we also examined two enzymes of the tricarboxylic acid cycle, malate dehydrogenase (MDH) (E. C. 1.1.1.37) and isocitrate dehydrogenase (ICDH) (E. C. 1.1.1.42).

Materials and Methods

*Thiocapsa roseopersicina* strain 6311 (DSM* 219) was grown anaerobically in the light (incandescent lamp, 1000 lux) at 28 °C in 1 l-screw cap bottles. The

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modified Pfennig's medium [8] was used containing either Na₂S · 9 H₂O (0.075% w/v = 3.1 mM) as electron donor and a varying nitrogen source as indicated, or an organic substrate (10 mM) instead of sulfide with 6 mM NH₄Cl as the only nitrogen source; in the latter case 0.04% Na₂SO₄ were added. The pH was 7.2. Unless otherwise stated, sulfide-oxidizing cultures were fed twice with Na₂S solution and incubated until they were free of sulfur.

Growth was measured as optical density of sulfur-free cultures at 650 nm with a Bausch & Lomb Spectronic 20 photometer, or by protein determination of whole cells according to Schmidt et al. [9].

For the preparation of cell-free extracts cells were harvested by centrifugation, washed twice with 0.05 M Tris-HCl buffer, pH 7.6, containing 2 mM 2-mercaptoethanol, and passed twice through an Amino French pressure cell at 140 MPA (= 20000 psi). After centrifugation at 38000 × g for for 15 min and 100000 × g for 2 h the supernatant was used in enzyme assays.

The activities of the dehydrogenases and of GOGAT were measured spectrophotometrically with an Eppendorf 1101 M photometer by recording the initial rate of conversion of NADH or NADP(H) at 366 nm. GDH (aminating) and GOGAT were tested as described previously [4] except that the pH was 7.6; the GOGAT reaction was started by NADH addition [10]. GDH (deaminating) was assayed according to Bachofen and Neeracher [11], MDH was tested after Ochoa [12] and ICDH after Ochoa [13], using 0.05 M Tris-HCl buffer, pH 7.6 and 7.4, respectively. GS activity was determined at pH 7.4 by the Mn²⁺-dependent γ-glutamyl transferase assay [4, 14], and by the Mg²⁺-dependent biosynthetic assay after Woolfolk et al. [15] measuring phosphate production by the method of Taussky and Shorr [16]. (The synthetic assay with hydroxylamine instead of ammonia [17] revealed similar results.) The enzyme assays were carried out at 30 °C. Glutaminase was tested at 37 °C in Tris-HCl buffer, pH 8.5, by the method of Meister [18]; released ammonia was determined with GDH from beef liver (Boehringer 127086) according to Kun and Kearney [19]. The protein content of cell-free extracts was determined by the biuret method in the modification of Beisenherz et al. [20]. Specific enzyme activities are expressed as milliunits (mU) per mg protein. One milliunit catalyzed the conversion of one nmol of substrate per min.

In the supernatant culture liquid ammonia was determined by the Berthelot reaction [21] and L-glutamine by the glutamyl transfer reaction with GS prepared from *Escherichia coli* [14].

### Results and Discussion

Table I shows the specific activities of the ammonia-assimilating enzymes in cell-free extracts from batch cultures of *Thiocapsa roseopersicina* grown on sulfide with various nitrogen sources. Cultures grown with an excess of ammonia exhibited a rather high activity of – NADPH-dependent – GDH (aminating). (The deamination activity was about

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Relative growth 1 [%]</th>
<th>NH₃ content of culture supernatant [mM]</th>
<th>Specific activities [milliunits/mg protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl (6 mM)</td>
<td>100</td>
<td>2.3</td>
<td>102 220 0 15 31</td>
</tr>
<tr>
<td>NH₄Cl (30 mM)</td>
<td>64</td>
<td>23.1</td>
<td>93 98 0 0 5 n.d.</td>
</tr>
<tr>
<td>N₂ (30 mM)</td>
<td>75</td>
<td>0.05</td>
<td>6 1200 261 31 n.d.</td>
</tr>
<tr>
<td>Glutamate (30 mM)</td>
<td>75</td>
<td>0.2</td>
<td>2 850 204 19 n.d.</td>
</tr>
<tr>
<td>Glutamine (6 mM)</td>
<td>114</td>
<td>0.9</td>
<td>96 620 14 22 128</td>
</tr>
<tr>
<td>Glutamine (20 mM)</td>
<td>114</td>
<td>3.5</td>
<td>157 375 0 19 156</td>
</tr>
</tbody>
</table>

