Purification and partial characterization of glutamate synthase from *Rhodospirillum rubrum* grown under nitrogen-fixing conditions

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Glutamate synthase, a key enzyme in ammonia assimilation, has been purified from the photosynthetic bacterium *Rhodospirillum rubrum*. The purification procedure involves ion-exchange chromatography, affinity chromatography and gel filtration. The recovery in the procedure is high (62%) and the specific activity is 21 μmol of NADPH oxidized/min per mg. The enzyme is specific for its substrates, and no activity was demonstrated with NADH or NH₄⁺ ions substituting for NADPH and glutamine respectively. The enzyme is composed of two dissimilar subunits with molecular masses of 53 and 152 kDa, and it is shown that Cl⁻ ions have an effect on the aggregation of the enzyme. Kₘ values for the substrates are: NADPH, 16 μM; 2-oxoglutarate, 10 μM; and glutamine, 65 μM. The enzyme is inhibited by amidotransferase inhibitors at micromolar concentrations. The role of the enzyme in the metabolic regulation of nitrogenase is discussed.

**INTRODUCTION**

In nitrogen-fixing organisms, ammonia assimilation takes place via the reactions catalysed by glutamine synthetase and glutamate synthase, as suggested by Nagatani et al. 20 years ago [1]. Since then this assimilatory pathway has been demonstrated in a number of bacteria and the individual enzymes studied. Glutamine synthetase, especially from *Escherichia coli*, has been characterized in great detail and the elucidation of its regulation by the adenylylation cascade mechanism has given new insight into metabolic regulation [2]. The other enzyme of the pathway, glutamate synthase, was discovered by Tempest and co-workers in *Klebsiella aerogenes* [3]. The enzyme from *E. coli* has been described in great detail, but it is not as well characterized as glutamine synthetase. The *E. coli* enzyme is an oligomeric iron–sulphur flavoprotein, composed of two different kinds of subunits with molecular masses of 53 kDa and 135 kDa respectively (reviewed in ref. [4]). The protomer, composed of one of each subunit, contains one FAD and one FMN in addition to eight iron and eight acid-labile sulphur atoms [4]. Glutamate synthase from *Azospirillum brasilense* is the only enzyme isolated from a nitrogen-fixing organism that has been characterized in any detail [5,6]. Its properties are very similar to those of the *E. coli* enzyme, although some significant dissimilarities in the amino acid composition and the N-terminal sequence of the smaller subunit have been reported [6].

In nitrogen-fixing phototrophic purple bacteria the operation of glutamate synthetase and glutamate synthase has already been implied in the original report on this assimilatory pathway [1] and was further substantiated in studies by Brown & Herbert [7,8]. Glutamine synthetase has been purified and characterized from *Rhodospirillum rubrum* [9–12], *Rhodopseudomonas palustris* [13,14], *Rhodobacter capsulatus* [15,16] and *Rhodobacter sphaeroides* [17]. Enzymes from these organisms exhibit essentially the same properties as the *E. coli* enzyme, including the regulation through adenylylation. One possible exception is *Rps. rubrum* in which the regulation seems to be more complex [11,12,18,19].

The second assimilatory enzyme, glutamate synthase, has attracted little attention. Its participation in nitrogen metabolism in phototrophs was confirmed in studies on glutamate synthase in *Rps. rubrum* [20] and a partial purification of the enzyme from this organism has also been reported [21].

In *Rps. rubrum* and a number of other phototrophs, in addition to the transcriptional control, nitrogenase is also regulated on the metabolic level (reviewed in ref. [22]). This metabolic regulation is manifested as a decrease in nitrogenase activity on addition of NH₄⁺ ions, glutamine, asparagine or oxygen or when cells are shifted to darkness [23,24]. The effect, which has been termed ‘switch-off’ [25], is due to the reversible inactivation of the Fe-protein (dinitrogen reductase) of nitrogenase by ADP-ribosylation of a specific arginine residue [26]. One major question concerning this regulatory system is the identity of the signal between the ‘switch-off’ effectors and the two enzymes that catalyse the inactivation and the activation respectively. We have previously proposed that changes in the NAD⁺/NADH ratio are part of this signal [27]. Glutamate synthase has a central role in this model which can be explained as follows: when NH₄⁺ ions are added as ‘switch-off’ effector, the flux through the glutamine synthetase–glutamate synthase reactions increases with the concomitant generation of NADP⁺, which is then re-reduced in the transhydrogenase reaction, thus increasing the concentration of oxidized NAD. NAD⁺ is the donor of the ADP-ribose moiety in the reaction leading to inactivation of the Fe-protein, and the enzyme catalysing this reaction has a surprisingly high Kₘ for NAD⁺ (2 mM) *in vitro* [22]. In order to study glutamate synthase and its role in this regulation we have purified this enzyme from *Rps. rubrum* and partially characterized it.

