Molecular and kinetic characterization of glutamate synthase from the phototrophic bacterium *Rhodobacter capsulatus* E1F1

M. ISABEL IGEÑO, FRANCISCO J. CABALLERO and FRANCISCO CASTILLO*

Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Córdoba,
14071 Córdoba, Spain

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Glutamate synthase (GOGAT) from the phototrophic non-sulphur purple bacterium *Rhodobacter capsulatus* E1F1 has been purified to electrophoretic homogeneity by affinity chromatography. The native protein consisted of two different subunits of 175 and 53 kDa and contained 4 mol FAD, 4 mol iron and 4 mol labile sulphide per mol of dimer enzyme. The enzyme used NADPH as the electron donor and was inhibited by iron-chelating and thiol group reagents. GOGAT exhibited NAD(P)H-diaphorase activity which used sodium ferricyanide, cytochrome *c* and dichlorophenol indophenol as alternative electron acceptors. By contrast, glutaminase activity was not detected in purified GOGAT. The amino acid composition was quite different from that of other bacterial GOGATs, and the protein presented different aggregation states depending on the ionic strength. Two major multimeric active species with Stokes' radii of 6.18 and 7.32 nm could be separated by gel-filtration of protein solutions made in 0.5 M-KCl, whereas in the absence of salt, the maximal GOGAT activity corresponded to an oligomer with Stokes' radius of 6.80 nm. The enzyme exhibited apparent negative cooperativity for glutamine, and was competitively inhibited by L-glutamate and NADP⁺.

Introduction

Glutamate synthase (GOGAT) was first described by Tempest *et al.* (1970) as a key enzyme in ammonia assimilation by bacteria, and was later found in other organisms (Lea & Mifflin, 1975; Mifflin & Lea, 1976; Stewart *et al.*, 1980). The enzyme catalyses the reductive transfer of the amide group of L-Gln to 2-oxoglutarate, forming L-Glu:

The enzyme has been purified and characterized from eubacteria including *Escherichia coli* (Miller & Stadtman, 1972), *Azospirillum brasilense* (Ratti *et al.*, 1985) and *Aerobacter aerogenes* (Trotta *et al.*, 1974). The active enzyme is composed of a small subunit of relatively constant molecular mass (about 50 kDa) and a large subunit of molecular mass ranging from 135 kDa

(*E. coli*; Miller & Stadtman, 1972) to 200 kDa (*Thiobacillus thioparus*; Adachi & Suzuki, 1977). GOGATs from bacteria are iron-sulphur flavoproteins, with the exception of that of *Clostridium pasteurianum*, which apparently possesses a different quaternary structure and lacks iron and flavin as prosthetic groups (Singhal *et al.*, 1989). There have been only a few reports containing partial characterizations of GOGAT from phototrophic bacteria (Yelton & Yoch, 1981; Caballero *et al.*, 1989*b*; Carlberg & Nordlund, 1991).

The phototrophic nonsulphur purple bacterium *Rhodobacter capsulatus* E1F1 reduces inorganic nitrogen to ammonium which, as in other *Rhodospirillaceae* strains, is assimilated to nitrogen compounds through the glutamine synthetase (GS)/GOGAT cycle (Johansson & Gest, 1976; Alef & Zumft, 1981; Caballero *et al.*, 1985). When the bacterium is grown with reduced nitrogen compounds, ammonium is incorporated into carbon skeletons by an alternative pathway which includes L-alanine dehydrogenase and L-alanine:2-oxoglutarate aminotransferase activities (Moreno-Vivián *et al.*, 1983; Caballero *et al.*, 1989*a*; Moreno-Vivián *et al.*, 1990). This alternative pathway is also induced in L-methionine DL-sulphoxime (MSX)-treated bacterial cells, which have an inactivated GS (Moreno-Vivián *et al.*, 1983; Romero *et al.*, 1985). As described for other *Rhodospirillaceae*

* Author for correspondence.

Abbreviations: DCPIP, dichlorophenol indophenol; DTE, dithioerythritol; GOGAT, glutamate synthase; GS, glutamine synthetase; MSX, L-methionine DL-sulphoximine; pHMB, 4-(hydroxymercuri)benzoate.

strains (Johansson & Gest, 1976; Herbert *et al.*, 1978; Madigan & Cox, 1982), *R. capsulatus* E1F1 lacks glutamate dehydrogenase activity. An apparent glutamate dehydrogenase activity observed in crude extracts of *R. capsulatus* E1F1 was actually due to the coupling of L-alanine:2-oxoglutarate aminotransferase and L-alanine dehydrogenase activities (Castillo *et al.*, 1991). This L-alanine dehydrogenase has been purified and characterized at the molecular level (Caballero *et al.*, 1989a).