1 Optical density (650 nm) of sulfur-free cultures after the second feeding, calculated for a growth time of 7 days and expressed as percent of the absorbance of cultures on 6 mM NH₄Cl, which was 0.7 corresponding to a cell protein content of 0.15 mg/ml culture liquid.
2 GS-T = GS transferase activity.
3 GS-B = biosynthetic GS activity.
4 Medium and Na₂S solution were filter-sterilized with N₂ pressure.
5 n.d. = not determined.
one tenth of the amination activity under all growth conditions.) The transferase activity of the GS (GS-T) was rather low, and no biosynthetic GS activity (GS-B) could be detected. Under ammonia limitation (growth with molecular nitrogen as sole nitrogen source), however, a very low level of GDH activity was observed, while the GS transferase activity increased five- to tenfold and also a high biosynthetic activity was recorded. Cultures grown on L-glutamate under an argon atmosphere showed similar growth rates and enzyme activities as N₂-grown cells. It therefore appears as if glutamate might not be able to enter the cell at any significant rate. Accordingly, growth and enzyme activities on glutamate would be due to utilization of atmospheric N₂ dissolved during preparation of the medium.

When *Thiocapsa roseopersicina* was cultivated at a low exogenous ammonia level (initial concentration 2 mM) in the presence of N₂, the cells at first contained high GDH activity and relatively low GS (transferase) activity (Fig. 1). As growth proceeded and the ammonia content of the medium decreased, the GDH activity started to decline at about 1.2 mM NH₄⁺ reaching 25% of its initial level after eight days of incubation. The GS activity remained constant until the ammonia concentration had decreased to about 0.2 mM, and then showed a sharp increase.

The GOGAT activity (NADH-dependent) varied little with ammonia concentration and different nitrogen sources (Table I) except that at very high (30 mM) NH₄⁺ concentration no activity was detectable.

These findings indicate that in *Thiocapsa roseopersicina* — as in many other bacteria studied including phototrophic species [7, 22, 23] — ammonia is assimilated via GDH during growth at high NH₄⁺ concentrations, while under conditions of NH₄⁺ deficiency, as they occur during nitrogen fixation, ammonia assimilation proceeds via the GS/GOGAT system.

During growth of *Thiocapsa roseopersicina* on L-glutamine as sole source of nitrogen (Table I) the glutamine content of the medium decreased from 6 to 2 mM and from 20 to 10 mM, respectively. The ammonia concentration in the culture liquid at the end of incubation (Table I) was well above the values of nonenzymatic hydrolysis of glutamine under the same conditions which were 0.2 and 1.0 mM NH₄⁺, respectively. Indeed, in glutamine cultures a glutaminase activity (E.C. 3.5.1.2) four to five times higher than in NH₄Cl cultures could be demonstrated. The GDH activities were similar to those on NH₄Cl; the somewhat elevated activity on 20 mM L-glutamine together with an increase of ICDH activity (not shown in Table I) may indicate a catabolic function of GDH under these conditions. The resulting increase of the intracellular α-ketoglutarate level might account for the high GS transferase activities relatively to the ammonia content of the culture liquids (see below). According to our findings *Thiocapsa roseopersicina* may utilize L-glutamine via GOGAT as well as via glutaminase plus GDH.

Obviously, glutamine doesn't repress GS synthesis. The reduced GS activities on glutamine compared to those of N₂-grown cells apparently are due
Table II. Activities of ammonia-assimilating enzymes, MDH and ICDH in *Thiocapsa roseopersicina* grown on sulfide with N$_2$ or 6 mM NH$_4$Cl in the presence of alanine or asparagine.

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Amino acid added (6 mM)</th>
<th>Relative growth [%]</th>
<th>NH$_4$ content of culture supernatant [mM]</th>
<th>Specific activities [milliunits/mg protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GDH</td>
</tr>
<tr>
<td>N$_2$ $^4$</td>
<td>—</td>
<td>75</td>
<td>0.05</td>
<td>6</td>
</tr>
<tr>
<td>N$_2$</td>
<td>Alanine $^5$</td>
<td>32</td>
<td>0.1</td>
<td>32</td>
</tr>
<tr>
<td>N$_2$</td>
<td>Asparagine $^5$</td>
<td>46</td>
<td>0.06</td>
<td>47</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>—</td>
<td>100</td>
<td>2.3</td>
<td>102</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>Alanine $^6$</td>
<td>70</td>
<td>2.4</td>
<td>45</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>Asparagine $^6$</td>
<td>7</td>
<td>3.8</td>
<td>72</td>
</tr>
</tbody>
</table>

$^1$ $-$ $^4$ See the legend to Table I.

$^5$ Added at the second feeding.

$^6$ Added before inoculation.

Ammonia, besides controlling the GS, also plays a part in the regulation of GDH, however in the inverse sense: A high GDH activity corresponds to a high NH$_4^+$ concentration and vice versa. There seems to be no direct regulatory relationship between GS and GDH as demonstrated in *Enterobacter aerogenes* [28]. The regulation of GOGAT obviously is rather incomplete in *Thiocapsa roseopersicina*. The enzyme appears to be repressed by very high ammonia concentrations and by certain amino acids.