**MATERIALS AND METHODS**

**Materials**

DEAE-Sepharose FF, 2',5'-ADP-Sepharose 4B, Sepharcl S-300 HR and standards for gel filtration were purchased from Pharmacia-LKB. 2-Oxoglutarate, phenylmethanesulphonyl fluoride and NADPH were from Boehringer-Mannheim. 2-Mercaptoethanol, Mes, glutamine, FAD, FMN, 6-diazo-5-oxo-L-norleucine (DON) and azaserine were from Sigma. Rainbow standard proteins for SDS/PAGE were from Amersham, and all chemicals for polyacrylamide gels were from Bio-Rad. All other chemicals were of analytical grade available commercially.

**Growth of cells**

*Rps. rubrum*, A.T.C.C. 1170, was grown photohetero-
trophically under an atmosphere of N\textsubscript{2}/CO\textsubscript{2} (19:1, v/v) in the medium of Ormerod et al. [28] with the omission of NH\textsubscript{4}\textsuperscript{+} ions. Cells were harvested by filtration in a Pellicon Cassette (Millipore) and then frozen and stored in liquid nitrogen until used.

**Preparation of cell extract**

Typically 250 g of frozen cells were thawed in 550 ml of 0.1 M-Tris/HCl, pH 8.0, containing 5 mM-2-mercaptoethanol, 1 mM-EDTA and 1 mM-2-oxoglutarate. Cells were broken by passage through a Ribi Cell Fractionator (Sorvall) at 138 MPa, phenylmethanesulphonyl fluoride (to 75 \(\mu\)g/ml) was added and cell debris removed by centrifugation at 20000 \(g\) for 25 min. Chromatophores were removed by centrifugation at 110000 \(g\) for 90 min and the supernatant was used for the purification of glutamate synthase.

**Purification of glutamate synthase**

All purification steps were performed at 5 \(^\circ\)C and all buffers contained 5 mM-2-mercaptoethanol, 1 mM-EDTA and 1 mM-2-oxoglutarate. The supernatant was made 30 \% in (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and after being stirred for 15 min in the cold, centrifuged at 20000 \(g\) for 20 min. (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} was added to the supernatant to give a concentration of 47 \% and after 15 min of being stirred in the cold, the precipitate was again collected by centrifugation at 20000 \(g\) for 20 min. The pellet was dissolved in 420 ml of 25 mM-Tris/HCl, pH 7.8, and applied to a column (3.2 cm \(\times\) 7.5 cm) of DEAE-Sepharose FF equilibrated in the same buffer containing 0.15 M-KCl. After being washed with 2 bed volumes of equilibration buffer, glutamate synthase was eluted with a linear gradient of 0.15-0.4 M-KCl in the same buffer (300 + 300 ml). The active fractions were pooled and made 47 \% in (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}. The pellet obtained after 20 min centrifugation at 20000 \(g\) was dissolved in 60 ml of 50 mM-Mes, pH 6.5, and dialysed against three changes, 45 min each, of 800 ml of this buffer. The dialysed material was centrifuged at 20000 \(g\) for 20 min and used in the next purification step.

The supernatant was diluted to 100 ml with 50 mM-Mes, pH 6.5, and loaded on to a column (1.0 cm \(\times\) 8.5 cm) of 2'S-ADP-Sepharose 4B equilibrated in the same Mes buffer. After being washed with 50 ml of equilibration buffer, glutamate synthase was eluted with a linear gradient of 0-50 \(\mu\)M-NADPH in 25 mM-Tris/HCl, pH 7.5 (25 + 25 ml). The active fractions were pooled and concentrated by (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} precipitation (47 \%) as described before. The pellet was dissolved in 2 ml of 25 mM-Tris/HCl, pH 7.5, containing 0.15 M-KCl.

The final purification step was gel filtration on a column (1.4 cm \(\times\) 70 cm) of Sephacryl S-300 HR in 25 mM-Tris/HCl, pH 7.5, containing 0.15 M-KCl, developed at 7.5 ml/h. Fractions containing high activity were pooled, frozen and stored as pellets in liquid nitrogen.