Although GS from phototrophic bacteria has been exhaustively characterized (Alef & Zumft, 1981; Nordlund *et al.*, 1985; Caballero *et al.*, 1985, 1987), less is known of GOGAT. GOGAT has been purified and partially characterized only from *Rhodospirillum rubrum* (Yelton & Yoch, 1981; Carlberg & Nordlund, 1991), a phototrophic bacterium which is not very closely related to *R. capsulatus* as deduced from 16S rRNA sequence analysis (Woese, 1987). GOGATs from heterotrophic bacteria are well-studied, dimeric enzymes of similar structural properties, quaternary structure and subunit size (Miller & Stadtman, 1972; Trotta *et al.*, 1974; Schreier & Bernlohr, 1984; Ratti *et al.*, 1985). By contrast, GOGATs from photosynthetic eukaryotic organisms are composed of a single polypeptide chain which has an unusually high molecular mass of 150–200 kDa (Galván *et al.*, 1984; Avila *et al.*, 1987). The large subunit from the GOGAT of *A. aerogenes* has been isolated and exhibits both glutaminase and NADPH oxidase activities (Trotta *et al.*, 1974). This subunit also contains flavin and non-haem iron in *E. coli* (Mäntsälä & Zalkin, 1976a) and seems to catalyse NH_4^+ -dependent L-glutamate synthesis. In addition, bacterial GOGATs exhibit several aggregation states depending on ionic strength (Miller & Stadtman, 1972; Yelton & Yoch, 1981; Carlberg & Nordlund, 1991). In *E. coli*, two structural genes, *gltB* and *gltD*, form an operon with a regulatory gene, *gltF* (Castaño *et al.*, 1988). The reaction mechanism of GOGAT has been extensively studied in *E. coli* (Mäntsälä & Zalkin, 1976a, b) and *A. brasilense* (Ratti *et al.*, 1987; Vanoni *et al.*, 1990, 1991a, b, 1992).

Glutamate biosynthesis and utilization in diazotrophic bacteria constitute a metabolic branch point with regulatory significance controlling glutamine concentration (and therefore the intracellular C/N balance) as well as several biochemical pathways related to the production of osmolytes (Madkour *et al.*, 1990). GOGAT from *Rsp. rubrum* has been proposed as being involved in regulating nitrogenase activity (Carlberg & Nordlund, 1991). *R. capsulatus* E1F1 is a moderate halophilic diazotroph whose nitrogenase, nitrate reductase and GS seem to be controlled by C/N status (Moreno-Vivián *et al.*, 1990). Unlike GS, GOGAT from phototrophic bacteria is not regulated through an interconversion mechanism, although enzyme synthesis

does increase in the presence of glutamine (Caballero *et al.*, 1989b).

This work presents the characterization of an enzyme with an unusual prosthetic group composition with respect to other bacterial GOGATs. We have also studied the kinetic parameters of the enzyme, which can be related to a possible regulatory role of GOGAT at the confluence of carbon and nitrogen fluxes in bacterial metabolism.

Methods

Organism and growth conditions. *Rhodobacter capsulatus* E1F1 was cultured phototrophically with DL-malate as the carbon source and L-glutamine as the nitrogen source, as described previously (Caballero *et al.*, 1989b).

Purification of GOGAT. All purification steps were performed at 4 °C. (i) *Cell extracts.* Cells were harvested at the mid-exponential phase of growth by centrifugation at 20000 g for 15 min, washed with 50 mM-MOPS/KOH buffer (pH 7.5), and resuspended in the same buffer containing 2.5% (v/v) glycerol and 2 mM-DTE (buffer A). Cell suspensions were sonicated in a Vibracell sonifier (20 × 5 s at 90 W) and, after centrifugation of sonicated cells at 15000 g for 15 min, the supernatant was further clarified by ultracentrifugation at 100000 g for 90 min to eliminate the chromatophore fraction. The clear supernatant was used as the starting material for enzyme purification.

(ii) *Ammonium sulphate precipitation.* Ammonium sulphate (194 mg ml⁻¹) was added to the cell extract with constant stirring for 20 min to bring it to 35% saturation. The solution was allowed to stand for 15 min and then centrifuged at 36000 g for 15 min. The pellet was discarded and the supernatant adjusted to 50% ammonium sulphate saturation by adding 87 mg ml⁻¹. After 15 min stirring, the solution was allowed to stand for 15 min and then centrifuged as above; the pellet, containing most of the enzyme activity, was resuspended in a minimal volume of buffer A.