Not only nitrogenous compounds, but also carboxylic acids and sugars affect the enzymes of ammonia assimilation in *Thiocapsa roseopersicina* (Table III), possibly by changing the intracellular concentration of a-ketoglutarate. The bacteria are able to grow in the absence of reduced sulfur compounds using...
Table III. Activities of ammonia-assimilating enzymes, MDH and ICDH in T. roseopersicina grown on different organic substrates as the only electron donors and 6 mM NH₄Cl as nitrogen source.

<table>
<thead>
<tr>
<th>Electron donor (10 mM)</th>
<th>Relative growth [%]</th>
<th>NH₄⁺ content of culture supernatant [mM]</th>
<th>Specific activities [milliunits/mg protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GDH</td>
</tr>
<tr>
<td>Acetate</td>
<td>250</td>
<td>1.1</td>
<td>158</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>200</td>
<td>1.3</td>
<td>171</td>
</tr>
<tr>
<td>Dl-Malate</td>
<td>250</td>
<td>1.0</td>
<td>70</td>
</tr>
<tr>
<td>Fructose</td>
<td>400</td>
<td>0.1</td>
<td>56</td>
</tr>
<tr>
<td>Sulfide</td>
<td>100</td>
<td>2.3</td>
<td>102</td>
</tr>
</tbody>
</table>

1–3 See the legend to Table I, except that there was no “feeding” of cultures growing on organic substrates.

simple organic substrates as the only photosynthetic electron donors. In addition, the organic compounds are utilized as supplementary or even predominant carbon sources besides CO₂ fixed via the Calvin cycle [29, 30]. This also accounts for the considerably higher growth yields in the presence of the organic substrates.

T. roseopersicina lacks α-ketoglutarate dehydrogenase and also isocitrate lyase [31] and therefore possesses neither a complete tricarboxylic acid cycle nor a glyoxylate cycle. The incomplete tricarboxylic acid cycle mainly serves biosynthetic functions, first of all the generation of α-ketoglutarate. As the organic substrates enter the cycle by different routes, their degradation may lead to different α-ketoglutarate concentrations in the cell. Variations in the activities of MDH and ICDH may reflect such differences in substrate catabolism.

After growth on the organic substrates (in the presence of 6 mM NH₄Cl) the ICDH activities always were somewhat higher than after lithoautotrophic growth (Table III). With the exception of acetate, the MDH activities were considerably elevated, the increase being exceptionally high on pyruvate.

In T. roseopersicina pyruvate is carboxylated to oxaloacetate by pyruvate carboxylase or — after phosphorylation — by phosphoenolpyruvate carboxykinase [32]. Oxaloacetate then may be converted to α-ketoglutarate by reverse flow through the “dicarboxylic acid pathway” via malate and succinate (high MDH activity) and by α-ketoglutarate synthase [33, 34]. Acetate also can enter the pathway of pyruvate metabolism via pyruvate synthase [33, 34]. On the other hand, it may be partly oxidized via citrate by the reactions of the “tricarboxylic acid pathway” [30] equally yielding α-ketoglutarate. The existence of this latter route is supported by the high ICDH activities and low MDH activities found in acetate-grown cells.

A high intracellular level of α-ketoglutarate has been reported to cause both an induction of anaerobic, NADPH-dependent GDH [35–38] and a derepression and activation of GS [39–41]. Accordingly we observed slightly increased GDH activities and high GS transferase activities in cells grown on acetate or pyruvate; the biosynthetic GS activities, however, remained on a very low level.

T. roseopersicina failed to grow on α-ketoglutarate (10 mM) in the absence of any other electron donors. When added to a medium containing sulfide α-ketoglutarate had no effect on the growth rate as well as on the activities of GDH and GS. Possibly α-ketoglutarate — like glutamate — is not taken up into the cell.

Cultures grown on malate or fructose also showed increased GS transferase activities, but rather low GDH activities (Table III). In the case of fructose, this partly may be due to the very low ammonia content of the culture medium at the end of incubation. However, in fructose-grown cells the biosynthetic GS activity was remarkably high as compared to the transferase activity. Furthermore, this was the only case in which GOGAT activity was clearly elevated.

Malate can be oxidized by MDH which, however, showed only medium activity in cells grown on this substrate. On the other hand, the conversion of malate to α-ketoglutarate may involve the dicarboxylic acid pathway. Fructose is catabolized by T. roseopersicina via fructose-1-phosphate and the Embden-Meyerhof pathway [42]; it may yield α-
ketoglutarate via pyruvate and citrate synthase as well as via phosphoenolpyruvate, phosphoenolpyruvate carboxykinase [32] and the dicarboxylic acid pathway. Accordingly, both ICDH and MDH showed rather high activities. The exceptionally high synthetic GS activities on fructose. Moreover, several intermediates of the Embden-Meyerhof pathway, such as fructose-1,6-bisphosphate and 3-phosphoglycerate, may inhibit the adenylylation of GS [43].

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