**Enzyme assay**

Glutamate synthase activity was measured spectrophotometrically at 340 nm as the oxidation of NADPH [29]. The standard reaction mixture contained 50 mM-Tris/HCl, pH 7.5, 1 mM-EDTA, 5 mM-glutamine, 2.5 mM-2-oxoglutarate and 0.1 mM-NADPH in a total volume of 1.0 ml. Initial reaction rates were measured and 1 unit = 1 \(\mu\)mol of NADPH oxidized/min, using \(E_{100}^\text{max}\) = 6.22 \(\times\) 10\(^4\) M\(^{-1}\) cm\(^{-1}\) for NADPH.

**Analytical methods**

SDS/PAGE was performed as described by Laemmli [30] [10.2 \% (w/v) polyacrylamide/2.6 \% (w/v) cross-linker]. The following precoloured molecular-mass markers were used: myosin (200 kDa), phosphorylase \(b\) (92.5 kDa), BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa).

Determination of the molecular mass of glutamate synthase was done by gel filtration using the same column as in the last purification step, but with buffers as described in the text. Reference proteins used were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and aldolase (158 kDa).

FAD and FMN were determined by analysis using h.p.l.c. as described by Light et al. [31] after boiling the samples for 7 min in the dark. Iron was determined using the method of Fish [32]. Protein content was determined by the method of Bradford [33], using the Bio-Rad Protein Assay kit with BSA as standard.

**RESULTS AND DISCUSSION**

The results of a representative purification of glutamate synthase from *Rsp. rubrum* grown under nitrogen-fixing conditions are shown in Table 1. The specific activity of the purified enzyme is about the same as that reported for the enzyme from *A. brasiliense* (19.5 units/mg) [5] and somewhat lower than for the *E. coli* enzyme (26 units/mg) [29,34]. The enzyme was close to homogeneous (98 \%) as judged by SDS/PAGE after staining with Coomassie Brilliant Blue. The procedure is completed within 4 days and leads to a substantially higher recovery (62 \%) than other published protocols [5,29,34-37]. In contrast with a previous report on glutamate synthase from *Rsp. rubrum* [21], the enzyme was quite stable in our hands and could be kept in the gel-filtration buffer for at least 1 month at 5 \(^\circ\)C, without significant loss in activity.

SDS/PAGE of the purified enzyme showed two subunits in equal amount having molecular masses of 53 and 152 kDa respectively, indicating a protomer with molecular mass of around 200 kDa. This is similar to most glutamate synthases from prokaryotes, which have been reported to be composed of two dissimilar subunits, one smaller (50-75 kDa) and one larger (135-175 kDa). Recently, however, the purification of glutamate synthase from *Clostridium pasteurianum* was reported, and this enzyme is composed of two of each five polypeptides with molecular masses 91, 86, 68, 31 and 17.5 kDa [38].

The reported number of protomers in the native enzyme from different bacteria varies between one and four, corresponding to molecular masses around 200 kDa and 800 kDa respectively [5,6,21,29,35-37]. For the purified glutamate synthase from *Rsp. rubrum* we obtained a molecular mass of 810 kDa in 25 mM-Tris/HCl, pH 7.5, containing 0.1 M-KCl and 375 kDa in 25 mM-Tris/HCl, pH 7.5 (without KCl) or in 60 mM-potassium phosphate, pH 7.5. This is, in principle, in agreement with a previous report on the *Rsp. rubrum* enzyme [21]. Since the ionic strength in the phosphate buffer is about the same as in the Tris+KCl buffer, we suggest that the 800 kDa form is mainly due to the presence of Cl\textsuperscript{−}. This is supported by the fact that in the other studies where the 800 kDa form was obtained, determinations were made in the presence of 0.1 M-KCl [29,35] or 0.1 M-NaCl [21]. Miller & Stadman [29] reported that, when a crude (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} precipitate of the *E. coli* enzyme was used, a more slowly migrating form was obtained in sedimentation analysis [29]. This determination was also performed in the presence of 0.1 M-KCl, but it is possible that the presence of sulphate counteracts the effect of Cl\textsuperscript{−}. These authors also showed that, at high protein concentration, the enzyme to some extent also appeared as in a form with a molecular mass less than 800 kDa [29]. The *A. brasiliense* enzyme behaves somewhat differently, in that gel filtration at high ionic strength (not specified in the report) leads to the formation of a 190 kDa form [6]. The enzyme from *Bacillus licheniformis* has been shown not to aggregate under a number of different conditions tested, including vari-
Glutamate synthase from *Rhodospirillum rubrum*