(iii) *First gel-filtration.* The 35–50% saturated ammonium sulphate fraction was applied to a Sephacryl S-300 HR column (100 × 2.6 cm) equilibrated with buffer A. Proteins were eluted with the same buffer at a flow rate of 50 ml h⁻¹, and fractions containing GOGAT activity were pooled.

(iv) *Affinity chromatography.* The pooled fractions from Sephacryl chromatography were passed through a Red-120 agarose (22 × 1.6 cm) column equilibrated with buffer A. After washing with the same buffer A containing 0.1 M-KCl, GOGAT activity was eluted by using a linear gradient of 100 ml 0.1–0.5 M-KCl.

(v) *Second gel-filtration.* Fractions containing enzyme activity were pooled and concentrated by adding solid ammonium sulphate up to 60% saturation (361 mg ml⁻¹). The pellet was resuspended in an appropriate volume of buffer A and a concentrated solution was applied to a Sephacryl S-300 HR column (90 × 1.6 cm) previously equilibrated with the same buffer at a flow rate of 50 ml h⁻¹.

(vi) *Anion-exchange chromatography.* Pooled active fractions from step (v) were applied to a DEAE-Sephacel column (6 × 1.7 cm) equilibrated with buffer A. After washing with 20 ml of the same buffer, the enzyme was eluted with 100 ml of a linear gradient of 0–0.5 M-KCl in buffer A at a flow rate of 25 ml h⁻¹. Fractions containing GOGAT activity were pooled and concentrated by ultrafiltration in Centricon-30 vials (Amicon).

Enzyme activities. Glutamate synthase [L-glutamate:NADP⁺ oxidoreductase (transaminating); EC 1.4.1.13] activity was determined spectrophotometrically at 340 nm as previously described

(Caballero *et al.*, 1989b). Diaphorase activity was measured spectrophotometrically in purified preparations by following the oxidation rate of NAD(P)H at 340 nm, as described by Márquez *et al.* (1984). The reaction mixture contained (ml⁻¹): 100 µmol MOPS/KOH buffer (pH 7.6), 0.3 µmol NAD(P)H, 0.6 µmol of electron acceptor. Diaphorase activity was also measured electrophoretically as described by Wang & Raper (1970).

One unit of enzyme activity is defined as the amount of enzyme which catalyses the disappearance of 1 µmol NAD(P)H min⁻¹.

Determination of molecular parameters. Stokes' radius, α , and the diffusion coefficient, $D_{20,w}$, were determined (at 4 °C) as described by Siegel & Monty (1966) and Pundak & Eisenberg (1981), respectively, using a Sephacryl S-300 HR column (36 × 2.4 cm) equilibrated with buffer A. The following standards (in nm and m² s⁻¹ × 10¹¹) were used: bovine pancreas ribonuclease (1.8; 1.19); bovine serum albumin (3.55; 0.59); yeast alcohol dehydrogenase (4.61; 0.47); bovine liver catalase (5.22; 0.41); and horse spleen apoferritin (7.8; 0.36).

The sedimentation coefficient, $s_{20,w}$, was obtained by the method of Martin & Ames (1961) using a linear sucrose gradient (5–20%) at 4 °C. The following standards from Sigma were used (in S): horse heart cytochrome *c* (1.83); bovine serum albumin (4.65); yeast alcohol dehydrogenase (7.65); bovine liver catalase (11.35); horse spleen apoferritin (17.6); and bovine thyroglobulin (19.45).

The relative frictional quotient, f/f_0 , was calculated according to Brewer *et al.* (1974).

Electrophoresis. Non-denaturing- and SDS-PAGE were performed as described previously (Laemmli, 1970). For determination of the molecular mass of GOGAT subunits, the following standards from Sigma were used (in kDa): bovine erythrocyte carbonic anhydrase (29); rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36); egg albumin (45); bovine serum albumin (66); rabbit muscle phosphorylase b (97.4); *E. coli* β -galactosidase (116); and rabbit muscle myosin (205).

Amino acid composition. The amino acid analysis of purified GOGAT was carried out in a Waters HPLC, using the Waters PICO-TAG method, after hydrolysis in 6 M-HCl by a standard procedure. Tryptophan was determined after alkaline hydrolysis with 4 M-NaOH.

Analytical procedures. Protein was routinely estimated according to Bradford (1976) with bovine serum albumin as a standard. When necessary, the values of protein content were also obtained by the Lowry method and by the method of Smith *et al.* (1985).

Flavin content was determined in pure enzyme preparations, after thermal deproteinization, by analysing the emission fluorescence spectrum in a Hitachi-Perkin-Elmer, model 650-40, spectrofluorimeter. FMN and FAD were identified by a reverse-phase HPLC method. Homogeneous enzyme preparations were heated at 120 °C in the dark, centrifuged to eliminate coagulated proteins, and the supernatants were injected in a Spheri-5 ODS (Brownlee Laboratories) HPLC column (5 µ) previously equilibrated with 10 mM-potassium phosphate buffer (pH 7.0). Samples were eluted by using a gradient of 0–50% (v/v) methanol in the same phosphate buffer for 20 min with a flow rate of 1 ml min⁻¹. Either FMN or FAD from Sigma was used as a standard.

The iron content of the purified enzyme was determined according to Doeg & Ziegler (1962), based on the reaction between *o*-phenanthroline and the Fe(II) released from the protein after acid treatment.

Labile sulphide was determined by the formation of methylene blue from *N,N*-dimethyl-*p*-phenylenediamine (Beinert, 1983).

Results and Discussion

In *R. capsulatus* EIF1, GOGAT was strictly dependent on NADPH as the electron donor and L-glutamine as the nitrogen donor. In contrast to reports on other bacterial

GOGATs (Trotta *et al.*, 1974; Mäntsälä & Zalkin, 1976b; Ratti *et al.*, 1985), the purified enzyme from *R. capsulatus* EIF1 neither used ammonia as nitrogen donor nor showed glutaminase activity. A high NAD(P)H-diaphorase activity, which used sodium ferricyanide, *p*-nitro blue tetrazolium, cytochrome *c* and DCPIP as alternative substrates, is linked to GOGAT in *R. capsulatus* EIF1 (Table 1). It is worth noting that this activity used both pyridine nucleotides as the electron donor, although NADH was considerably less effective. NADH-GOGAT from green algae also exhibits diaphorase activity with NAD(P)H as reductant (Márquez *et al.*, 1984).

The purification of GOGAT from *R. capsulatus* EIF1 is a simple, high-yielding procedure, which includes gel-filtration and affinity and anion-exchange chromatographies (Table 2). The specific activity of the purified fraction was similar to that described for other bacterial GOGATs (Schreier & Bernlohr, 1984; Ratti *et al.*, 1985; Carlberg & Nordlund, 1991).

Protein fractions from the DEAE-Sephacel column (step vi of purification) showed two protein bands in SDS-PAGE corresponding to molecular masses of 175 kDa (subunit α) and 53 kDa (subunit β). When subjected to non-denaturing PAGE, purified preparations were resolved into a major band of 230 kDa, exhibiting both diaphorase and GOGAT activities, and a minor protein band corresponding to the large subunit that showed no activity (data not shown); this indicated that the small subunit is essential for both activities.

The molecular masses of the GOGAT subunits from *R. capsulatus* EIF1 (53 and 175 kDa) were similar to those of the *A. aerogenes* enzyme (Trotta *et al.*, 1974), whereas GOGATs from *E. coli*, *A. brasilense* and *Nocardia mediterranea* possess a small subunit of about 50 kDa and a large subunit of about 140 kDa (Miller & Stadtman, 1972; Ratti *et al.*, 1985; Mei & Jiao, 1988). A

Table 1. *Electron acceptors for the NAD(P)H-diaphorase activity of GOGAT from R. capsulatus EIF1*

Diaphorase activity was determined as indicated in Methods by using NADPH or NADH as electron donor, and the indicated electron acceptor. A purified preparation of 21 U GOGAT activity mg⁻¹ was used. Data represent the mean of four experiments.

Electron acceptor	Diaphorase activity (U mg ⁻¹)	
	NADPH	NADH
None	0	0
DCPIP	68.33 ± 1.07	13.87 ± 0.72
Cytochrome <i>c</i>	10.96 ± 0.86	11.05 ± 1.34
<i>p</i> -Nitro blue tetrazolium chloride	8.23 ± 1.44	0
Ferricyanide	2.37 ± 0.56	0

Table 2. Purification of GOGAT from 50 g (fresh weight) of *R. capsulatus* E1F1

Purification steps (i)–(iv) were performed four times on a small scale, using 200 ml of crude extract each time, and the resulting purified fractions pooled and used as starting material for steps (v) and (vi).