Table 1. Purification of glutamate synthase from 250 g of *Rsp. rubrum*

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>550</td>
<td>195</td>
<td>10615</td>
<td>0.018</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>47% (NH₄)₂SO₄</td>
<td>420</td>
<td>182</td>
<td>2982</td>
<td>0.061</td>
<td>3.4</td>
<td>94</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>78</td>
<td>154</td>
<td>70</td>
<td>2.2</td>
<td>122</td>
<td>79</td>
</tr>
<tr>
<td>2',5'-ADP-Sepharose</td>
<td>9.5</td>
<td>132</td>
<td>8.6</td>
<td>15.4</td>
<td>856</td>
<td>68</td>
</tr>
<tr>
<td>Sephacryl S-300</td>
<td>12.3</td>
<td>121</td>
<td>5.8</td>
<td>20.9</td>
<td>1161</td>
<td>62</td>
</tr>
</tbody>
</table>

Table 2. Effect of amino acids on the activity of glutamate synthase from *Rsp. rubrum*

<table>
<thead>
<tr>
<th>Amino acid added</th>
<th>Activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(5 mM)</td>
<td></td>
</tr>
<tr>
<td>Aspartate</td>
<td>100</td>
</tr>
<tr>
<td>Glutamate</td>
<td>109</td>
</tr>
<tr>
<td>Methionine</td>
<td>92</td>
</tr>
</tbody>
</table>

The assay was run as described in the Materials and methods section with two different concentrations of glutamine in the assay.

It is clear that the inhibitors on purified glutamate synthase from *Rsp. rubrum*, and the results are shown in Fig. 1 and Table 3. The results confirm that the effect of these inhibitors can be partially prevented by glutamine [42], since the degree of inhibition with glutamine present during the 1 min preincubation of enzyme and inhibitor is lower than when glutamine is absent. It is clear that DON is the more potent inhibitor with an IC₅₀ of 10 mM in the absence of glutamine during preincubation and 35 µM when glutamine is included. The corresponding values for azaserine are 50 and 215 µM. In the experiments shown in Fig. 1, the inhibitor was added to the reaction mixture 1 min before the initiation of the reaction by addition of glutamine or 2-oxoglutarate. However, if the enzyme was preincubated for 10 min with the inhibitor present, before the start of the reaction, stronger inhibition was obtained as shown in Table 3, confirming that DON and azaserine are irreversible inhibitors of glutamate synthase [42]. Our results show that these inhibitors can be valuable tools in studying the role of glutamate synthase in the metabolic regulation of nitrogenase in *Rsp. rubrum*, and the results in previous reports [41,43,44] show that the inhibitors are taken up by the cells. However, interpretation of effects obtained on whole cells with these inhibitors must also consider the possibility that the effects are due to inhibition of other amidotransferases, especially when long incubation times are used.

This investigation has shown that glutamate synthase from *Rsp. rubrum* can be easily purified in a stable form which will make further studies on the role of this enzyme in ammonia assimilation and in the metabolic regulation of nitrogenase.

Glutamate synthase has been reported to be very specific for its substrates, and this was verified for the *Rsp. rubrum* enzyme. The activity with NH₄⁺ ions (10 mM) instead of glutamate was less than 2%, and no activity was obtained with NADH (0.1 mM) replacing NADPH. NAD⁺ has been shown to be competitive with NADPH, and we obtained 54% inhibition with 1 mM. On the other hand, NAD⁺ had no effect.

Glutamate synthase from *E. coli* has been shown to be susceptible to inhibition by certain amino acids, which is consistent with its role in nitrogen metabolism [29]. Table 2 shows the effect of methionine, aspartate and glutamate on the purified *Rsp. rubrum* enzyme. The effect was negligible in the presence of 5 mM-glutamate but methionine and aspartate showed considerable inhibition at 0.25 mM-glutamate. Since these two amino acids are not effectors of glutamate synthetase [2], their inhibitory effect on glutamate synthase makes ammonia assimilation even more sensitive to feedback regulation. Glutamate, which has been shown to be competitive with glutamine [40], is less inhibitory. The low degree of inhibition allows a high rate in the glutamate synthase reaction even at high glutamate/glutamine ratios in the cell, which is in line with the suggestion by Kanemoto & Ludden [41] that the glutamine pool is more tightly regulated than the glutamate pool in *Rsp. rubrum*.