Purification step	Vol. (ml)	Protein (mg)	GOGAT activity		Purification (-fold)	Yield (%)
			Specific (U mg ⁻¹)	Total (U)		
(i) Cell-free extract	200	1160	0.05	58	1	100
(ii) Ammonium sulphate (35–50 %)	18	367	0.15	55	3	95
(iii) Sephacryl S-300 HR	82	145	0.33	48	7	83
(iv) Red-120 agarose	25	7.21	5.83	42	116	72
(v) Sephacryl S-300 HR	30	14.38	9.04	130	181	56
(vi) DEAE-Sephacel	7	4.9	21.22	104	425	45

Fig. 1. Effect of KCl on the aggregation of GOGAT protomers from *R. capsulatus* E1F1. Partially purified GOGAT solutions were passed through a Sephacryl S-300 HR column (100 × 1.6 cm) equilibrated with buffer A (b), or with the same buffer containing 0.5 M-KCl (a). Fractions (1.7 ml) were collected at a rate of 50 ml h⁻¹.

large subunit of 152 kDa has been reported in GOGAT from *Rsp. rubrum* (Carlberg & Nordlund, 1991).

Like GOGATs from other bacteria (Miller & Stadtman, 1972; Yelton & Yoch, 1981; Carlberg & Nordlund, 1991), the enzyme from *R. capsulatus* E1F1 exhibited different aggregation states, as deduced from gel-filtration profiles, depending on the ionic strength (Fig. 1). A quaternary structure including a large and a small subunit could be deduced from the molecular mass of the native protein in *R. capsulatus* E1F1 in the absence of KCl, whereas in the presence of salt, different protein superaggregates could be observed (Fig. 1). Thus, two major oligomeric species with Stokes' radii of 7.32 and 6.18 nm were obtained from Sephacryl S-300 HR filtration of a GOGAT preparation in buffer A containing 0.5 M-KCl (Fig. 1a). However, in the absence of salt, a major protein peak corresponding to a Stokes'

radius of 6.80 nm could be observed in the Sephacryl S-300 HR eluate (Fig. 1b). Other minor components were also detected in both preparations (Figs 1a and b). As deduced from the staining for GOGAT and diaphorase activities in non-denaturing polyacrylamide gels, the different superaggregation states were functional. An overestimation of the molecular masses of these oligomeric species was obtained from gel-filtration data due to the high frictional quotient of native GOGAT of *R. capsulatus* E1F1 (1.60) indicative of a non-spherical shape. A more accurate value was obtained from hydrodynamic data of the protein as described by Siegel & Monty (1966). Thus, a molecular mass of about 243 kDa (quaternary structure $\alpha\beta$) for the native protein was estimated by combining data from gel-filtration and sedimentation experiments performed in the absence of KCl (Table 3). Nevertheless, taking into account a Stokes' radius of 6.80 nm obtained by filtration of the protein through a Sephacryl S-300 HR column equilibrated in buffer A, a molecular mass of 455 kDa was calculated for GOGAT from *R. capsulatus* E1F1. This may be the correct value if it is assumed that the superaggregate has a spherical shape and a quaternary structure of $\alpha_2\beta_2$. A quaternary structure of $\alpha_4\beta_4$ has been reported for GOGATs from *E. coli* (Miller & Stadtman, 1972) and *Rsp. rubrum* in 0.1–0.4 M-NaCl (Yelton & Yoch, 1981; Carlberg & Nordlund, 1991), although the enzyme from *Rps. rubrum* also exhibits the tetrameric, $\alpha_2\beta_2$ form (Carlberg & Nordlund, 1991). Sedimentation coefficients of glutamate synthase from *A. aerogenes* and *Bacillus licheniformis* (Trotta *et al.*, 1974; Schreier & Bernlohr, 1984) were higher than that found in the *R. capsulatus* E1F1 enzyme, which could be due to different aggregation states of the protein from these bacteria.

GOGAT from *R. capsulatus* E1F1 is an iron-sulphur flavoprotein which contains 4 mol FAD, 4 mol iron and 4 mol labile sulphide per mol of enzyme (Table 3). The flavin prosthetic group was identified in native purified

Table 3. Molecular and kinetic parameters of GOGAT from *R. capsulatus* E1F1

Stokes' radius (nm)	7.32/6.18*
Sedimentation coefficient, $s_{20,w}$ (S)	6.80†
Diffusion coefficient, $D_{20,w} \times 10^{11}$ ($\text{m}^2 \text{s}^{-1}$)	7.6
	3.4/3.7*
Frictional quotient, f/f_0	3.5†
	1.60
Molecular mass (kDa):	
native enzyme	228–243
subunits	175; 53
Type of monomer	2
Number of subunits	2
Activation energy (kJ mol^{-1})	25.15
Q_{10} (25–35 °C)	1.39
Optimum pH	7.5
Apparent K_m (mM):	
NADPH	0.020
L-Gln	0.45/3.4
2-oxoglutarate	0.015
Hill coefficient (for L-Gln)	0.5
Absorption maxima (nm)	278; 372; 443
Prosthetic groups [$\text{mol (mol enzyme)}^{-1}$]:	
Fe	3.9 ± 0.4
FAD	3.7 ± 0.2
Labile sulphide	4.1 ± 0.5

* In the presence of 0.5 M-KCl.

† In the absence of KCl.

Fig. 2. Photochemical reduction of purified GOGAT from *R. capsulatus* E1F1. Spectra of the enzyme (0.7 mg ml^{-1}) were recorded anaerobically in buffer A without DTE (a and b) or in the same buffer containing 1 mM-EDTA and $5 \mu\text{M}$ -5-deazariboflavin (c). (a) Native enzyme; (b) native enzyme in an expanded range; (c) fully reduced enzyme after blue light irradiation.

GOGAT. HPLC chromatograms of boiled purified protein fractions showed peaks with retention times corresponding to FAD, which was also identified by analysing the spectrum of the flavin peak with a diode-

Fig. 3. Fluorescence emission spectra from the flavin component of purified GOGAT from *R. capsulatus* E1F1. Emission spectra were recorded by illuminating the pure enzyme preparations (0.16 mg ml^{-1}) with 450 nm wavelength light. (a) Intact native enzyme; (b) the same after 20 min boiling; (c) the same after 6 M-urea treatment.

array system coupled to the HPLC. An unusual flavin content of 4 mol FAD per mol of enzyme was calculated from fluorimetric studies. This value was also estimated from UV-visible spectroscopic data using an ϵ_{448} of $11400 \text{ M}^{-1} \text{ cm}^{-1}$ for FAD and an ϵ_{448} of $10600 \text{ M}^{-1} \text{ cm}^{-1}$ for the iron-sulphur cluster. Figure 2 shows the UV-visible spectra of oxidized and reduced forms of GOGAT. The native oxidized protein showed absorption maxima at 278, 372 and 443 nm (Fig. 2 and Table 3). The absorption spectrum of GOGAT in the visible region was completely bleached by EDTA/5-deazariboflavin photoreduction under anaerobic conditions. The fluorescence emission at 530 nm of the native enzyme was considerably enhanced in the denatured enzyme (Fig. 3) probably due to a quenching effect of the apoprotein, as described in *B. licheniformis* (Schreier & Bernlohr, 1984).

By contrast, the *A. aerogenes* enzyme contains 1 mol FAD and 1 mol FMN per mol of enzyme, both located in the large subunit (Trotta *et al.*, 1974). GOGATs from *A. brasilense* and *Rsp. rubrum* also possess FAD and FMN as prosthetic groups (Ratti *et al.*, 1985; Carlberg & Nordlund, 1991). The iron content of the *R. capsulatus* E1F1 enzyme was less than that described in *Rsp. rubrum* and *A. brasilense* (Carlberg & Nordlund, 1991; Ratti *et al.*, 1985). An A_{278}/A_{443} ratio of 5.7 was calculated from the UV-visible spectrum of purified oxidized *R. capsulatus* E1F1 GOGAT. Taking into account that this enzyme contained a high amount of FAD, this A_{278}/A_{443} ratio can be explained on the basis of the high content of tyrosine residues, which could account for a high

Table 4. Effect of some reagents on the activity of GOGAT from *R. capsulatus* E1F1

Purified enzyme was assayed as described in Methods in the presence of the reagents at the indicated concentrations. Other metal chelators such as CN^- , N_3^- , C_2H_2 , CO, NO, O_2 were without effect. Percentage inhibition was calculated with respect to control activity in the absence of any added inhibitor (21 U mg^{-1}). Data represent the mean of four experiments.

Inhibitor	Concn (mM)	Inhibition (%)
Atebrin	0.2	12.21 ± 1.82
	0.5	27.34 ± 2.08
Salicylhydroxamate	1	6.27 ± 1.03
	10	90.42 ± 3.21
<i>o</i> -Phenanthroline	1	15.83 ± 1.33
	10	72.11 ± 1.09
2,2'-Dipyridyl	0.2	22.42 ± 1.73
	0.5	59.80 ± 1.87

Table 5. Effect of thiol reagents on GOGAT activity of *R. capsulatus* E1F1

Purified enzyme was dialysed to remove DTE present in buffer A. Dialysed enzyme was preincubated for 10 min in the presence of the reagents at the indicated concentrations, and assayed as described in Methods. To allow complete reactivation of enzyme treated with pHMB, an additional period of 5 min incubation with DTE was required. Specific activity of 100% corresponded to 21 U mg^{-1} . Data represent the mean of four experiments.