The two glutamine analogues DON and azaserine are irreversible inhibitors of glutamine-utilizing amidotransferases [42] and have been used in studies concerning the metabolic regulation of nitrogenase in *Rsp. rubrum* to determine the role of different nitrogen metabolites in the "switch-off" effect [41,43,44]. However, in these studies it was not shown that the effects observed were due to inhibition of glutamate synthase, e.g. it was not demonstrated that the inhibitor, at the concentrations used, was in fact inhibiting glutamate synthase. We have investigated the effect of these inhibitors on purified glutamate synthase from *Rsp. rubrum*, and the results are shown in Fig. 1 and Table 3. The results confirm that the effect of these inhibitors can be partially prevented by glutamine [42], since the degree of inhibition with glutamine present during the 1 min preincubation of enzyme and inhibitor is lower than when glutamine is absent. It is clear that DON is the more potent inhibitor with an IC₅₀ of 10 mM in the absence of glutamine during preincubation and 35 µM when glutamine is included. The corresponding values for azaserine are 50 and 215 µM. In the experiments shown in Fig. 1, the inhibitor was added to the reaction mixture 1 min before the initiation of the reaction by addition of glutamine or 2-oxoglutarate. However, if the enzyme was preincubated for 10 min with the inhibitor present, before the start of the reaction, stronger inhibition was obtained as shown in Table 3, confirming that DON and azaserine are irreversible inhibitors of glutamate synthase [42]. Our results show that these inhibitors can be valuable tools in studying the role of glutamate synthase in the metabolic regulation of nitrogenase in *Rsp. rubrum*, and the results in previous reports [41,43,44] show that the inhibitors are taken up by the cells. However, interpretation of effects obtained on whole cells with these inhibitors must also consider the possibility that the effects are due to inhibition of other amidotransferases, especially when long incubation times are used.

This investigation has shown that glutamate synthase from *Rsp. rubrum* can be easily purified in a stable form which will make further studies on the role of this enzyme in ammonia assimilation and in the metabolic regulation of nitrogenase.
Fig. 1. Effect of DON and azaserine on glutamate synthase from *Rsp. rubrum*.

The assay was run in the standard assay system described in the Materials and methods section. The enzyme was incubated for 1 min with DON or azaserine in the presence of 0.5 mM-glutamine (closed symbols) or 2.5 mM-2-oxoglutarate (open symbols) before the reaction was initiated by the addition of 2.5 mM-2-oxoglutarate or 0.5 mM-glutamine respectively. Symbols: △ DON; □ azaserine.

Table 3. Effect of DON and azaserine on glutamate synthase from *Rsp. rubrum*

The experiment was run as shown in Fig. 1, except that the enzyme was preincubated for 10 min with the inhibitor before initiation of the reaction with either 2.5 mM-2-oxoglutarate or 0.5 mM-glutamine. The values in parentheses are taken from Fig. 1 and show percentage inhibition after 1 min preincubation with the inhibitor.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>0.5 mM-Glutamine</th>
<th>2.5 mM-2-Oxoglutarate</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON (2.5 μM)</td>
<td>35 (12)</td>
<td>73 (24)</td>
</tr>
<tr>
<td>Azaserine (12.5 μM)</td>
<td>38 (12)</td>
<td>69 (25)</td>
</tr>
</tbody>
</table>

...possible. However, to understand its role in these processes, its co-ordination with glutamine synthetase must be considered. The specific activity of glutamate synthase in cell extracts as shown in Table 1 is about 5% of the activity of glutamine synthetase in comparable extracts [12]. Using the *K*ₘ values of glutamate synthase from *Rsp. rubrum*, it can be assumed that the production of glutamine in the glutamate synthetase reaction will be regulating the rate of glutamate synthase. A more thorough evaluation will require more information about the absolute concentration of 2-oxoglutarate and glutamine in phototrophs, the possible effect of the physiological milieu on kinetic constants and the physical interaction between glutamine synthetase and glutamate synthase. However, it is quite conceivable that a rapid increase in glutamine concentration, imposed by using NH₄⁺ ions or glutamine as 'switch-off' effector, would lead to a rapid increase in the NAD(P)⁺ concentration as a result of increased flux through the glutamate synthase reaction, giving this enzyme a central role in the metabolic regulation of nitrogenase.

This investigation was supported by grants from the Swedish Natural Science Research Council and Stiftelsen Futura to S.N.

Received 18 January 1991/8 April 1991; accepted 16 April 1991

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