Reagent	Concn (mM)	Activity (%)
Iodoacetate	1	91.67 ± 1.29
	5	88.31 ± 1.44
<i>N</i> -Ethylmaleimide	1	39.75 ± 1.71
	5	10.38 ± 2.14
pHMB	0.1	39.57 ± 0.97
	0.5	5.33 ± 1.03
pHMB/DTE	0.1/10	99.37 ± 0.72

absorption band at 278 nm (Fig. 2). Bacterial GOGATs presented A_{278}/A_{443} values ranging from 4.8 in *A. brasilense* (Ratti *et al.*, 1985) to about 20 in *N. mediterranei* (Mei & Jiao, 1988).

Purified GOGAT was inhibited by atebrin and 2,2'-dipyridyl, whereas salicylhydroxamate and *o*-phenanthroline inhibited only at high concentrations (Table 4). Sodium cyanide, sodium azide, NO, CO and acetylene showed no effects. The enzyme was also inactivated by a low concentration of thiol group reagents such as pHMB, the activity being almost completely recovered after incubation of inactivated enzyme with DTE (Table 5).

The GOGAT from *T. thioparus* was inhibited by reagents that would imply a catalytic role for both flavin and iron (Adachi & Suzuki, 1977). The enzyme from *R. capsulatus* E1F1 required high concentrations of these reagents to be completely inhibited (Table 4), thus

Table 6. Amino acid composition of GOGATs from various organisms

Samples of purified GOGAT from *R. capsulatus* E1F1 (about 0.3 mg protein) were hydrolysed in evacuated glass tubes at 110°C for 24 h with 6 M-HCl. After hydrolysis, the samples were evaporated in vacuum, derivatized with phenylisothiocyanate and analysed in a Waters HPLC according to the Waters PICO-TAG amino acid analysis system. Tryptophan was determined after alkaline hydrolysis with 4 M-NaOH.

Residue	<i>R. capsulatus</i> *	<i>E. coli</i> †	<i>A. brasilense</i> ‡
Alanine	206	176	176
Arginine	156	144	114
Aspartic acid	84	193	174
Half-cystine	59	38	38
Glutamic acid	37	190	203
Glycine	161	183	207
Histidine	58	41	44
Isoleucine	88	104	86
Leucine	162	170	159
Lysine	77	96	82
Methionine	90	47	36
Phenylalanine	54	75	55
Proline	109	96	65
Serine	117	111	90
Threonine	135	100	105
Tryptophan	9	20	13
Tyrosine	90	56	34
Valine	128	143	129

* Data from this study. Values are expressed as residues per 228 kDa dimer.

† Data from Oliver *et al.* (1987). Values are expressed as residues per 218.5 kDa dimer.

‡ Data from Vanoni *et al.* (1990). Values are expressed as residues per 196.5 kDa dimer, and were rounded to the nearest whole residue.

indicating that the prosthetic groups of GOGAT are not easily accessible to the chemical reagents used. By contrast, the strongly inactivating effect of pHMB implies that some -SH groups essential for enzyme activity and/or stability are probably more exposed.

GOGAT from *R. capsulatus* E1F1 contained an unusual amount of carboxylic and amidic amino acids, as well as of methionine and tyrosine. When compared with other GOGATs, a relatively high amount of cysteine residues (0.24 per kDa) was found in the *R. capsulatus* E1F1 enzyme (Table 6; Oliver *et al.*, 1987; Vanoni *et al.*, 1990). GS, the first enzyme of the main ammonium assimilation pathway, has a very similar ratio (0.22 cysteine residues per kDa, see Caballero *et al.*, 1985). Besides the role of cysteine in iron-sulphur clusters, this amino acid has been reported as a key residue in binding substrates of *E. coli* GOGAT (Mäntsälä & Zalkin, 1976b; Gosset *et al.*, 1989).

In *R. capsulatus* E1F1, apparent K_m values of GOGAT for 2-oxoglutarate (15 μM) and NADPH (20 μM) were of the same order as those described for other bacterial GOGATs (Yelton & Yoch, 1981; Ratti *et al.*, 1985). Non-hyperbolic saturation kinetics for L-Gln has been

Fig. 4. Double reciprocal plots of initial velocity versus substrate concentration in the GOGAT reaction. Initial velocities (in $\mu\text{mol min}^{-1}$) of purified GOGAT from *R. capsulatus* E1F1 were measured at the L-Gln concentrations indicated in the Figure. In each case, the concentrations of the other substrates were saturating. The inset shows the replot of the data according to the Hill representation. Each point represents the mean of four experiments.

Table 7. Effect of several compounds on the GOGAT activity from *R. capsulatus* E1F1

Enzyme activity was determined with pure enzyme preparations, as described in Methods, in the presence of the different metabolites at the indicated concentrations. Other L-amino acids tested were without effect. Specific activity of 100% corresponded to $20.5 \text{ U (mg protein)}^{-1}$. Data represent the mean of four experiments.

Addition	Concn (mM)	Inhibition (%)
L-Asp	1	20.40 ± 1.14
	10	37.06 ± 1.17
Fumarate	1	17.64 ± 1.67
	10	29.43 ± 1.52
Isocitrate	1	14.64 ± 1.16
	10	22.17 ± 2.61
Oxaloacetate	1	17.17 ± 1.82
	10	27.45 ± 1.38
ATP	1	0.25 ± 1.04
	10	37.83 ± 1.33
ADP	1	24.02 ± 2.16
	10	52.38 ± 1.60
AMP	1	0.32 ± 0.95
	10	35.20 ± 1.79
Azaserine	1	26.78 ± 1.28
	10	90.25 ± 0.96

observed in GOGAT from *R. capsulatus* E1F1, whereas hyperbolic curves were observed for 2-oxoglutarate and NADPH. Non-hyperbolic kinetics has also been described in *B. licheniformis* (Schreier & Bernlohr, 1984) and *Bacillus megaterium* (Hemmilä & Mäntsä, 1978)

contrary to what was reported for the GOGAT from *A. brasilense*, which showed hyperbolic kinetics for L-Gln, NADPH and 2-oxoglutarate (Ratti *et al.*, 1985). *B. licheniformis* and *Rsp. rubrum* enzymes also presented hyperbolic kinetics for L-Gln, but with low K_m values ($65 \mu\text{M}$ and $8 \mu\text{M}$, respectively) (Schreier & Bernlohr, 1984; Carlberg & Nordlund, 1991). The two apparent K_m values for L-Gln (0.45 and 3.4 mM) exhibited by GOGAT from *R. capsulatus* can be interpreted in terms of negative cooperativity as deduced from the Hill plot, the value of the Hill coefficient ($h = 0.5$; Fig. 4) and the concave form of the Scatchard curve (not shown). Negative cooperativity in GOGAT is not unexpected, taking into account that the enzyme forms spontaneously polymeric superaggregates which probably present several glutamine binding sites (at least one site per functional dimer). Taking into account that the L-Gln concentration inside these cells is probably subjected to rapid and strong fluctuations due to the regulation of GS activity by the adenylation cascade (Caballero *et al.*, 1985), kinetic regulation of GOGAT by L-Gln could not be discarded. However, the Scatchard plot is only a qualitative approach to studies on negative cooperativity (Dahlquist, 1978), therefore a concave form of the plot due to site heterogeneity caused by different aggregation states cannot be ruled out in our case.

We have not observed a significant inhibition of GOGAT by amino and carboxylic acids (Table 7), at least in the physiological intracellular concentrations of these compounds in *R. capsulatus* E1F1, which were always of the micromolar range (results not shown). Nevertheless, the enzyme was 64% inhibited by 1 mM -MSX. GOGAT from the phototrophic bacterium *Rhodospseudomonas acidophila* was also strongly inhibited by this glutamate analogue (Herbert *et al.*, 1978). The inhibition of GOGAT by high concentrations of amino and carboxylic acids has been reported in other bacteria (Adachi & Suzuki, 1977; Mei & Jiao, 1988; Ertan, 1992).

GOGAT from *R. capsulatus* E1F1 was strongly inhibited by the products of the reaction, L-Glu and NADP^+ , which were competitive inhibitors with respect to L-Gln and NADPH, respectively, as deduced from the corresponding Cornish-Bowden plots. From the Lineweaver-Burk plots, K_i values of 10 mM -L-Glu and 0.1 mM - NADP^+ were calculated. This result is consistent with MSX inhibition reported above. Product inhibition of GOGAT in *R. capsulatus* could also have physiological significance, especially in the case of the competitive inhibition of NADP^+ , which gave a low K_i value (0.1 mM). Inhibition of GOGAT by L-Glu and NADP^+ has also been reported in other bacterial enzymes (Sung *et al.*, 1984; Ratti *et al.*, 1985; Matsuoka & Kimura, 1986). Since we have not measured the intracellular concentrations of NADP^+ and NADPH under different

metabolic conditions, we cannot conclude that the NADP⁺/NADPH ratio has an *in vivo* regulatory significance in the GOGAT activity of *R. capsulatus* E1F1.

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